DEVELOPMENT OF A MICROENCAPSULATED ANTHRAX SPORE VACCINE IN THE MOUSE MODEL FOR ORAL ADMINISTRATION IN WILDLIFE

A Dissertation

by

JAMIE SUZANNE BENN FELIX

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Walter Cook
Co-Chair of Committee,	Allison C. Rice-Ficht
Committee Members,	Thomas A. Ficht
	Yi Xu
Head of Department,	Ramesh Vemulapalli

December 2019

Major Subject: Biomedical Sciences

ABSTRACT

Anthrax (*Bacillus anthracis*) is a zoonotic disease endemic to environments worldwide. Spores, the dormant form of the bacteria, can survive for decades in nature's harshest environments and maintain their viability to cause disease. Outbreaks are common in free-ranging livestock and wildlife, thus making anthrax an economically and ecologically important disease. The currently available vaccine to protect livestock is a suspension of *B. anthracis* Sterne Strain 34F2 spores in saponin (Sterne vaccine). However, it is only available as a subcutaneous injection which is an impractical method of prevention for wildlife. Oral vaccination is the ideal method for free-ranging wildlife, but the Sterne vaccine has never been thoroughly evaluated for oral administration. The current study evaluated the antibody titers induced in mice by subcutaneous or oral vaccination with three different doses of the Sterne vaccine. Results presented here demonstrate that the Sterne vaccine is only effective as a subcutaneous injection and that an alternate oral anthrax vaccine formulation must be developed, therefore we evaluated the immunogenicity and *in vitro* protective abilities of a controlled release vehicle when orally administered in mice. Microencapsulated *B. anthracis* Sterne strain 34F2 spores (Sterne spores), coated with poly-L-lysine and a non-immunogenic eggshell precursor protein, vitelline protein B, stimulated antibody production after subcutaneous and oral vaccination. This antibody response was shown to be protective against *in vitro* anthrax toxin challenge encouraging further development of a new oral anthrax vaccine formulation. We also investigated the stability of these microcapsules as a function of

the Sterne spore dose and coating formulation. An increased Sterne spore dose greatly improved the measured antibody titer following subcutaneous injection while modifying the ratio of poly-L-lysine to vitelline protein B exhibited dose response effects with respect to microcapsule stability in gastrointestinal environments. Taken together, these results suggest that microencapsulation of Sterne spores in a controlled delivery vehicle can enhance the immune response following oral vaccination, therefore promising efficient and protective oral vaccination of free-ranging livestock and wildlife.

DEDICATION

To my husband, Jared.

Thank you for your love, patience and never-ending support and faith in me, especially throughout the last five months during what should have been our honeymoon phase.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Walt Cook, and my committee members, Dr. Allison Rice-Ficht and Dr. Thomas Ficht, for their guidance and support throughout the course of this research. I would also like to thank my fourth committee member, Dr. Yi Xu, for sharing her knowledge of the Sterne strain with me and Dr. Michael Criscitiello for his encouragement, advice and for always being a listening ear.

I am forever indebted to Dr. Sankar Chaki who has played an essential role in the development of my laboratory skills. I would have been lost without him. His daily support, encouragement, positive attitude, mentoring and dedication to my project helped make the biggest challenges seem manageable.

I'm also grateful for my lab mates and friends throughout the years: to Daniel Garcia for teaching me the encapsulation process and how to conduct mouse experiments at Texas A&M, to Gabrielle Free for helping measure hundreds of microcapsule images and joining me for Spore Therapy along with Anna George. To Ana Lucia Cabello, Omar Khalaf, Luciana de Costa Fachini and Slim Zriba for making tedious days in the lab enjoyable.

Finally, I would like to thank my family and friends for their continuous support and encouragement.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a dissertation committee consisting of Walter E. Cook (advisor) of the Department of Veterinary Pathobiology, Allison C. Rice-Ficht (co-advisor) jointly of the Department of Veterinary Pathobiology and Department of Cellular and Molecular Medicine, Thomas A. Ficht of the Department of Veterinary Pathobiology and Yi Xu of the Texas A&M Health Science Center Institute of Biosciences & Technology. Additional collaborators included Sankar P. Chaki and Michael Criscitiello from the Department of Veterinary Pathobiology. Gabrielle Free assisted with microcapsule image measurements. All other work conducted for the dissertation was completed by the student independently.

Graduate study and research costs were supported by faculty start-up funding provided to Walter E. Cook by Texas A&M University. Research costs were also supported by the American Association of Zoo Veterinarians Wild Animal Health Fund and the Texas A&M CVM Graduate Student Research Trainee Grant. Vials of the Anthrax Spore Vaccine were generously donated by Dr. Randall Berrier at Colorado Serum Company.

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

CONTRIBUTORS AND FUNDING SOURCES vi

TABLE OF CONTENTS vii

LIST OF FIGURES x

LIST OF TABLES xii

1. INTRODUCTION TO WILDLIFE ANTHRAX 1

1.1. Historical significance 1
1.2. Susceptibility by species 5
1.2.1. Ruminants 7
1.2.2. Equines 10
1.2.3. Swine 11
1.2.4. Carnivores 12
1.2.5. Other animals 13
1.3. Anthrax ecology 14
1.4. Conclusion 16

2. *BACILLUS ANTHRACIS* STERNE STRAIN 34F2 VACCINE ANTIBODY DOSE RESPONSE BY SUBCUTANEOUS AND ORAL ADMINISTRATION 17

2.1. Introduction 17
2.2. Materials and Methods 20
2.2.1. Mice 20
2.2.2. Bacterial strains 21
2.2.3. Vaccine preparation 23
2.2.4. Vaccination 24
2.2.5. Detection of anthrax-specific antibody levels 24
2.3. Results 26
2.3.1. Anti-anthrax antibody response 26

2.4. Discussion 28

3. PROTECTIVE ANTIBODY RESPONSE FOLLOWING ORAL VACCINATION WITH MICROENCAPSULATED *BACILLUS ANTHRACIS* STERNE STRAIN 34F2 SPORES 32

3.1. Introduction 32
3.2. Materials and Methods 35
3.2.1. Preparation of Sterne spores 35
3.2.2. Sterne spore response to simulated gastrointestinal environments 35
3.2.3. Vaccine preparation 36
3.2.4. Mouse immunizations 39
3.2.5. Detection of anthrax-specific antibody titers 41
3.2.6. Lethal toxin neutralization assays 41
3.2.7. Statistical analysis 43
3.2.8. Surveying white-tailed deer serum for anti-anthrax antibodies 43
3.2.9. Interpretation of white-tailed deer serum ELISAs 44
3.3. Results 44
3.3.1. Sterne spore stability in simulated gastrointestinal environments 44
3.3.2. Microcapsule vaccines induce anthrax specific antibody titers 45
3.3.3. Microencapsulated Sterne spores induce toxin neutralizing antibodies 47
3.3.4. Seroprevalence of anti-anthrax antibodies in white-tailed deer serum from the East Foundation property 49
3.4. Discussion 50

4. STABILITY OF ALTERNATE FORMULATIONS FOR ALGINATE MICROCAPSULES AS ORAL VACCINE DELIVERY VEHICLES 56

4.1. Introduction 56
4.2. Materials and Methods 58
4.2.1. Expression and purification of vitelline protein B 58
4.2.2. Preparation of Sterne spores 62
4.2.3. Vaccine preparation 63
4.2.4. Mouse vaccination 66
4.2.5. Bacterial release from microcapsules 67
4.2.6. Detection of anthrax-specific IgG and IgA 68
4.2.7. Toxin neutralization assays 69
4.2.8. Characterization of microcapsules in simulated gastrointestinal environments 70
4.2.9. Statistical analysis 70
4.3. Results 71
4.3.1. Evidence of controlled release from microcapsules 71
4.3.2. Anti-anthrax protective antigen IgG and IgA 74
4.3.3. LeTx neutralization assay 78

4.3.4. Comparison of microcapsule formulations in gastrointestinal environments 79
4.4. Discussion 91

5. CONCLUSION 101

5.1. Summary 101
5.2. Future Directions 103

REFERENCES 104

APPENDIX A NOMENCLATURE 120

APPENDIX B VPB MICROCAPSULE FORMULATION ORIGINS 121

LIST OF FIGURES

Page

Figure 1.1. Illustration of *Bacillus anthracis* plasmids. 3

Figure 2.1. Carcass pile discovered on a previously poorly managed ranch in Uvalde, Texas. 19

Figure 2.2. *Bacillus anthracis* Sterne strain 34F2 spore preparation for vaccination. 22

Figure 2.3. Heat treated and filter purified *Bacillus anthracis* Sterne strain 34F2 spores. 23

Figure 2.4. IgG responses against subcutaneously injected *Bacillus anthracis* Sterne strain 34F2 spores. 27

Figure 2.5. IgG responses against oral inoculation of *Bacillus anthracis* Sterne strain 34F2 spores. 28

Figure 3.1. Explanation of vaccine dose calculations for microencapsulation. 37

Figure 3.2. Illustration of microcapsules produced for vaccination of BALBc/J mice.....39

Figure 3.3. Sterne spore titer response to simulated gastrointestinal environments. 45

Figure 3.4. IgG responses from subcutaneously injected Empty Capsules, Sterne Vaccine and Spore Capsules. 46

Figure 3.5. IgG responses from orally administered Empty Capsules, Sterne Vaccine and Spore Capsules. 47

Figure 3.6. *In vitro* toxin neutralizing abilities of antibodies from subcutaneous and orally administered Sterne Vaccine and Spore Capsules. 48

Figure 3.7. Map of the East Foundation property ranches. 50

Figure 4.1. IPTG time course induction of VpB. 59

Figure 4.2. Purified VpB on SDS-PAGE at a molecular weight of ~31 kD. 62

Figure 4.3. *In vitro* release from VpB Shell and VpB Core & Shell capsules. 72

Figure 4.4. Sterne spore entrapment in VpB Shell and VpB Core & Shell capsules. 73

Figure 4.5. Serum IgG responses from subcutaneously injected VpB Shell capsules and Sterne vaccine. 75

Figure 4.6. Serum IgG responses from orally inoculated VpB Shell or VpB Core & Shell capsules. 76

Figure 4.7. Serum IgA responses from subcutaneously and orally inoculated VpB Shell or VpB Core & Shell capsules. 77

Figure 4.8. Fecal IgA responses from subcutaneously and orally inoculated VpB Shell or VpB Core & Shell capsules. 78

Figure 4.9. Toxin neutralizing abilities of VpB Shell and VpB Core & Shell capsules. 79

Figure 4.10. Uncoated microcapsule response to simulated gastrointestinal environments. 82

Figure 4.11. Low VpB $CaCl_2$ and Low VpB MOPS capsule diameter changes to simulated gastrointestinal environments. 83

Figure 4.12. Mid VpB $CaCl_2$ and Mid VpB MOPS capsule diameter changes to simulated gastrointestinal environments. 84

Figure 4.13. Low, Mid and High VpB $CaCl_2$ capsule diameter changes to simulated gastrointestinal environments. 85

Figure 4.14. Microscope images of the Low VpB $CaCl_2$ capsule response to simulated gastrointestinal environments. 86

Figure 4.15. Microscope images of the Low VpB MOPS capsule response to simulated gastrointestinal environments. 87

Figure 4.16. Microscope images of the Mid VpB $CaCl_2$ capsule response to simulated gastrointestinal environments. 88

Figure 4.17. Microscope images of the Mid VpB MOPS capsule response to simulated gastrointestinal environments. 89

Figure 4.18. Microscope images of the High VpB $CaCl_2$ capsule response to simulated gastrointestinal environments. 90

LIST OF TABLES

Page

Table 2.1. Overview of the vaccination groups used in this study to evaluate the Sterne Vaccine in BALBc/J mice. 21

Table 3.1. Vaccination groups to assess the efficacy of microencapsulated Sterne spores as an oral vaccine. 40

Table 3.2. Seroprevalence of anti-anthrax protective antigen (PA83) in serum from white-tailed deer on the East Foundation Ranch. 49

Table 4.1. Microcapsule formulations prepared to assess the microcapsule efficacy, release rate and stability in gastrointestinal environment. 65

Table 4.2. Vaccination groups testing two microcapsule formulations with higher Sterne spore doses 67

1. INTRODUCTION TO WILDLIFE ANTHRAX

1.1. Historical significance

Anthrax is a zoonotic disease caused by the spore forming bacterium, *Bacillus anthracis*. In the last few decades it has gained public attention as a potential bioweapon but very few are aware of the burden it places on our wildlife. To fully understand the influence anthrax has on wildlife, it is helpful to review its historical significance, another lesser known aspect of the disease.

In its dormant spore form anthrax is uniquely suited to survive for decades in nature's harshest environments, thus explaining how it has continued to affect humans and animals alike for millennia. While the first documented outbreak of anthrax is believed to have caused the 5th and 6th plagues of Egypt over three millennia ago, it has been suggested that anthrax afflicted the founders of agriculture in Mesopotamia some 5000 years BCE [1,2]. A review of early literature reveals indications of anthrax outbreaks in ancient Egypt, Greece, and Asia, and some even suggest it may have contributed to the fall of Rome [1,2]. Periodic anthrax outbreaks continued throughout history as it utilized wildlife, livestock and human hosts to spread all over the world.

By the 19th century, scientists and doctors started realizing the connection between the etiological agent and the manifested disease. The exact details from the beginning of this discovery period remain uncertain to this day, except for the work by Robert Koch in 1877 [1]. Frequently credited as the founder of modern bacteriology, Koch experimented with *B. anthracis* and described the bacteria's sporulating

characteristics while establishing the four criteria to identify the causative agent of a disease [1]. Around the same time that Koch's Postulates were outlined, Louis Pasteur was performing his initial experiments for an anthrax vaccine. In 1881, Pasteur performed his famous public experiment in which he challenged two groups of animals with *B. anthracis*, only one of which had previously received his experimental vaccine [1]. Consequently, only the vaccinated animals survived meaning Pasteur had successfully produced the first vaccine against anthrax and demonstrated its efficacy. The pioneering work of Koch and Pasteur laid the foundation for modern microbiology, immunology and vaccinology while verifying the historical significance of anthrax by simply confirming that anthrax was a disease worth studying.

Anthrax continued to make history during the 19th and 20th centuries when it proved itself to be an economically important disease by causing large scale mortalities, with one outbreak resulting in the deaths of up to 60,000 animals [3,4]. By the 1930s, the majority of anthrax research was focused on improving the Pasteur vaccine to prevent costly livestock outbreaks [1]. A fully virulent *B. anthracis* cell contains two main virulence plasmids, pXO1 and pXO2 (Figure 1.1). The pXO1 plasmid encodes the three toxin components: edema factor, lethal factor and protective antigen while the pXO2 plasmid encodes the poly-γ-D-glutamic acid capsule [5]. When infecting a host, the poly-γ-D-glutamic acid capsule has the critical job of preventing phagocytosis while edema factor and lethal factor both pair separately with protective antigen to produce edema toxin and lethal toxin. Fatal anthrax infections involve rapid production of destructive amounts of toxin that eventually result in septicemia, toxic shock and host

death. In 1937, Max Sterne discovered what is now aptly named, the Sterne strain, a live attenuated strain of *B. anthracis* that had naturally lost the pXO2 plasmid. The Sterne strain was no longer capable of producing the poly-γ-D-glutamic acid capsule but it did retain the pXO1 plasmid and was able to produce both functioning toxins.

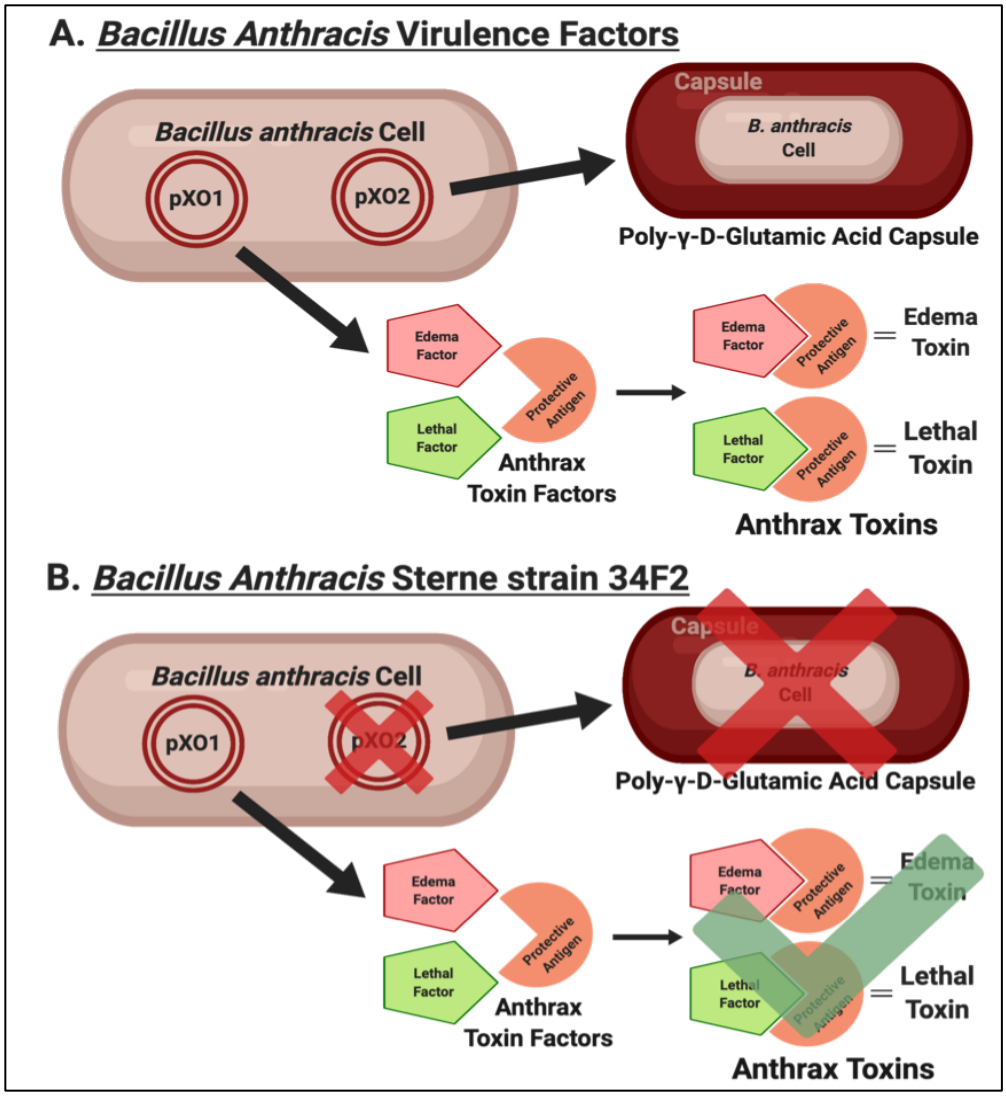

Figure 1.1. Illustration of *Bacillus anthracis* plasmids. (A) A fully virulent *B. anthracis* cell containing both the pXO1 and pXO2 plasmids. (B) *B. anthracis* Sterne strain 34F2 cell containing only the pXO1 plasmid. Created with BioRender.com.

Thus, the Sterne strain was able to induce protection by stimulating antibodies primarily against protective antigen but also against all toxin components [5,6]. Shortly after the discovery the Sterne vaccine was formulated as an effective livestock vaccine consisting of Sterne spores suspended in saponin and it is still in use to this day. Given the lack of knowledge regarding anthrax and wildlife conservation at the time the Sterne vaccine was not produced with large scale wildlife vaccination needs in mind [1]. In fact, the earliest estimates of global wildlife anthrax incidence are from just before the breakthrough of the Sterne vaccine and are overwhelmingly dependent on imported contaminated animal products [1].

Under the impression that anthrax outbreaks in livestock could be prevented now that there was a protective vaccine on the market, anthrax research shifted to understanding the transmission of the disease [1]. By the middle of World War II, the British started experimenting with biological warfare on Gruinard Island off the coast of Scotland in 1942. The true durability of anthrax spores wasn't completely appreciated until viable spores were still found on the island 48 years later. Gruinard Island was only confirmed decontaminated after 280 tons of formaldehyde was applied to every inch of the island over a period of 4 years [7].

Despite the historical and economic significance of this disease, anthrax was recently described as an "undervalued" and "neglected" zoonosis [8,9]. Since the 1940s, a great deal of research has been published regarding the pathogenicity and cellular biology of anthrax, but comparatively little has been done regarding anthrax in wildlife. The Gruinard Island experiments may have hinted at the extent of dormant spore

survival, but anthrax spores have also previously isolated from animal bones estimated to be about 200 years old [10]. Given the extreme resilience of the anthrax spore, and that it is endemic or hyperendemic worldwide, any eradication effort would be nearly impossible [11]. Therefore, it is essential that the prevalence and effect of anthrax in wildlife be defined, starting with this review which attempts to summarize all current knowledge regarding the burden anthrax places on our wildlife.

1.2. Susceptibility by species

Over the course of history, anthrax infections have been described in hundreds of species around the world. Since the commercialization of the Sterne vaccine, we have experienced a global decrease in livestock anthrax but not in wildlife anthrax [1,4]. There is an estimated global distribution and suitability for environmental anthrax and many animals on anthrax prone landscapes are not vaccinated [11,12]. Within Canada and the United States, enzootic regions continue to be a problem in Alberta and southern Saskatchewan, and in North Dakota, South Dakota, Montana, Minnesota, Nebraska, Nevada and Texas [4,12–21].

Thorough and descriptive data for anthrax infections in all domestic and wildlife species does not exist but anthrax is believed to be capable of affecting all mammals with varying degrees of virulence. Across all species with extremely severe infections, it has been observed that seemingly healthy animals suffer a short period of disorientation followed by sudden death [12]. Herbivores are undoubtedly the most susceptible, but

omnivores and carnivores can also be affected. There are even suggestions of anthrax infections in birds, although these reports are lacking sufficient detail [12].

Back in 1967, Lincoln et al. suggested that anthrax will have a characteristic symptomology and impact for every species [22]. Research since then has been mildly productive in defining some of those patterns, but many remain unclear with only theory to suggest why one is different from another. The following section attempts to summarize the varying levels of mammal susceptibility, but it is important to note that the majority of the available data on anthrax in animals is an assortment of wildlife and exotic cases in nature, captivity (zoos, wildlife sanctuaries, wildlife rehabilitation facilities, etc.), experimental and domestic situations. Additionally, it must be kept in mind that lack of data does not imply resistance to infection but more likely lack of observation and reporting. Similarly, more reports of infection do not confirm increased susceptibility as much as it does more frequent observation.

Lastly, we must be cautious when making comparisons between species. With such a limited supply of records it is probable that data for one species in the wild is being compared to data for another species in experimental or captivity conditions. This must be taken into consideration because it is possible for the same species to have different susceptibilities under natural or experimental situations [12]. For example, species susceptibility data could be reporting increased sensitivity in one species due to experimental infection by subcutaneous inoculation as opposed to the more likely natural oral route of infection, as is the case with a lethal dose study of the Vollum strain in sheep [12]. Likewise, Sterne found that cattle are extremely vulnerable to infection in

nature, yet resistant to experimental infection, while sheep are readily infected experimentally but not so easily infected in nature. Sterne also observed goats as even more vulnerable than sheep in experimental infections despite accounting for fewer cases of infection in nature [12]. Variability between experimental and natural conditions could be a result of many different factors, such as reduced immunogenicity of *B. anthracis* strains on subculture or variations in the infectious dose of different *B. anthracis* strains, the latter of which has not yet been studied in any species except mice [12,23–25]. It may be beneficial to complete some preliminary interspecies variability studies with the Sterne strain as that also has variable effects that could correlate with variability in fully virulent strains. The difference in the immunogenicity may be due to species specific reactions to saponin, but Colorado Serum, the North American distributor of the Sterne vaccine, has observed bison establishing a better immune response after a 2 ml dose compared to the generally recommended 1 ml dose whereas cattle have a good anamnestic response with a 3 week booster dose (Berrier 2019, personal communication).

1.2.1. Ruminants

Ruminants are by far the most susceptible of the herbivores and are generally exposed by ingesting or inhaling anthrax spores while grazing. Particularly susceptible wildlife species include deer, bison, gnu, gazelles, kudu, antelopes and reindeer [8]. For the sake of comparison, domesticated species, in descending order of susceptibility, are cattle, sheep, horses, pigs, goats and camels [8]. Anthrax in ruminants may be acute

febrile infections without localization or hyperacute infections, with sudden death occurring before any clinical signs are observed [8,12]. Hyperacute infections are most commonly reported in sheep, which can die in as little as a few minutes, however this data point is likely under experimental conditions, as discussed above [8]. In the event of an acute infection, signs may include loss of appetite, ruminal stasis, rapid breathing, congested and/or hemorrhaging mucous membranes, high fever, cutaneous edema, muscle tremors and convulsions, extravasation of blood from body openings, and eventually death after 2-3 days due to septicemia [8]. The most documented anthrax cases in North America are in white-tailed deer in the southern US and in bison in northern Canada [12,18,20,26,27].

Within the ruminant suborder there is a great amount of variability regarding interspecies susceptibility [12]. One theory to explain the inconsistency is the difference between grazers and browsers. Grazers, such as bison, wildebeest and zebra have a tendency to pull plants out of the ground and ingest a lot of soil while grazing. In contrast, browsers, like kudu and white-tailed deer, take in very little soil by feeding on leaves and shoots of high-growing, woody plants. Some have suggested that anthrax may have a greater influence on grazers because their feeding method is more likely to expose them to anthrax spores in the soil [8,12]. At first, it seems like a plausible explanation for the disease burden in bison, wildebeest and zebra but it doesn't account for the high infection rates in kudu and white-tailed deer. In Etosha National Park, grazing zebras are more frequently affected than browsing kudu, yet in Kruger National Park, browsing kudu are more frequently affected than grazing zebra [21,28]. This

specific example directly opposes the browsers vs. grazers explanation but another plausible explanation could be found in the fly transmission pathway phenomena.

Previous studies have associated anthrax transmission with necrophagic fly transmission pathways in Kruger National Park and the hyper enzootic range in western Texas [18,29,30]. Necrophagic (blowflies) flies feed on spore-infested carcasses, then relocate to nearby vegetation to vomit excess fluid and defecate. Both actions release enough spores on the vegetation that eventual browsers, such as kudu or white-tailed deer, can be infected with anthrax themselves. In this way, blowflies act as case multipliers of anthrax. Since this phenomenon has only been observed (so far) in Kruger, but not Etosha National Park, it's possible that blowflies are responsible for the higher anthrax prevalence in Kruger kudus than in Etosha kudus [12,21].

The haemophagic (biting) fly pathway is another factor to consider with regard to ruminant species infection variability [26]. This space multiplier phenomenon involves biting flies feeding on infected or dead animals and then transmitting spores to a healthy animal via contaminated mouthparts [21]. Incidence rates in one species may be higher than another due to factors such as species behavior or vector feeding preferences, but it could also be attributed to differences in animal coat patterns. For example, it is currently believed that the primary function of zebra stripes is to prevent the attack of biting flies, thus it could be postulated that for one reason or another the biting flies in Etosha are not as deterred by the zebra stripes as they are in Kruger [31].

Unfortunately, these fly transmission pathway theories can also only partially justify contradictions in ruminant susceptibility. In the enzootic range in western Texas

where the fly transmission pathways are active, both goats and white-tailed deer are browsers and they both feed in the same areas, yet there is a strong infection bias towards white-tailed deer leaving goats unaffected [12,18,30]. A possible, but as of yet unexplored, theory to account for this variation in susceptibility may be related to *B. anthracis* strain differences, however it seems unlikely considering isolates from similar geographic locations are nearly identical [12,32]. Aside from species specific immune responses to the same strain, other plausible explanations may be related to blowfly, white-tailed deer and goat preferences. For example, blowflies may have a host preference for the blood meal, as is observed in blood-feeding mosquitoes [21,33]. Blowflies may also have a vegetation preference for their post-blood meal shenanigans, similar to mosquitoes that have vegetation preferences for their daytime resting [34]. If these preferences happen to correlate with different browsing tastes between white-tailed deer and goats, then it may reveal a plausible explanation for the infection bias.

1.2.2. Equines

The next most susceptible group of animals are equines typically displaying acute infections and dying after 2-3 days, although recovery is possible [12]. Domestic horses generally develop colic and intestinal lesions, muscle tremors, high fever, rapid breathing and heart rate, cyanosis and cutaneous edemas in cases transmitted by biting flies [8,12]. Currently there is a lack of information about anthrax in wild equine species in North America as the most recent clinical course description available from 1959 is likely from a domestic horse in South Africa [12]. However, as there are several wild

herds within the North American enzootic regions mentioned previously [12,35–37] it is possible that anthrax infections do occur in these herds but are not witnessed or reported. As for equine species in African countries, there is sufficient data available discussing anthrax prevalence in zebras but nothing describing the clinical course of infection. Thus, it is believed that infected equids in North America and infected zebras in Etosha or Kruger National Park would exhibit similar clinical signs as observed in domestic horses [8,12,38,39].

1.2.3. Swine

Swine are considered somewhat resistant to anthrax infections [8,12], which may have developed as an evolutionary adaptation associated with their rooting behavior. It is evidenced by localized infections, manifesting as pharyngeal or intestinal porcine anthrax [12]. The pharyngeal form is associated with scavenging or feeding of carcasses and would be the most probable cause of infection in feral hogs [12]. The intestinal form would likely be limited to domesticated swine as it is believed to be associated with contaminated mineral supplements [12]. The pharyngeal form is characterized by ulcerative stomatitis, laryngitis, fever, cyanosis and edematous swelling of the parotid region that may extend to the neck and chest resulting in anorexia and dysphagia [8,12]. The intestinal form is less noticeable, and animals frequently recover after experiencing some anorexia, vomiting, diarrhea or constipation [8,12]. When the symptoms are severe, death can occur within 3-7 days but for animals that only experience retropharyngeal or mesenteric lymph node inflammation, recovery is common [8,12].

Despite the apparent resistance, herd outbreaks can still cause significant mortalities [8,12]. Again, descriptive epidemiological data is very limited but considering anthrax has been observed in warthogs in Kruger National Park and the Serengeti, as well as in wild boar in the Ukraine, it is likely that feral hogs across North America can be infected with anthrax and would undergo similar courses of infection [4,38,40,41].

1.2.4. Carnivores

In general, carnivores are resistant to anthrax infection, but infection, spontaneous recovery and/or death can still occur [8,12]. If infected, they show signs of acute gastroenteritis and oro-pharyngitis after consuming large amounts of infected meat [8]. Other cases have reported anorexia, lethargy, unilateral facial and/or submaxillary swelling, labored breathing and discharge (sometimes bloody) from natural orifices [12,42]. Resistance in wild carnivores in enzootic areas results from acquired humoral immunity from frequent exposure to carcasses of anthrax victims [43]. Interestingly, in African national parks, carnivore antibody titers are reflective of their habits and of anthrax activity in the area, but data such as this is not available for North American carnivore species [12]. Of the minimal data that is available, there is one report of an anthrax mortality in a mountain lion [44]. Other authors observed multiple wolf interactions with infected carcasses. Some studies confirmed survival, but not necessarily absent infection, in exposed wolves, yet others neglected to investigate it [14,20,45]. Anthrax data from African carnivores can be loosely applied to North American carnivores, but inconsistencies from African lions and cheetahs may

discourage this. For example, despite the presumed resistance to infection, African lions have experienced increased anthrax infections possibly as a consequence of simultaneously increased incidences of canine distemper which damages the immune system [12,43]. Also, cheetahs in Namibia appear to be much more susceptible to anthrax, likely because they don't scavenge as other carnivores do and therefore can't build up immunity [46].

1.2.5. Other animals

As discussed earlier, anthrax causes the most infections and mortalities in ruminants, yet it should be expected that after thousands of years of existence the full list of species affected by anthrax is extensive. A few additional highlights are discussed here, but a complete list of species reported is available in the World Health Organization's most recent edition of the anthrax guidelines, *Anthrax in humans and animals* [12].

Unsurprisingly since they are our closest genetic relatives, anthrax or anthrax-like infections have been reported in several species of great apes [47–49]. Regarding other species especially close to humans, on an emotional scale as opposed to the genetic scale, there are instances of anthrax affecting domestic dogs and cats [12,50]. Likely due to their carnivorous relatives, they are generally considered resistant to infection, so it typically isn't lethal but can manifest as inflammation and swelling of the throat, stomach, intestines, lips, jowls, tongue and gums [12].

In 1988-1989, a large outbreak of anthrax in hippopotami in Zambia was reported, interestingly, at the same time as an increased mortality rate in crocodiles [12,51]. While the added deaths were not investigated bacteriologically and crocodile experts did not believe they were related to anthrax, previous reports of potential anthrax infections or associated deaths in other cold-blooded animals suggest that it may be an area to consider in the future as we strive to characterize wildlife anthrax [12].

Birds are another non-mammalian species to contemplate. Sterne claimed avian anthrax usually resulted in sudden death, or with less acute infections, he observed carbuncular lesions on the comb and/or extremities [12]. Granted, the data and support are lacking, but the reliable sources discuss anthrax in ostriches in Etosha National Park, eagles in captivity and a vulture in Kruger National Park, among others [4,12,52,53]. Not only are birds possibly susceptible to anthrax, they are also suspected of transmitting spores various distances as a mechanical vector for the disease [12,28,54].

1.3. Anthrax ecology

Direct horizontal transmission of anthrax between infected and healthy animals of the same or different species is not common [8], so when discussing wildlife anthrax is it essential to also review anthrax ecology. Following a lethal infection, vegetative cells in an anthrax carcass will sporulate upon exposure to oxygen [8,21]. Of course, it is rare for carcasses to be left completely undisturbed in nature, so it can be expected that some subsequent exposure or infection may result from carcass scavenging. If the

carcass remains are not disposed of properly, then carcass decomposition will return the anthrax spores to the environment to start the cycle of infection over again.

While waiting to infect the next host, anthrax spores survive best in semi-arid soils rich in calcium and organic matter with a pH between 6 and 8 [21]. The full timeline of spore dormancy in the environment remains unknown, with some suggesting there may be cycles of germination and sporulation outside of an animal host [21]. Conversely, spores recently thawed out of the Siberian permafrost suggesting that these soil types and germination/sporulation cycles may be irrelevant to spore endurance [55]. Others have suggested that spores may be involved in plant establishment and growth rates which could promote spore transmission [56]. These examples just barely skim the surface of the available research in this area, however more research is always needed to fully understand the spore and soil interaction, which could eventually lead to better anthrax landscape management and reduced outbreaks.

Anthrax outbreaks have also been linked to water movements as it is believed that ground water can carry spores to the surface, even if a carcass is deeply buried [8]. On a larger scale, rainwater can both move spores down into the soil and stir spores up in the soil and transmit them to another area in run-off [21]. The same is also possible in run-off from un-buried carcasses or blowfly contaminated vegetation. This relationship with water encourages a seasonal pattern of anthrax outbreaks that are more frequent after a rainy spring followed by a hot, dry summer as mostly recently experienced this year (2019) in Texas [4,19,21,40,57,58]. Coincidently, rainy weather encourages increased hatching of biting flies [4]. It is also highly reasonable to postulate that water

has some other undiscovered effect on the anthrax spore cycle, further emphasizing the importance of anthrax ecology in predicting and preventing anthrax outbreaks in wildlife.

1.4. Conclusion

Bacillus anthracis is an ancient bacterium that has plagued humans, livestock and wildlife for thousands of years. While most of its effects are not readily noticeable today, anthrax has had a significant role in shaping human and animal history. Development of an effective livestock vaccine has contributed to a global reduction in the disease for humans and livestock, but wildlife remains unprotected. There is also very little information regarding the influence anthrax has on wildlife worldwide and we are unfamiliar with how and why anthrax affects one species more than another. Furthermore, the ecology of anthrax is exceptionally complex and presents an infinite number of questions that can only be answered by more research. The threat of bioterrorism has dominated most research related to this disease and the remaining resources are presumably not sufficient for adequately characterizing wildlife anthrax and anthrax ecology. A global effort should be made to ensure resource availability for wildlife anthrax research so that eventual programs can be established for wildlife conservation purposes.

2. *BACILLUS ANTHRACIS* STERNE STRAIN 34F2 VACCINE ANTIBODY DOSE RESPONSE BY SUBCUTANEOUS AND ORAL ADMINISTRATION*

2.1. Introduction

Anthrax is an ancient, zoonotic disease caused by the spore forming bacterium, *Bacillus anthracis*. It gained public attention as a potential bioweapon because in its dormant spore form it is uniquely suited to survive for decades in nature's harshest environments but the importance of anthrax as a zoonosis has been undervalued [8,9]. Since its first documented outbreak over three millennia ago, anthrax has utilized livestock and human hosts to spread all over the world while proving itself to be an economically important disease by causing large scale mortalities, with one outbreak resulting in the deaths of up to 60,000 animals [3]. Luckily, the discovery of the pXO2 negative strain in 1937 resulted in the live attenuated, non-capsulated strain, *B. anthracis* Sterne strain 34F2 (Sterne spores) [6]. Shortly thereafter, the Sterne vaccine was developed by suspending Sterne spores in saponin and routine parenteral vaccination of domesticated livestock with the Sterne vaccine ever since has prevented additional livestock mortalities of this scale [23].

Unfortunately, the same cannot be said for free-ranging wildlife where large outbreaks still occur. In the last 82 years since the Sterne strain was discovered, national parks and wildlife reserves have become more popular, more widespread and more valuable to locals and tourists worldwide [59,60]; however, anthrax prevalence is

undetermined in these areas so it is probable that additional smaller outbreaks occur more frequently without proper documentation [4]. For example, specifically in the United States, the Edwards Plateau region of Texas spans more than 1.5 million acres and experiences yearly outbreaks that can be exacerbated by cool, wet weather followed by hot, dry conditions [21]. This same region of Texas also happens to include upwards of 68% of the state's nearly 500 exotic animal reserves that are important economic and conservation resources, according to a 1992 report by Texas Parks and Wildlife Department [61]. While environmental conditions may help predict anthrax epizootics, the predictability isn't beneficial if the only method of anthrax prevention is to individually inject thousands of animals ranging freely over thousands of acres [19].

The resilience of the anthrax spore ensures that there will always be sporadic large-scale outbreaks in wildlife along with more common small-scale losses, and currently management options are limited. While surrounding livestock can be quarantined and vaccinated by subcutaneous injection with the commercially available formulations of the Sterne vaccine, this is impossible with free-ranging wildlife. Also, animal carcasses need proper disposal to prevent further spread of infection [12]. The best method of disposal is incineration on site but this requires a great amount of fuel, is expensive and time consuming, and may not be tolerated when the risk for wildfire is high, as it often is when outbreaks occur [4]. The inability to vaccinate animals coupled with poor outbreak management and documentation practices can result in spore-laden carcass piles that further exacerbate the risk of infection (Figure 2.1).

Figure 2.1. Carcass pile discovered on a previously poorly managed ranch in Uvalde, Texas [62, Reprinted]**. Ranch is now under new management and carcass pile has been disposed of properly. Photo courtesy of G. Staack.**

The most common method of anthrax prevention is to prophylactically administer wildlife feed laced with an antibiotic, such as chlortetracycline [4]. The long-term effectiveness of this strategy is unconfirmed, and investigations suggest it is significantly less effective than adequately timed vaccination [63,64]. Furthermore, this practice is illegal and recently under more scrutiny with implementation of the Veterinary Feed Directive which prohibits the off-label use of antibiotics in feed [65]. In

an effort to protect their herds from inevitable anthrax outbreaks, there are anecdotal accounts of wildlife and ranch managers resorting to pouring the commercial vaccine over feed with no solid scientific evidence to suggest it is truly an effective vaccination strategy (Davis 2019, personal communication), therefore the present study was designed to compare the immunogenicity of the Sterne vaccine at three doses following subcutaneous and oral vaccination.

2.2. Materials and Methods

2.2.1. Mice

A total of 64, four- to six-week-old female BALBc/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival at the animal facility, mice were randomly distributed into eight groups (Table 2.1) and allowed to acclimate for at least a week prior to any manipulation. All animal care and experimental procedures were performed in compliance with the Texas A&M University Institutional Animal Care and Use Committee regulations.

Route	Group (n=8)	Inoculation Volume	Spores/ ml	Spores/Mouse	Blood Collection (days post-vaccination)
SC	Control	0.2 ml	PBS	-	0, 3, 8, 15, 22, 31, 43, 55
	Sterne Dose 1	0.2 ml	$5x10^4$	$1x10^4$	0, 3, 8, 15, 22, 31, 43, 55
	Sterne Dose 2	0.2 ml	$5x10^6$	$1x10^6$	0, 3, 8, 15, 22, 31, 43, 55
	Sterne Dose 3	0.2 ml	$5x10^8$	$1x10^8$	0, 3, 8, 15, 22, 31, 43, 55
Oral	Control	0.2 ml	PBS	-	0, 3, 8, 15, 22, 31, 43, 55
	Sterne Dose 1	0.2 ml	$5x10^4$	$1x10^4$	0, 3, 8, 15, 22, 31, 43, 55
	Sterne Dose 2	0.2 ml	$5x10^6$	$1x10^6$	0, 3, 8, 15, 22, 31, 43, 55
	Sterne Dose 3	0.2 ml	$5x10^8$	$1x10^8$	0, 3, 8, 15, 22, 31, 43, 55

SC = subcutaneous, Sterne = *Bacillus anthracis* Sterne strain 34F2 spores in saponin

Table 2.1. Overview of the vaccination groups used in this study to evaluate the Sterne Vaccine in BALBc/J mice [62, Reprinted]**.**

2.2.2. Bacterial strains

All bacteria used in this experiment were live attenuated *B. anthracis* Sterne strain 34F2 spores (Sterne spores) that were cultured from a new vial of the Anthrax Spore Vaccine (ASV) from Colorado Serum Company (Denver, CO, USA), the commercial producer of the Sterne vaccine in North America. Sterne spores were prepared following the protocol illustrated in Figure 2.2. Briefly, 5 ml of LB broth was inoculated with 200 µl of the ASV and cultured overnight at 37°C with shaking. The overnight growth was collected by centrifugation at 3800 rpm, resuspended in 1 ml of fresh LB broth, plated onto LB agar (100 µl per plate) and incubated at 37°C for 6 days to sporulate. Sterne spores were harvested from the plates and washed repeatedly with 25 ml of sterile water. The harvested Sterne spores were further purified by heating at

68°C for 1 hour and filtering through a 3.1 µm filter to kill and remove any remaining vegetative cells (Figure 2.3). Final Sterne spore concentrations were determined by serial dilutions and plating on LB agar and then final spore stocks were stored in sterile water.

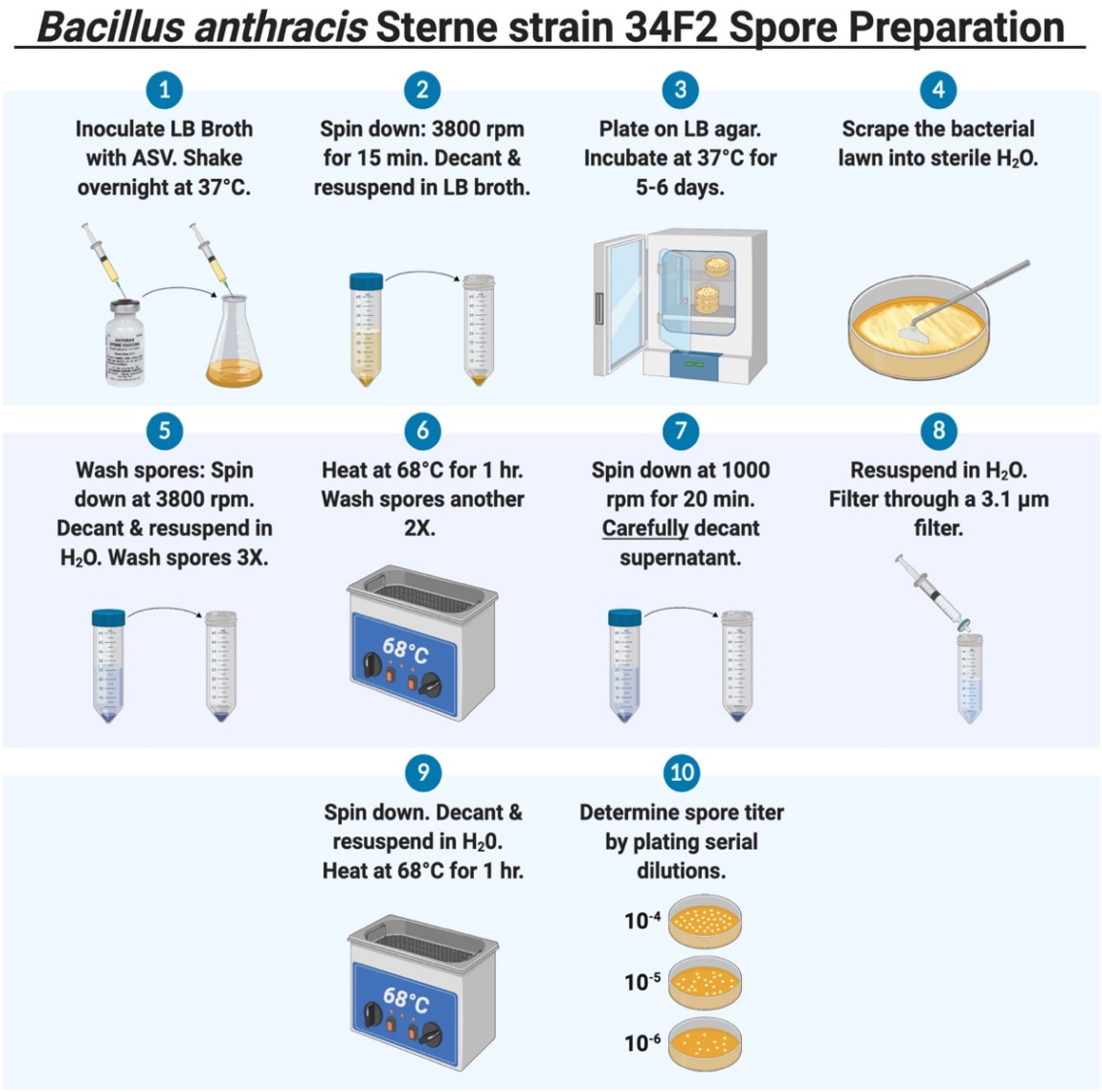

Figure 2.2. *Bacillus anthracis* Sterne strain 34F2 spore preparation for vaccination. Created with BioRender.com.

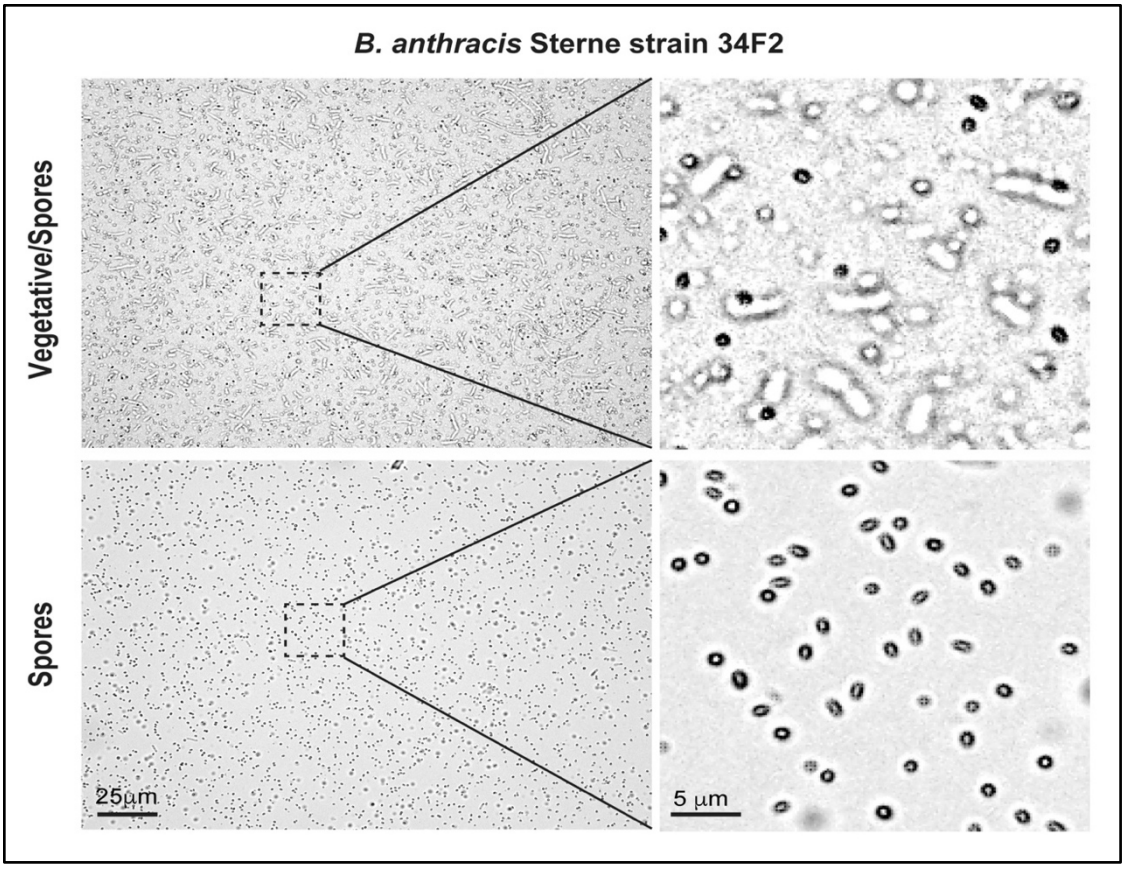

Figure 2.3. Heat treated and filter purified *Bacillus anthracis* Sterne strain 34F2 spores [62, Reprinted]. Representative brightfield images in the top panel show a mixture of vegetative and sporulated *B. anthracis* Sterne strain 34F2. Images in the bottom panel show pure spores devoid of vegetative bacteria obtained after heating at 68°C and filtering through a 3.1 µm filter. The magnified images in the right panel correspond to the segmented square selections in the left panel.

2.2.3. Vaccine preparation

The ASV is commercially distributed for use in cattle, sheep, goats, swine and horses with a recommended 1 ml dose of between $4x10^6$ and $6x10^6$ viable Sterne spores in saponin (Berrier 2016, personal communication). This experiment investigated the dose response to subcutaneous or oral vaccination with three doses of Sterne spores in saponin. The lowest dose was $5x10^4$ spores/ml, the middle dose was $5x10^6$ spores/ml and

the highest dose was $5x10^8$ spores/ml. For the purposes of this experiment, the dose of the ASV was assumed to be $5x10^6$ spores/ml and was used as the middle dose exactly as it was received from Colorado Serum Company. The lower and higher doses were made as similar as possible to the commercialized product to ensure that any variation in response was solely due to the different bacterial doses. Additional bottles of the ASV were centrifuged and filtered to remove all Sterne spores from the ASV, resulting in pure, commercial quality saponin. The purified saponin was plated on LB agar to confirm all commercial Sterne spores had been removed, and then it was used to resuspend lab-prepared Sterne spores at the target concentrations of $5x10^4$ and $5x10^4$ spores/ml.

2.2.4. Vaccination

Mice (n=8) were vaccinated subcutaneously or by oral gavage with 0.2 ml of sterile PBS or one of the following Sterne vaccine doses: $5x10^4$ spores/ml, $5x10^6$ spores/ml or $5x10^8$ spores/ml (Table 2.1). Antibody responses were evaluated in blood samples that were collected three to seven days prior to vaccination, three to five days after vaccination and then every seven to ten days for eight weeks.

2.2.5. Detection of anthrax-specific antibody levels

Serum was isolated from collected blood samples and analyzed by ELISA to quantify the anthrax-specific IgG levels induced by each vaccine dose following subcutaneous or oral vaccination. Corning 96-well EIA/RIA high binding plates

(Corning Inc., Corning, NY, USA) were coated with 100 ng per well of anthrax protective antigen (List Biological Laboratories Inc., Campbell, CA, USA) in carbonate bicarbonate buffer, pH 9.6. Plates were incubated at 37°C for 1 hour, then overnight at 4°C and used within four days. All plates were washed three times with phosphate buffered saline containing 0.5% Tween 20 (PBST) and then blocked for 1 hour at 37°C with 100 µl per well of 1% Bovine Serum Albumin (w/v) in PBST (1% BSA). After blocking, plates were washed and incubated with 100 µl per well of diluted serum at 37°C for 1 hour. Serum samples were diluted in 1% BSA at 1:3,000 for subcutaneously immunized mice and 1:500 for orally immunized mice. Plates were washed with PBST, then incubated at 37°C for 1 hour with 100 µl per well of Anti-Mouse IgG (H+L) Antibody (SeraCare, Milford, MA, USA) diluted 1:5000 in 1% BSA according to the supplier's recommendations. Plates were washed five times with PBST prior to application of 100 µl of TMB/E Substrate (Sigma-Aldrich, St. Louis, MO, USA). The reaction was stopped after 15 minutes by adding 100 µl of 0.5 M H_2SO_4. Optical density of each well was read at 450 nm using a Tecan Infinite F50 Plate Reader. Samples from each mouse, at each time point were run in duplicate. Absorbance values are reported as the average value for all duplicates in the vaccination group. Statistical analysis was performed using ANOVA followed by the Tukey-Kramer HSD test with p-values <0.05 considered significant (JMP Pro 14, Cary, NC, USA).

2.3. Results

2.3.1. Anti-anthrax antibody response

The anti-anthrax specific antibody titers in response to subcutaneous or oral vaccination with three doses of the Sterne vaccine were measured by ELISA. The mean absorbance values for each subcutaneous vaccination group are illustrated in Figure 2.4. All three vaccine doses stimulated a gradual increase in antibody levels with each time point and all measured antibody levels for the $5x10^4$ spores/ml and $5x10^8$ spores/ml groups are significantly different from control starting at 22 days post vaccination ($p<0.001$). A clear dose response can be observed when comparing the $5x10^4$ spores/ml group with the $5x10^6$ spores/ml and the $5x10^8$ spores/ml groups separately. The $5x10^6$ spores/ml group resulted in a faster antibody response that was significantly different from control starting at 15 days post vaccination, seven days earlier than the response from both the $5x10^4$ spores/ml and the $5x10^8$ spores/ml groups ($p<0.01$). The $5x10^6$ spores/ml group also had significantly higher antibody titers than the $5x10^8$ spores/ml group starting at 15 days post vaccination ($p<0.01$). In contrast, ELISA results from the oral vaccination groups did not reveal any antibody response following oral vaccination with any dose of the Sterne vaccine, despite using a dilution that was four times more concentrated than the subcutaneous serum (Figure 2.5).

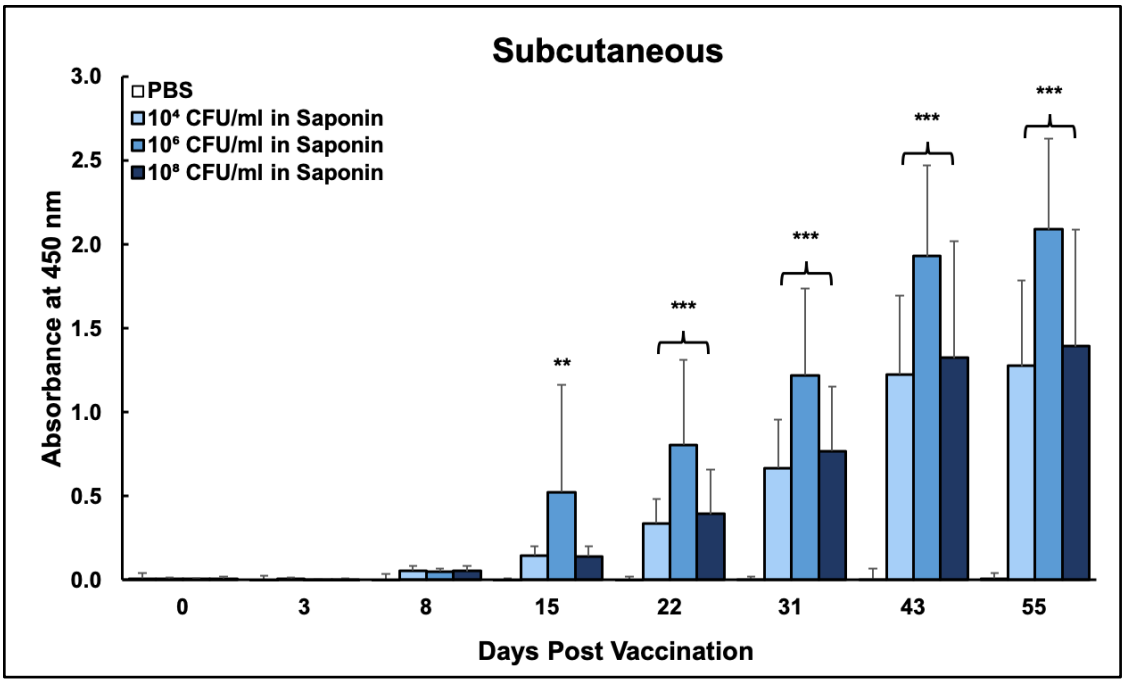

Figure 2.4. IgG responses against subcutaneously injected *Bacillus anthracis* Sterne strain 34F2 spores [62, Reprinted]. BALBc/J mice were inoculated with 0.2 ml of $5x10^4$ spores/ml, $5x10^6$ spores/ml or $5x10^8$ spores/ml of *B. anthracis* Sterne strain 34F2 spores in saponin. Control mice received 0.2 ml of sterile PBS. Blood samples were collected three to seven days prior to vaccination, three to five days after vaccination and then every seven to ten days for eight weeks. Serum was isolated from all blood samples and the antibody titer was determined by ELISA at a dilution of 1:3,000. Results are shown as average absorbance values + the standard deviations at 450 nm (, $p<0.01$ and ***, $p<0.001$).**

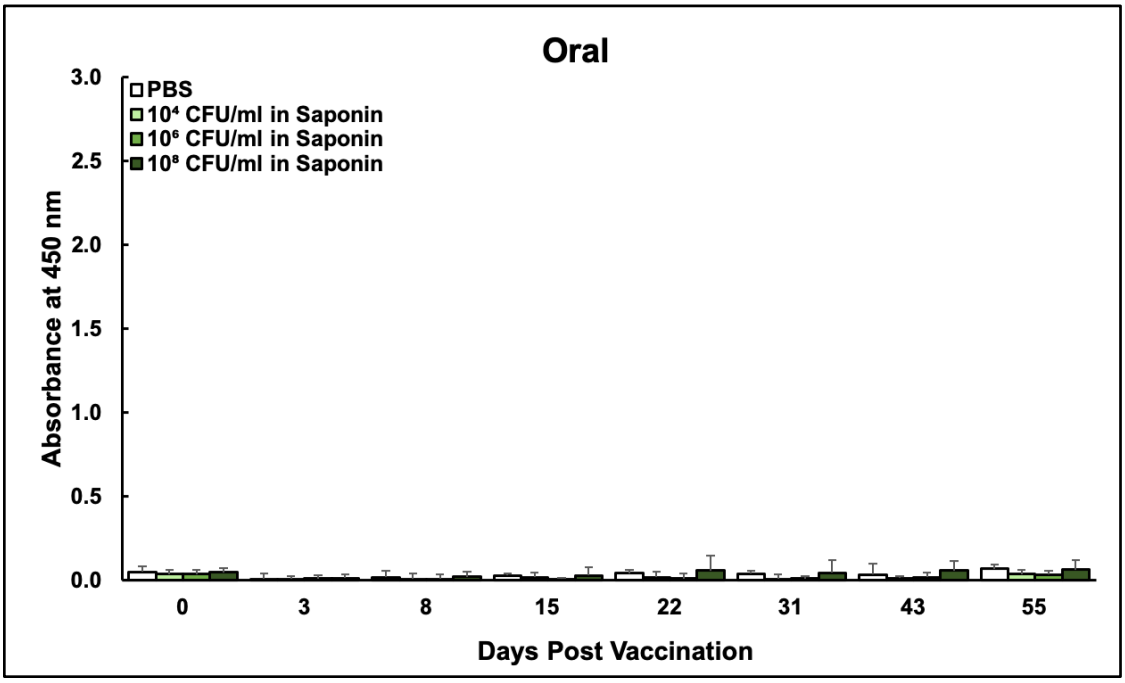

Figure 2.5. IgG responses against oral inoculation of *Bacillus anthracis* Sterne strain 34F2 spores [62, Reprinted]. BALBc/J mice were inoculated with 0.2 ml of $5x10^4$ spores/ml, $5x10^6$ spores/ml or $5x10^8$ spores/ml of *B. anthracis* Sterne strain 34F2 spores in saponin. Control mice received 0.2 ml of sterile PBS. Blood samples were collected three to seven days prior to vaccination, three to five days after vaccination and then every seven to ten days for eight weeks. Serum was isolated from all blood samples and the antibody titer was determined by ELISA at a dilution of 1:500. Results are shown as average absorbance values + the standard deviations at 450 nm (, $p<0.01$ and ***, $p<0.001$).**

2.4. Discussion

For over 80 years, the Sterne vaccine formulation of live attenuated *B. anthracis* Sterne strain 34F2 spores in saponin has been manufactured and distributed worldwide as a subcutaneous livestock vaccine. Results presented here confirm its ability to stimulate anthrax specific antibody production after subcutaneous injection with three different doses (Figure 2.4). Antibody titers from each of the three vaccine doses showed

gradual increases over time as expected. Interestingly, the middle dose, $5x10^6$ spores/ml in saponin, induced a faster antibody response by seven days and a stronger antibody response when compared to the higher dose, $5x10^8$ spores/ml in saponin. Normally a 100-fold increase in inoculated spores would be expected to induce the stronger antibody response, however the inoculant for the middle dose was used directly from a new ASV vial whereas the spores for the higher dose were prepared by culturing them out of an ASV vial. This discrepancy can likely be attributed to reduced immunogenicity on subculture of non-capsulated *B. anthracis* strains, such as the Sterne strain [12,23].

As evidenced by the antibody titers observed from the subcutaneously vaccinated mice, as well as decades of vaccination history, parenteral inoculation with Sterne spores can protect an animal from anthrax, however the Sterne vaccine has never been thoroughly evaluated as an oral vaccine [1,12,66]. Thus, the current study compared the antibody response of subcutaneously inoculated versus orally administered Sterne spores in mice. In contrast to the robust immune responses induced by the subcutaneous injections, no antibody response was detected in mice following oral administration in this study (Figure 2.5). Even inoculating mice with a lower or higher dose of Sterne spores failed to induce any antibody titer. A previous experiment with goats found a similar pattern when comparing subcutaneous injections and oral inoculation with Sterne spores mixed with scarifying agents. Upon secondary exposure to Sterne spores by subcutaneous injection, an antibody response was observed in the goats that were previously vaccinated orally [67] but it is likely this secondary response was due solely to the method of subcutaneous challenge.

All-encompassing data for anthrax infections in wildlife is essentially non-existent, but it is believed that anthrax is capable of affecting all mammals with varying degrees of morbidity and mortality [4]. Infections have been reported in over 50 African wildlife species, including threatened and endangered species such as the black rhinoceros, African elephant, cheetah and wild dog, with dozens more known to be susceptible [4,28,40,68,69]. In North America, anthrax has been documented in white-tailed deer, elk, bison, bears, and other native and exotic species [13,14,18]. Inconvenient outbreaks in wildlife cause economic, ecological and conservational burdens worldwide [3,11,12]. Despite the availability of a safe and effective animal vaccine, a recently proposed model for the global distribution and suitability of anthrax found that most livestock and wildlife at risk of exposure are not vaccinated [11]. This may be due to the fact that most commercial animal vaccines use the same formulation developed by Max Sterne in 1939 which is outdated, can cause adverse reactions, requires yearly boosters and requires refrigerated storage [70,71]. The most prominent reason, however, is because the existing method of subcutaneous injection with the Sterne vaccine is not a feasible method of vaccination for free-ranging livestock and wildlife, so many anthrax vaccine reviews have called for a new anthrax vaccine, specifically an oral anthrax vaccine [4,12,70]. Sharing in that opinion are ranch managers in the Edwards Plateau region of Texas who cannot practically vaccinate all of their animals by injection, so some have improvised by pouring the Sterne vaccine over feed, even doing so every other week for the entire spring before the typical anthrax season by one account. While this specific narrative claims that this tedious vaccination

strategy appeared to be effective (Davis 2019, personal communication), the results of the current study suggest it may have been circumstantial. Desperate attempts such as these to protect their herds, coupled with the absence of antibody titer following oral inoculation in this study, further emphasize the urgent need for an anthrax vaccine specifically formulated for oral use in wildlife.

In the time since the Sterne vaccine was discovered, the human population has nearly tripled and interactions with wildlife have drastically changed [72]. Humans, domesticated livestock and wildlife have become more interconnected thus increasing the economic and intrinsic value of wildlife and overall wildlife health [73]. Sensitive wildlife conservation efforts are in a constant state of worry because they frequently involve unvaccinated animals on anthrax prone landscapes [11]. Furthermore, the success of wild animal reserves in these same areas can be limited by anthrax outbreaks since non-existent vaccination policies are known to affect the epidemiology and rate of transmission to humans [74]. Given the unsatisfactory performance of the orally administered Sterne vaccine in this study, future research efforts must focus their attention on developing a safe and effective oral anthrax vaccination protocol for use in wildlife as it will be the key to preventing anthrax epidemics in wildlife, as well as maintaining public and global wildlife health.

3. PROTECTIVE ANTIBODY RESPONSE FOLLOWING ORAL VACCINATION WITH MICROENCAPSULATED *BACILLUS ANTHRACIS* STERNE STRAIN 34F2 SPORES

3.1. Introduction

Anthrax infections have plagued humans and animals alike for millennia, possibly even causing the fifth and sixth plagues of Egypt [4]. The causative agent, *Bacillus anthracis*, has been studied since the beginning of microbiology but even after more than a century of scientific studies, the anthrax vaccination field has made little progress, especially with veterinary anthrax vaccines [1,4]. A recent study consolidated data from the last twenty years and found a worldwide distribution and suitability for environmental anthrax with reports of the disease on every habitable continent, and yet, most animals remain unvaccinated [11]. While it may be prudent to mention that the incidence of human infection can be decreased with adequate livestock vaccination policies, it should also be of great concern that free-ranging livestock and wildlife populations worldwide are unprotected against anthrax outbreaks that can cause catastrophic harm to sensitive wildlife conservation efforts [3,5,11,74].

A fully virulent *B. anthracis* cell contains two main virulence plasmids, pXO1 and pXO2. The current veterinary vaccine, historically referred to as the Sterne vaccine, uses *B. anthracis* Sterne strain 34F2 spores (Sterne spores) that have naturally lost the pXO2 plasmid and therefore can no longer produce poly-γ-D-glutamic acid capsule, also known as the anti-phagocytic capsule [5]. The original formulation of the Sterne

vaccine, which is still in use today, consists of Sterne spores suspended in saponin and has been used to vaccinate domesticated livestock against anthrax since its discovery in the late 1930's [4,12]. Despite decades of successful protection, the Sterne vaccine is outdated and impractical. It's been known to vary in its potency and cause adverse reactions, occasionally even death [70]. Logistically speaking, the Sterne vaccine is distributed as a subcutaneous injection which is a highly impractical method of vaccination for free-ranging livestock and wildlife [4]. Lack of a reasonable vaccination method is especially problematic for wildlife managers considering there are yearly anthrax outbreaks in national parks and other wildlife areas worldwide [8,21]. Even with these yearly outbreaks, the anthrax spore distribution in these areas is undetermined so it isn't possible to vaccinate wildlife based on an estimated risk of exposure [75]. The simplest way to protect wildlife in these areas would be via oral vaccination. However, after results from a previous study demonstrated that the Sterne vaccine is incapable of eliciting an immune response following oral vaccination the urgent need for an effective oral anthrax vaccine for wildlife has never been more evident [62].

Other research groups in the oral anthrax vaccination field have reported encouraging results from vaccines expressing a recombinant form of anthrax protective antigen in a variety of bacterial, viral or plant-based expression systems [76–79]. Any progress toward the development of an oral anthrax vaccine is a great achievement, but studies have suggested that anthrax spore associated antigens may also make important contributions to vaccine induced protection [80]. Since it is possible that exposure to a single recombinant antigen may not stimulate sufficient immune activity to protect

against fully virulent exposure [80], it may be advantageous to maintain the live attenuated format of the Sterne vaccine, but adapt it for oral use.

Recent experiments and reviews have emphasized the seemingly unlimited applications of controlled release vehicles, particularly with alginate encapsulation of bacteria [81–83]. Alginate is naturally indigestible in mammalian systems and can be implemented as a natural controlled release vehicle [84]. The beneficial characteristics of alginate can also be enhanced with the addition of a proteolysis resistant protein such as vitelline protein B (VpB), a non-immunogenic, eggshell precursor protein isolated from the parasite *Fasciola hepatica* [85]. Like alginate, VpB is resistant to enzymatic and chemical degradation and can further extend the already slow erosion of the alginate capsule [86]. Prior research in our laboratory has applied this encapsulation method to *Brucella spp.* and reported stronger, extended immune responses that correlated with enhanced protection against wild type challenge [87–90].

In the current study, we examined the immunogenicity of microencapsulated Sterne spores and observed a pronounced increase in the resulting antibody response from both subcutaneous and oral vaccination. Moreover, an *in vitro* toxin challenge revealed that the observed antibody titer was protective following oral vaccination suggesting that with further optimization, microencapsulated Sterne spores can be developed into an alternative anthrax vaccine formulation capable of efficient and protective vaccination of free-ranging livestock and wildlife.

3.2. Materials and Methods

3.2.1. Preparation of Sterne spores

All bacteria used in this experiment were cultured from a vial of the Anthrax Spore Vaccine (ASV) from Colorado Serum Company (Denver, CO, USA), the North American commercial producer of the Sterne vaccine. The ASV consists of live attenuated *B. anthracis* Sterne strain 34F2 spores in saponin which were isolated and cultured as described previously [62]. Briefly, a small volume of Luria Broth (LB) was inoculated with the ASV and cultured overnight at 37°C with shaking. The growth was pelleted by centrifugation at 3800 rpm for 15 minutes, resuspended in LB broth , then plated onto LB agar and incubated at 37°C for 6 days to sporulate. The full bacterial lawns were harvested from the plates and washed repeatedly with sterile water. Remaining vegetative cells were removed by heating at 68°C for 1 hour and filtering through a 3.1 μm filter resulting in a suspension of pure Sterne spores. The final Sterne spore concentration was estimated by plating serial dilutions on LB agar.

3.2.2. Sterne spore response to simulated gastrointestinal environments

B. anthracis Sterne strain 34F2 spores were exposed to simulated gastric or intestinal fluids (GI fluids) to fully comprehend the obstacles to oral vaccination. Simulated gastric fluids consisted of 0.2% (w/v) NaCl and were adjusted to pH 2 and 5 with 1 M HCl to mimic the range of pHs in a non-fasted stomach [91]. Simulated intestinal fluids were 0.68% (w/v) K_2HPO_4 adjusted to pH 7 and 8 with 0.2 M NaOH [91]. A Sterne spore stock solution was prepared at an arbitrary concentration of $3.4x10^6$

spores/ml. From this stock solution, 0.2 ml was used to inoculate $6.8x10^5$ total Sterne spores into 5 ml of each GI fluid and MOPS Buffer (10 mM MOPS, 0.85% NaCl [pH 7.4]) as a control for future vaccine conditions. The starting spore titer of each inoculated GI fluid was determined by plating serial dilutions on LB agar, then the samples were placed on an orbital shaker at 37°C. Sterile water, phosphate buffered saline (PBS) and LB broth were also inoculated as negative and positive controls (data not included here). After an overnight incubation, the resulting spore concentration in the GI fluids was determined by plating serial dilutions. Data are reported as the average total recovered colony forming units (CFU) from each buffer.

3.2.3. Vaccine preparation

3.2.3.1. Sterne vaccine

The ASV is distributed by Colorado Serum with a recommended 1 ml dose of between $4x10^6$ and $6x10^6$ viable Sterne spores in saponin for use in cattle, sheep, goats, swine and horses [62]. This dosage range was simplified to $5x10^6$ spores/ml for the purposes of this experiment and was used exactly as received from Colorado Serum Company.

3.2.3.2. Microencapsulation of *B. anthracis* Sterne strain 34F2 spores

Two different microcapsule vaccine formulations with the VpB shell were prepared for the mouse experiments in this study: (i) microcapsules containing $5x10^6$ spores/ml (Spore Capsules) and (ii) Empty microcapsules (Empty Capsules). A single

"encapsulation batch" produced 6 ml of microcapsules whereas the microcapsule vaccine was a 1:1 solution of the 6 ml of capsules suspended in 6 ml of MOPS buffer with a final volume of 12 ml, therefore the total number of Sterne spores to encapsulate was determined as shown in Figure 3.1.

Microencapsulation Bacterial Load Calculations

Strategy 1:

1. $Target\ Dose\ per\ Mouse:$
   $5x10^6\ spores/ml = \frac{X\ spores}{0.2\ ml\ vaccine/mouse} \rightarrow 1x10^6\ spores/ml$

2. $Total\ Number\ of\ Spores\ Required:$
   $1x10^6\ spores/mouse\ \times\ 20\ mice = 2x10^7$

3. $Total\ Vaccine\ Volume\ Required:$
   $0.2\ ml/mouse\ \times\ 20\ mice = 4\ ml$

4. $Number\ of\ spores\ needed\ for\ a\ full\ encapsulation\ batch:$
   $\frac{2x10^7\ spores}{4\ ml\ vaccine} = \frac{X\ spores}{12\ ml\ final\ microcapsule\ vaccine} \rightarrow 6x10^7 spores$

Strategy 2:

1. $Number\ of\ vaccine\ doses\ being\ produced:$
   $\frac{12\ ml\ final\ microcapsule\ vaccine}{0.2\ ml\ vaccine/mouse} = 60\ vaccine\ doses$

2. $Total\ Number\ of\ Spores\ Required:$
   $1x10^6\ spores/dose\ \times\ 60\ doses = 6x10^7$

Figure 3.1. Explanation of vaccine dose calculations for microencapsulation.

Microcapsules were prepared similar to previous studies [89]. Sodium alginate (NovaMatrix, Sandvika, Norway) was dissolved in MOPS buffer to a concentration of

1.5% (w/v) alginate. To make the Spore Capsules, $6x10^7$ Sterne spores, as determined by the most recent stock titer results, were resuspended in 1 ml of MOPS buffer and then mixed with 5 ml of 1.5% (w/v) alginate solution. Microcapsules were formed using a Nisco Encapsulator VARV1 unit (Nisco Engineering AG, Zurich, Switzerland). The spore + alginate solution was extruded through a 170 μm nozzle released directly into 40 ml of cross-linking solution (100 mM $CaCl_2$, 10mM MOPS) and stirred for 30 minutes. The capsules were thoroughly washed with MOPS and then coated in the proteolysis resistant protein coating by stirring for 30 minutes in 15 ml of cross-linking solution containing 1 mg of VpB and 7 mg of poly-L-lysine (PLL) in 100 mM $CaCl_2$, 10 mM MOPS. After another washing with MOPS, the capsules received an outer shell of 0.03% (w/v) alginate by mixing for 5 minutes. Final microcapsule vaccines (Figure 3.2) were washed and resuspended 1:1 in MOPS for storage at 4°C until use within 4 days. The Empty Capsules were also prepared as above but without any Sterne spores being added to the alginate. The resulting dose of viable Sterne spores in the microcapsule vaccine was determined by dissolving 1 ml of capsules in 50 mM sodium citrate, 0.45% NaCl, 10 mM MOPS prior to permanent cross-linking with the VpB shell.

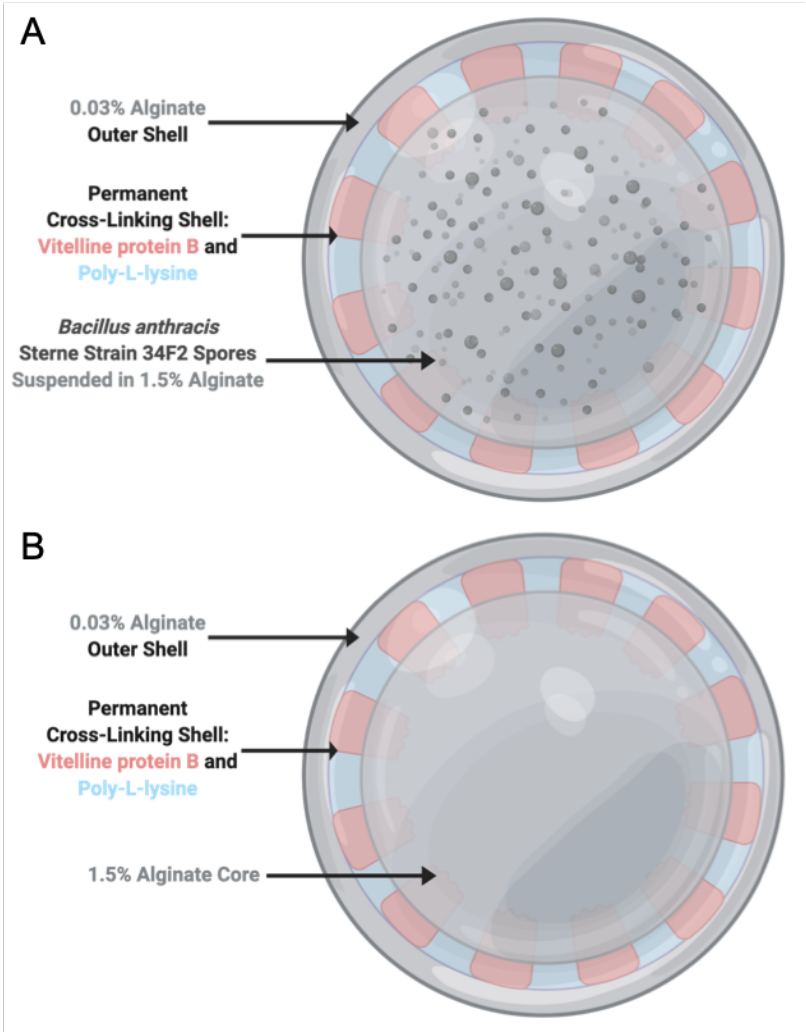

Figure 3.2. Illustration of microcapsules produced for vaccination of BALBc/J mice. (A) Spore Capsules loaded with Sterne spores and coated with the VpB and poly-L-lysine shell and (B) Empty Capsules coated with the VpB and poly-L-lysine shell. Created with BioRender.com.

3.2.4. Mouse immunizations

Female BALBc/J mice between four and six weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival at the animal facility, mice were randomly distributed into six groups of five mice each (Table 3.1) and allowed to acclimate for at least a week prior to any manipulation. All animal care and

experimental procedures were performed in compliance with the Texas A&M University Institutional Animal Care and Use Committee regulations (AUP # IACUC 2016-0112).

Mice were inoculated subcutaneously or by oral gavage with 0.2 ml of one of the three prepared vaccines: (i) Sterne vaccine, (ii) Spore Capsules and (iii) Empty Capsules (Table 3.1). All mice inoculated with either the Sterne vaccine or Spore Capsules received approximately $1x10^6$ spores/mouse. The Empty Capsules served as the unvaccinated control. Antibody responses were evaluated in blood samples that were collected three to seven days prior to vaccination and then every 10 to 14 days after vaccination for eight weeks.

Route	Group (n=5)	Inoculation Volume	Spores/ ml	Spores/ Mouse	Blood Collection (days post-vaccination)
SC	Empty Capsules	0.2 ml	-	-	0, 15, 31, 43, 55
	Sterne Vaccine	0.2 ml	$5x10^6$	$1x10^6$	0, 15, 31, 43, 55
	Spore Capsules	0.2 ml	$5x10^6$	$1x10^6$	0, 15, 31, 43, 55
Oral	Empty Capsules	0.2 ml	-	-	0, 15, 31, 43, 55
	Sterne Vaccine	0.2 ml	$5x10^6$	$1x10^6$	0, 15, 31, 43, 55
	Spore Capsules	0.2 ml	$5x10^6$	$1x10^6$	0, 15, 31, 43, 55

SC = subcutaneous
Sterne Vaccine = *B. anthracis* Sterne strain 34F2 spores in saponin
Spore Capsules = *B. anthracis* Sterne strain 34F2 microcapsules with PLL and VpB shell
Empty Capsules = Microcapsules with PLL and VpB shell (no bacteria)

Table 3.1. Vaccination groups to assess the efficacy of microencapsulated Sterne spores as an oral vaccine.

3.2.5. Detection of anthrax-specific antibody titers

Anthrax specific antibody levels were measured by ELISA as described previously [62]. High binding ELISA plates were coated with 100 ng per well of anthrax protective antigen in carbonate buffer, pH 9.6 and incubated at 37°C for 1 hour, then overnight at 4°C. The plates were washed 3-5 times with phosphate buffered saline containing 0.5% Tween 20 (PBST). This washing step was repeated between each of the following steps. Next, the plates were blocked for 1 hour at 37°C with 100 µl per well of 1% (w/v) bovine serum albumin in PBST (1% BSA). Serial dilutions of all serum samples were prepared in 1% BSA, loaded 100 µl per well and incubated for 1 hour at 37°C. The secondary antibody, Anti-Mouse IgG (H+L) (SeraCare, Milford, MA, USA) was diluted 1:5000 in 1% BSA and loaded 100 µl to a well with a 1 hour incubation at 37°C. TMB/E Substrate (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and the reaction was stopped after 12 minutes with the addition of 100µl of 0.5 M H_2SO_4. The optical density of all wells was read on a Tecan Infinite F50 Plate Reader at 450 nm. Samples from each time point, at each dilution were run in duplicate and are reported as average absorbance values for the vaccination group.

3.2.6. Lethal toxin neutralization assays

Toxin neutralization assays were performed to determine the ability of collected serum samples to inhibit the cytotoxicity of anthrax lethal toxin (LeTx) *in vitro* [77,78,92]. J774A.1 macrophages were cultured in Dulbecco's modified eagle medium (DMEM, Hyclone) with 10% (w/v) fetal bovine serum (FBS) and 1% (w/v) penicillin.

Upon reaching confluency, the cells were harvested and quantified using a hemocytometer, then brought to a final concentration of $5x10^4$ cells/ml. Cells were added to a 96-well flat-bottom tissue culture plate at 200 µl/well and incubated overnight at 37°C in 5% CO_2. LeTx was prepared by adding lethal factor (List Biological Laboratories Inc., Campbell, CA, USA) and protective antigen (List Biological Laboratories Inc., Campbell, CA, USA) to DMEM containing 10% FBS and no antibiotic at concentrations of 0.25 µg/ml and 0.1 µg/ml, respectively. The LeTx mixture was used to make serial dilutions of the collected mouse serum samples on a separate 96-well cell culture plate and then incubated for 1 hour at 37°C, 5% CO_2. The media was removed from the prepared macrophage plate and replaced with 100 µl/well of the serum LeTx mixture. After incubating for 4 hours at 37°C, 5% CO_2, 10 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Roche, Basel, Switzerland) was added to each well and incubated for another 4 hours at 37°C, 5% CO_2. Any remaining metabolically active cells reduced MTT, a yellow tetrazolium salt, to purple formazan crystals using NAD(P)H-dependent oxidoreductase enzymes. The insoluble formazan crystals were dissolved by adding 100 µl of solubilization solution (Roche, Basel, Switzerland) to each well and plates were incubated overnight at 37°C, 5% CO_2. The optical density of each well was read at 595 nm using a Tecan Infinite F50 Plate Reader. Cells that were exposed to only LeTx and no serum were used as a positive control. Cells that did not receive any LeTx or serum were used to determine 100% cell viability and 50% cell viability, also referred to as 50% protection. The LeTx neutralizing titer was defined as antibody titers resulting in 50% protection.

3.2.7. Statistical analysis

Differences between starting and ending titers for Sterne spore responses to GI fluids were determined by Student's t-tests with p-values <0.05 considered significant. Across all other experiments, results are expressed as mean values ± standard deviations for all replicates at each timepoint for each group. Statistical analysis was performed using ANOVA followed by the Tukey-Kramer HSD test with p-values <0.05 considered significant.

3.2.8. Surveying white-tailed deer serum for anti-anthrax antibodies

In an initial effort to estimate the risk of exposure in wildlife areas, we screened white-tailed deer serum outside of the Texas enzootic anthrax zone for antibodies against anthrax protective antigen. White-tailed deer serum samples were collected from live, presumably healthy animals in November 2018 at various locations on the East Foundation ranchlands. The seroprevalence of anti-anthrax protective antigen in collected serum samples was measured using a Sheep Anti-Anthrax Protective Antigen (PA83) IgG ELISA kit (Alpha Diagnostic International, San Antonio, TX, USA). ELISAs were performed exactly as instructed by the manufacturer. Serum samples were diluted 1:100 in Working Sample Diluent and each sample, control and calibrator was loaded onto the provided ELISA plate in duplicate wells of 100 µl. Plates were incubated at room temperature for 1 hour, then 100 µl of diluted Anti-Sheep IgG HRP was added to all wells for 30 minutes. TMB substrate was added to all wells and the reaction was stopped after 12 minutes and the reaction was stopped after 12 minutes

with 100 µl of Stop Solution. Optical density of all wells was read on a Tecan Infinite F50 Plate Reader at 450 nm.

3.2.9. Interpretation of white-tailed deer serum ELISAs

White-tailed deer serum ELISA results were normalized across plates using one of the kit calibrators then the OD's were evaluated against a Positive Index, as suggested by the supplier. The positive index was calculated as:

$$Positive\ Index =$$

$$Average\ Negative\ Control\ net\ OD\ (A450 - blank) + (2 \times Standard\ Deviation)$$

All sample ODs were then divided by the Positive Index. Values above a 1.0 were considered positive antibody activity.

3.3. Results

3.3.1. Sterne spore stability in simulated gastrointestinal environments

The unencapsulated Sterne spore response to simulated gastrointestinal conditions was observed to better understand and account for impairments while in transit through the stomach and intestines. The unencapsulated Sterne spore titer was severely reduced as a result of exposure to 0.2% NaCl (w/v) pH 2 ($p<0.01$) with no other significant responses observed from pH 5, 7 or 8 (Figure 3.3).

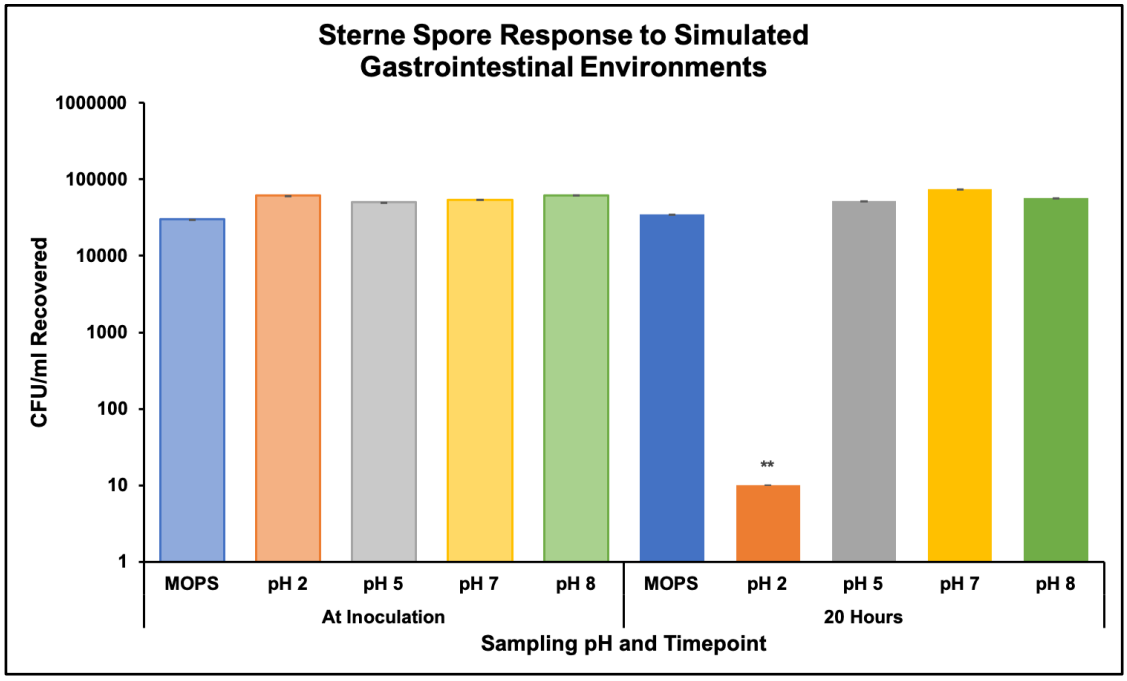

Figure 3.3. Sterne spore titer response to simulated gastrointestinal environments. Simulated gastric (0.2% (w/v) NaCl, pH 2 and pH 5) and intestinal (0.68% (w/v) KH_2PO_4, pH 7 and 8) fluids were inoculated with $6.8x10^5$ *Bacillus anthracis* Sterne strain 34F2 spores and incubated overnight at 37°C with shaking. The resulting viable bacterial titer in each solution was determined by plating serial dilutions. Differences between starting and resulting titers were determined by Student's t-tests with **, $p<0.01$.

3.3.2. Microcapsule vaccines induce anthrax specific antibody titers

Antibody levels against anthrax protective antigen were measured by ELISAs. Both vaccines containing Sterne spores elicited strong antibody responses starting at 15 days post subcutaneous vaccination (Figure 3.4). Similar to the responses observed in Figure 2.4, the Sterne vaccine exhibited a gradual increase with each timepoint. Despite being inoculated with the same dose of spores, the Spore Capsule group demonstrated higher antibody titers than the Sterne vaccine group at all timepoints past day 15. Additionally, the capsule vaccine displayed a drastic titer increase at 31 days post

vaccination. This same antibody titer spike was also observed from the orally administered Spore Capsules at 31 days post vaccination and it continued to increase like the titers observed from the subcutaneous vaccines (Figure 3.5). Both orally administered vaccines contained the same dose of Sterne spores, but the oral Sterne vaccine did not induce any antibody titer.

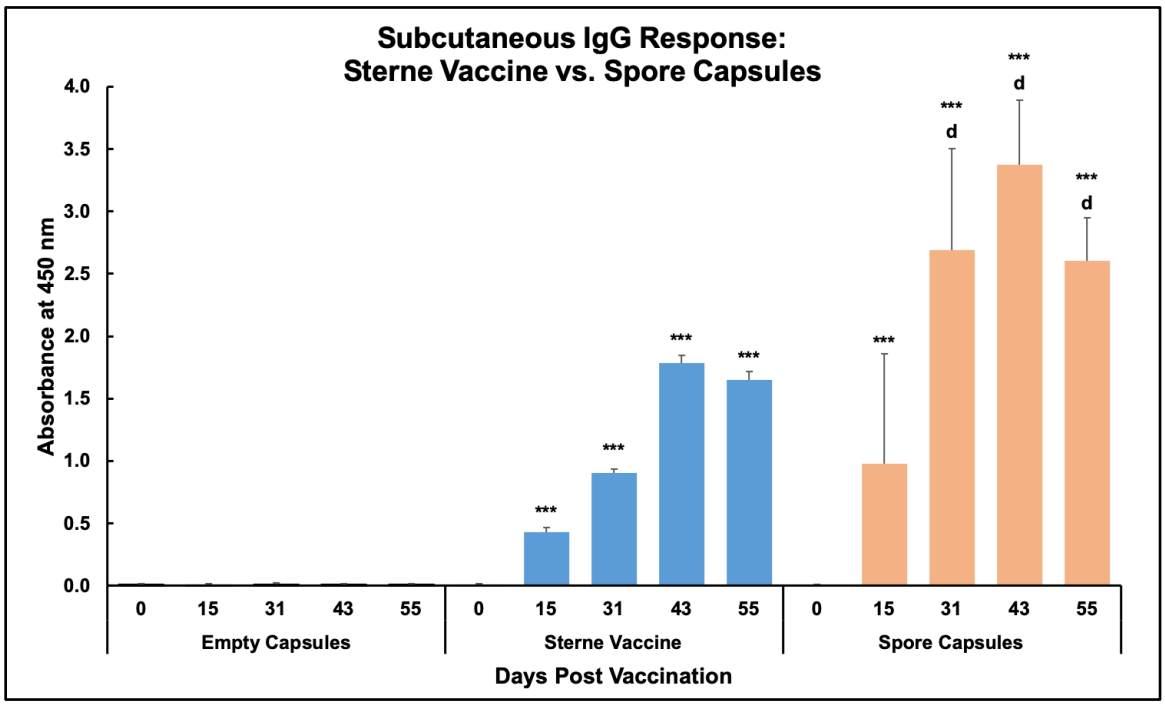

Figure 3.4. IgG responses from subcutaneously injected Empty Capsules, Sterne Vaccine and Spore Capsules. BALBc/J mice were inoculated with 10^6 *B. anthracis* Sterne strain 34F2 spores either unencapsulated (Sterne Vaccine) or encapsulated (Spore Capsules). Control groups received empty capsules. Serum samples were collected at 0, 15, 31, 43- and 55-days post vaccination and analyzed by ELISA. Antibody titers are shown from the 1:2000 dilution as mean absorbance at 450 nm ± standard deviation. Significant differences from pre-vaccination (Day 0) within the same group are identified as ***, $p<0.001$. Differences between the Sterne Vaccine and Spore Capsules titers at corresponding timepoints are identified with d, $p<0.0001$.

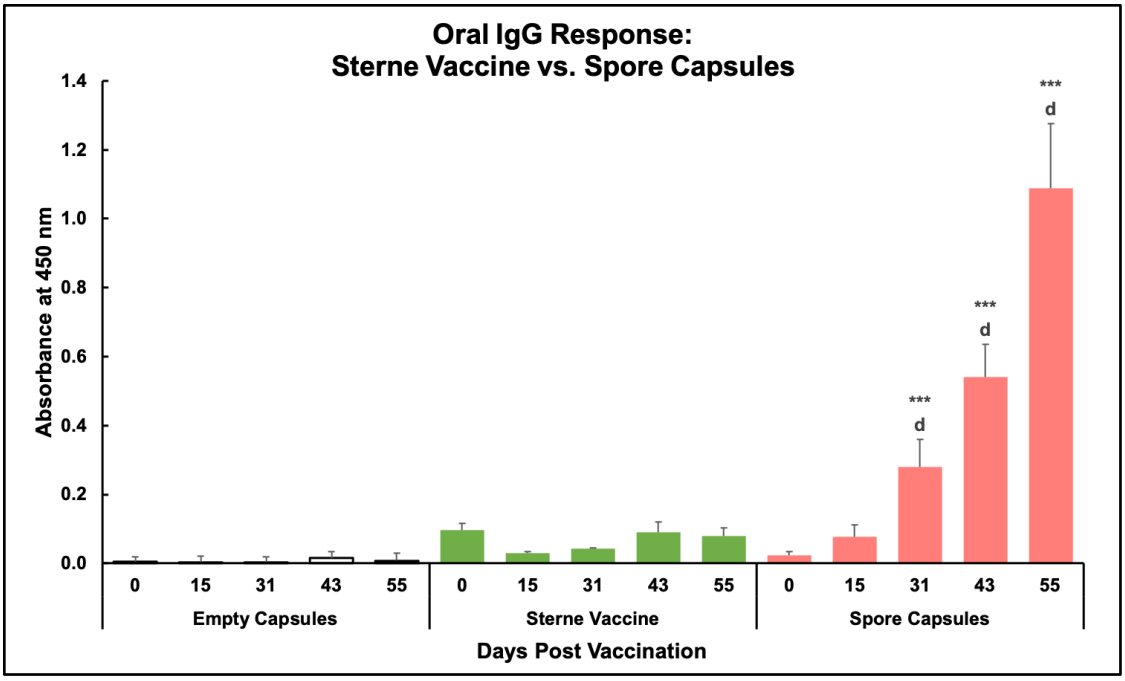

Figure 3.5. IgG responses from orally administered Empty Capsules, Sterne Vaccine and Spore Capsules. BALBc/J mice were inoculated with 10^6 *B. anthracis* Sterne strain 34F2 spores either unencapsulated (Sterne Vaccine) or encapsulated (Spore Capsules). Control groups received empty capsules. Serum samples were collected at 0, 15, 31, 43- and 55-days post vaccination and analyzed by ELISA. Antibody titers are shown from the 1:125 dilution as mean absorbance at 450 nm ± standard deviation. Significant differences from pre-vaccination (Day 0) within the same group are identified as ***, $p<0.001$. Differences between the Sterne Vaccine and Spore Capsules titers at corresponding timepoints are identified with d, $p<0.0001$.

3.3.3. Microencapsulated Sterne spores induce toxin neutralizing antibodies

LeTx neutralization assays evaluated the ability for vaccination induced antibody titers to protect J774A.1 cells from LeTx mediated killing. In agreement with the ELISA results, serum from both subcutaneous vaccines containing Sterne spores were able to prevent LeTx induced mortality *in vitro* at all measured timepoints (Figure 3.6). The Spore Capsule vaccine exhibited enhanced LeTx neutralizing abilities at 31- and 43-days

post vaccination. Strikingly, the oral capsule vaccine also resulted in toxin neutralizing effects at the same dilution as subcutaneously immunized mice. Serum from mice immunized orally with the Sterne vaccine did not provide any protection from LeTx challenge *in vitro*.

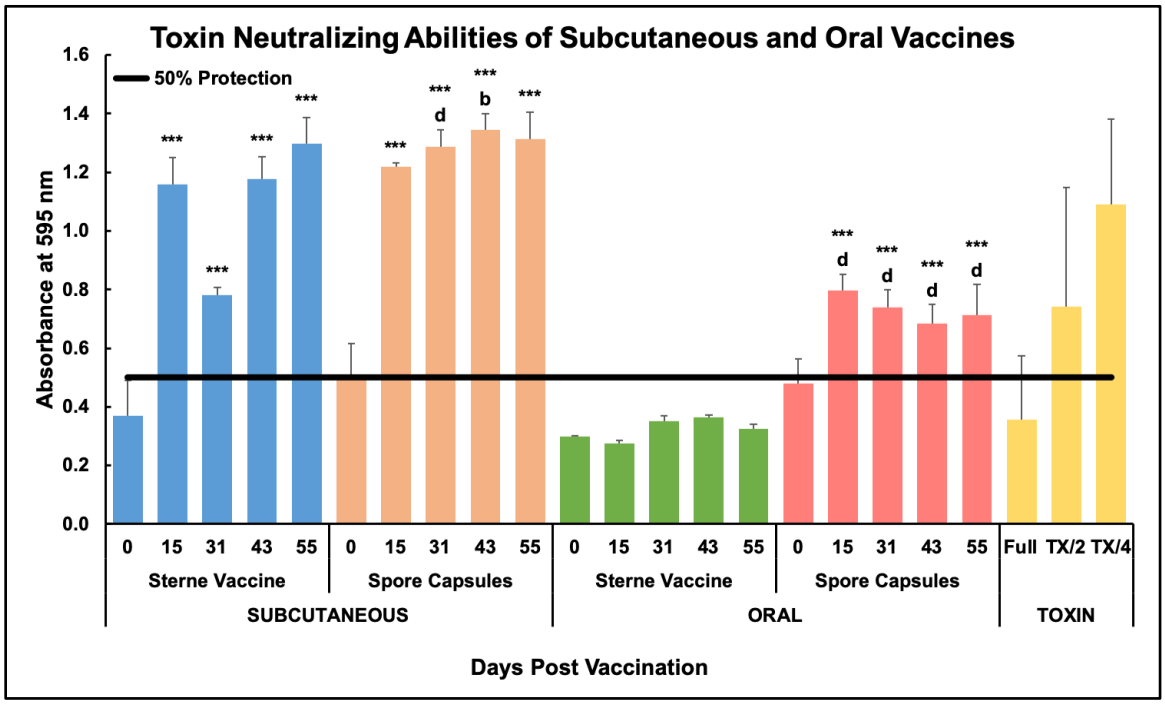

Figure 3.6. *In vitro* toxin neutralizing abilities of antibodies from subcutaneous and orally administered Sterne Vaccine and Spore Capsules. Serum was collected from mice at 0, 15, 31, 43- and 55-days post vaccination with 10^6 *B. anthracis* Sterne strain 34F2 spores either unencapsulated (Sterne Vaccine) or encapsulated (Spore Capsules). Control groups received empty capsules. Diluted serum samples were added to J774A.1 cells pre-incubated with LeTx and resulting cell viability was assessed with MTT dye. Data presented here represents the average OD + standard deviations for each group at each timepoint at a 1:50 dilution. LeTx serial dilutions are also included for reference. Significant differences from pre-vaccination (Day 0) within the same group are identified as ***, $p<0.001$. Differences between the Sterne Vaccine and Spore Capsules titers at corresponding timepoints are identified with b, $p<0.01$ and d, $p<0.0001$.

3.3.4. Seroprevalence of anti-anthrax antibodies in white-tailed deer serum from the East Foundation property

The seroprevalence of anthrax protective antigen antibodies was evaluated in white-tailed deer serum samples from the South Texas Plains and Gulf Coastal Plains regions of the East Foundation ranchlands (Figure 3.7). The estimated seroprevalence was over 80% for all sampling locations completed thus far, with the highest seroprevalence being 96.88% from the 64 samples collected at the San Antonio Viejo Ranch – E: Coloraditas & Aqua Verde location and the lowest being 80.00% from the 70 samples collected at the Buena Vista location (Table 3.2).

Sampling Location	Number of Samples	Seroprevalence %	Positive Antibody Activity	
			Minimum	Maximum
El Sauz – N	26	92.31%	1.05	3.31
SAVR – N: Coloraditas & Aqua Verde	58	89.66%	1.04	3.66
SAVR – E: Coloraditas & Aqua Verde	64	96.88%	1.04	4.11
SAVR – S: San Juan & Pipeline	40	85.00%	1.04	7.04
SAVR – W: San Juan & Pipeline	33	87.10%	1.09	3.27
Santa Rosa	32	87.50%	1.08	4.92
Buena Vista	70	80.00%	1.04	7.64

Table 3.2. Seroprevalence of anti-anthrax protective antigen (PA83) in serum from white-tailed deer on the East Foundation Ranch.

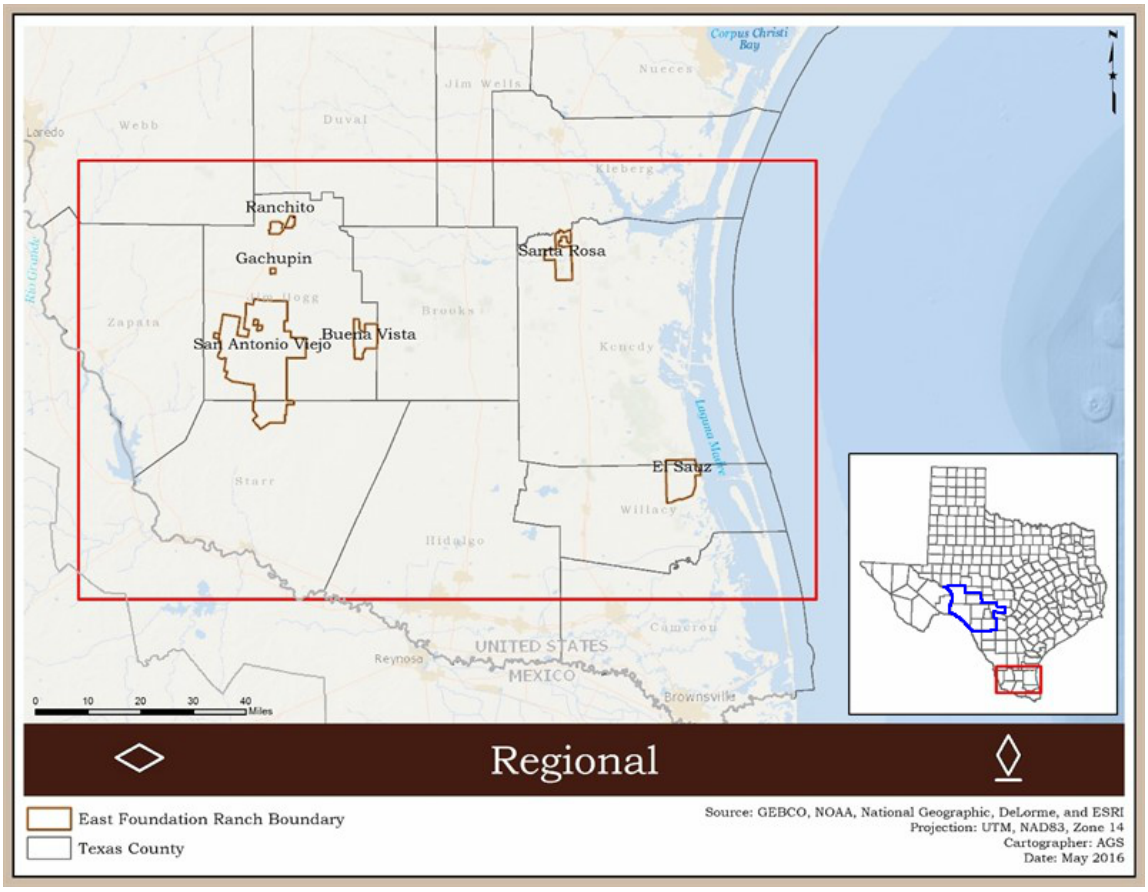

Figure 3.7. Map of the East Foundation property ranches. Specific ranches from which white-tailed deer serum samples were collected in November 2018 are labeled by name. Image downloaded from the East Foundation website and edited to reflect the current enzootic anthrax zone (inset, blue outline) [26,93]**.**

3.4. Discussion

An alternative anthrax vaccine formulation specifically for oral administration is urgently needed to protect animals worldwide from potentially catastrophic anthrax outbreaks [11,62]. Many wildlife health professionals have demanded a new veterinary anthrax vaccine because individual hand-injections for each and every animal is not a practical method of vaccination for free-ranging livestock and wildlife and a recent study

demonstrated that oral vaccination with the Sterne vaccine is not effective [4,62]. Also, sustained protection from the Sterne vaccine can only be achieved with yearly boosters which requires a yearly cycle of troublesome injections with the potential for adverse reaction [4]. To resolve the many issues associated with anthrax outbreaks and vaccination, we developed and evaluated a novel anthrax vaccine formulation for oral vaccination. Results of our study demonstrate that subcutaneous and oral vaccination with microencapsulated *B. anthracis* Sterne strain 34F2 spores can induce antibody production in the murine model and *in vitro* inactivation of *B. anthracis* lethal toxin.

Oral vaccination is a common goal throughout the entire vaccinology field but there are still a limited number of oral vaccines approved for animal, and human, use because the main obstacle facing oral vaccination is, ironically, oral vaccination itself [94–96]. The principle of oral vaccination is completely dependent on getting sensitive antigens through the harsh, gastric environment that was evolutionarily designed specifically to prevent that exact thing from happening. In contrast, gastrointestinal pathogens, such as anthrax, have also evolved over thousands of years to survive the gastric environment for eventual uptake in the small intestine but these pathogen survival strategies aren't typically conserved in live attenuated organisms, our most reliable vaccine format. Such is the case with *B. anthracis* Sterne strain 34F2. Upon exposure to a simulated gastric environment, there was a severe decrease in the viable Sterne spore titer (Figure 3.3). Given that the majority of anthrax infections in wildlife are gastrointestinal anthrax, this suggests that fully virulent anthrax spores are better equipped to survive in the gastric environment due to retention of the poly-γ-D-glutamic

acid capsule. Taken a step further, this also suggests that the poly-γ-D-glutamic acid capsule may be involved in much more than just preventing phagocytosis but has not yet been thoroughly researched. This also implies that development of an oral vaccine with Sterne spores must involve some protection as a substitute for the poly-γ-D-glutamic acid capsule.

A second challenge to oral vaccination, after having endured the harsh gastric environment, is to ensure antigen transport across the intestinal epithelia followed by antigen-presenting cell activation [96]. Advances in particulate vaccine delivery vehicles suggest that both issues can be resolved with microencapsulation. Prior studies have reported improved bacterial stability under acidic conditions due to microencapsulation in alginate [82,83,97,98]. Additionally, alginate capsules have demonstrated sustained release effects, as well as enhanced uptake and processing by antigen presenting cells [99,100]. These advantages proved beneficial in previous studies from our laboratory when microencapsulation of *Brucella spp.* in alginate microcapsules coated with VpB increased immune responses and reduced challenge organism recovery following oral vaccination [87–90]. Similar enhancements were observed in this study when we applied the same encapsulation method to *B. anthracis* Sterne strain 34F2.

Subcutaneous vaccination with Spore Capsules enhanced the observed antibody response even though mice received the same dose of spores as those vaccinated with the Sterne vaccine (Figure 3.4). The most encouraging data observed in this study was that of the orally administered microcapsules. ELISA results revealed a stark difference in the amount of antibody produced following oral vaccination with the Sterne vaccine

compared to Spore Capsules (Figure 3.5). To our knowledge, this is the first time a measurable antibody response has ever been recorded following oral vaccination with Sterne spores. The single prior attempt we are aware of involved mixing Sterne spores with scarifying agents for oral vaccination by way of tiny lacerations in the gums, tongue, oropharynx, etc. and observed limited success [67]. In contrast, results presented here were obtained from mice vaccinated by oral gavage which completely bypassed the oral mucosa. This suggests that microencapsulation with the VpB shell provides enough protection for Sterne spores to survive the gastric environment and progress into the small intestine to stimulate an immune response.

The advantages of the VpB capsules were also detected in results from toxin neutralization assays which are also considered a correlate of protection (Figure 3.6). Subcutaneous vaccination with Spore Capsules resulted in better protection for cultured macrophages at 31- and 43-days post vaccination when compared to the unencapsulated Sterne vaccine. Even more encouraging was the protection observed from the orally administered Spore Capsules. Oral antibody titers depicted in Figure 3.5 were produced from serum diluted 1:125 whereas the subcutaneous antibody titers depicted in Figure 3.4 were produced from serum diluted 1:2000. Despite being much less concentrated according to the ELISA results, the antibody titers induced by oral vaccination with Spore Capsules were considered protective against LeTx challenge at the same serum concentration as both subcutaneous antibody titers.

It is also possible that the antibody response due to oral vaccination with Spore Capsules had not yet peaked prior to the end of the experiment. In fact, a significant

antibody titer wasn't even detected until 31 days post vaccination. Given that the gastrointestinal emptying time for a mouse is less than 24 hours [101], these results suggest that VpB coated capsules containing Sterne spores may be demonstrating the mucoadhesive properties of alginate by adhering to the intestinal lumen to gradually release their bacterial load for at least two months [84,102]. Continued exposure resulting from extended capsule stability acts as a self-contained booster effect and it is possible that oral vaccination with a higher dose of microencapsulated Sterne spores, or even a booster dose of the same vaccine may further enhance the orally induced immune response.

In addition to developing a novel anthrax vaccine formulation, we also estimated the seroprevalence of antibodies in white-tailed deer serum from various locations on the East Foundation Ranch in south Texas (Table 3.2). Continued small surveys of potential wildlife exposure such as this may help identify at-risk areas to focus on for future vaccination policies. The current enzootic anthrax zone in Texas is mainly limited to the Edwards Plateau region which has been outlined in blue on the inset of Figure 3.7 [26]. Our seroprevalence screening suggested that the majority of all serum samples from the East Foundation Ranch had positive antibody activity against anthrax protective antigen despite being approximately 200-300 miles south of the enzootic anthrax zone. In the absence of an ELISA kit specifically for white-tailed deer, it's possible that the results from the selected ELISA kits are not fully reliable but the reproducibility of the readings suggests otherwise. Assuming the seroprevalence estimates are accurate, it is surprising to find such a high amount of antibody against anthrax in what was previously

considered a relatively anthrax-free zone. Future research should be implemented to determine if anthrax exposure is in fact occurring in this area, but not causing large outbreaks like in the Edwards Plateau region, or if this seroprevalence may be due to antibody cross-reactivity from another pathogen. For instance, the C2 toxin from *Clostridium botulinum,* another spore-forming bacterium, is able to bind to both anthrax lethal factor and edema factor. Conversely, anthrax protective antigen can transport *C. botulinum* C2I into target cells [103]. While cross-reactivity of anti-anthrax antibodies has not yet been suggested, the cross-reactivity of virulence factors implies it is possible and should be investigated further. It also calls for future research endeavors to determine if cross-reactivity can be advantageous for future anthrax vaccine formulations.

The findings of this study demonstrate the advantages and efficacy of Sterne spore microencapsulation. Following a single vaccination dose in mice, microencapsulated Sterne spores generated a significant antibody response via subcutaneous, but more importantly, oral vaccination, both of which were protective during *in vitro* LeTx challenge. Taken together, our study demonstrates for the first time the generation of protective antibody titers from oral vaccination with *B. anthracis* Sterne strain 34F2 spores and with further optimization, this microcapsule formulation has the potential to adapt the Sterne spore for effective oral vaccination of free-ranging livestock and wildlife.

4. STABILITY OF ALTERNATE FORMULATIONS FOR ALGINATE MICROCAPSULES AS ORAL VACCINE DELIVERY VEHICLES

4.1. Introduction

Without a reasonable method of wildlife vaccination, yearly anthrax outbreaks in wildlife pose economic, ecological and conservational burdens to wildlife health professionals around the world [11,12]. The live attenuated, non-capsulated Sterne strain has been the basis of the subcutaneously injected Sterne vaccine for nearly a century, yet the majority of anthrax vaccination research is focused on improving vaccines for human use with comparatively little effort being made for a new animal vaccine. Some vaccine research groups have been focusing on subunit vaccine development with recombinant anthrax antigens, however we are of the opinion that the seemingly infinite flexibility of alginate microencapsulation provides an ideal solution to the oral anthrax vaccine problem [76–78,81].

An abundance of alginate research has revealed the numerous advantages of the naturally occurring biopolymer [104]. Despite being indigestible in mammalian systems, it is still biocompatible because the indigestibility can be employed as a controlled release vehicle [84,105]. Additionally, the mild gelation conditions permit entrapment of the desired capsule load without significantly affecting the viability [84]. Post-gelation, the viability of the capsule load is maintained by stability of the microcapsule, particularly in gastric environments which has proven overwhelmingly beneficial for the development of probiotics [82]. Alginate has also demonstrated bio-adhesive properties

when interacting with mucosal tissues. Combined with the depot effect of alginate capsules, these bio-adhesive properties ensure that the capsule load is repeatedly released in close proximity to target cells [81]. In applications employing nanoparticles, the capsule load may even be released from within target cells because encapsulation can also lead to more efficient uptake and processing by antigen presenting cells [99,106].

Alginate microcapsules can be further tailored to individual applications by altering the capsule size, structure, load, layers and many other aspects of each capsule formulation. We recently used alginate encapsulation to adapt the Sterne strain for oral vaccination through development of a custom-made enteric delivery vehicle. We reported encouraging results from oral vaccination with microencapsulated *Bacillus anthracis* Sterne strain 34F2 spores coated with a mixture of poly-L-lysine (PLL) and vitelline protein B (VpB). PLL is a common microcapsule coating that enhances alginate capsule stability whereas VpB is a proteolysis resistant and non-immunogenic protein isolated from the eggshell of the parasitic liver fluke, *Fasciola hepatica* [85,86,107–109]. Previous research has demonstrated that incorporating VpB into the microcapsule coating can further enhance capsule stability in gastrointestinal environments and prolong the controlled release properties of the capsules [87–90]. This capsule formulation has already exhibited a neutralizing antibody response against anthrax lethal toxin following oral vaccination, however since alginate encapsulation allows for extreme customization, we evaluated the effect that formulation modifications, such as the Sterne spore dose, the PLL solvent and the amount of VpB, had on the microcapsule immunogenicity and stability.

4.2. Materials and Methods

4.2.1. Expression and purification of vitelline protein B

4.2.1.1. IPTG induction

VpB was previously cloned into the pET-SumoHis plasmid and transformed into *E. coli* BL21 for IPTG induction. Glycerol stock of the transformed *E. coli* carrying the VpB protein was streaked on an LB agar plate containing carbenicillin and incubated overnight at 37°C. A single colony from the plate was used to inoculate a 10 ml subculture of LB broth containing 50 µg/ml carbenicillin. The subculture was incubated overnight at 37°C with shaking, and then transferred to a full liter of LB broth with carbenicillin for continued growth prior to IPTG induction. To determine the optimal growth phase for IPTG induction, the OD was measured at 595 nm at various intervals and samples were collected to run on SDS-PAGE. Gels were stained with Coomassie blue for 30 minutes and destained with 10% acetic acid overnight (Figure 4.1). Once VpB cultures reached an OD of 0.2, IPTG was added with a final concentration of 1 mM and incubated at 30°C for the 4-hour induction. All bacteria were pelleted at 6000 rpm for 10 minutes at 4°C and stored at -20°C until lysis.

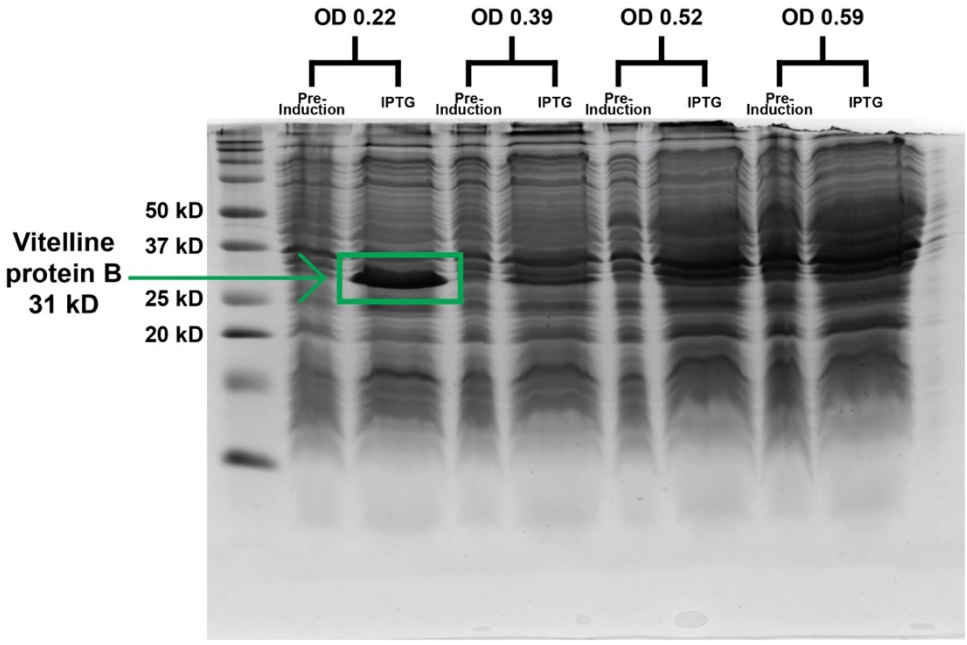

Figure 4.1. IPTG time course induction of VpB. The VpB containing *E. coli* was cultured as described in the text. IPTG was added to aliquots of the culture at various intervals to determine the best OD for future inductions. Resulting samples were evaluated by electrophoresis on 12.5% SDS-PAGE gels. Successfully induced VpB expression is outlined in green at OD=0.22.

4.2.1.2. Bacterial lysis

The bacterial pellet was thoroughly washed via resuspension in 25 ml of sterile water and centrifugation at 6000 rpm, 4°C. Rinsed pellets were resuspended in 20 ml of 6 M guanidine lysis buffer, pH 7.8 containing 200 µl lysozyme, 200 µl 0.1 M PMSF and 200 µl aprotinin, and placed on a tube rocker at 4°C for at least 30 minutes. The bacteria were sonicated on ice for a total of 6 minutes with 15 second intervals at 50% amplitude followed by 30 second cooling intervals. To ensure completely lysis, the lysate was returned to the tube rocker at 4°C overnight. The lysate was centrifuged at 10,000 rpm for 15 minutes at 4°C to remove all cellular debris.

4.2.1.3. His-tagged purification

VpB was purified from the bacterial lysate by his-tagged purification. The lysate supernatant was transferred to a new tube containing nickel chelating resin (G-Biosciences, St. Louis, MO, USA) prepared according to the supplier's recommendations. The lysate/resin mixture was placed on the tube rocker for at least 2 hours at 4°C for initial binding. Unwanted proteins and other compounds were removed from the lysate/resin mixer through a series of washes with 6 M guanidine at pH 7.8, pH 6 and pH 5.3 in that order. Each buffer was used for 2 washes which involved settling the resin by centrifugation at 800 rpm for 2-3 minutes, removing the previous supernatant, resuspending the resin in fresh guanidine buffer of the appropriate pH and shaking it for 5 minutes at 4°C before settling the resin again. After the last wash in pH 5.3, no more than 5 ml of 6 M guanidine pH 4 was added to the resin and it was incubated on the tube rocker at 4°C for at least 2 hours. The first VpB elution was collected as described above and replaced with another 5 ml of 6 M guanidine pH 4 for a second elution following overnight incubation on the tube rocker at 4°C.

4.2.1.4. Dialysis

Purified VpB elutions were dialyzed into 0.1 M acetate buffer, pH 4 to remove all guanidine salts from the final protein. Elutions were loaded into a Slide-A-Lyzer dialysis cassette with a 10K molecular weight cut off (ThermoFisher Scientific, Waltham, MA, USA). Cassettes were fully submerged in 0.1 M acetate buffer, pH 4 and dialyzed with gentle stirring at 4°C for at least 3 hours. The buffer was completely

replaced, and dialysis was repeated another 3 times until the remaining guanidine molarity was estimated to be less than 1 μM. Final purified and dialyzed VpB was collected from the cassette and stored at -20°C until use.

4.2.1.5. Evaluation of purified protein by SDS-PAGE

An aliquot of each batch of purified VpB was evaluated for purity by 12% SDS-PAGE. Gels were stained with Coomassie blue for 30 minutes and destained with 10% acetic acid overnight. Purified VpB was visualized at ~31 kD (Figure 4.2). VpB concentration was calculated using the protein absorbance at 280 nm and the molar extinction coefficient.

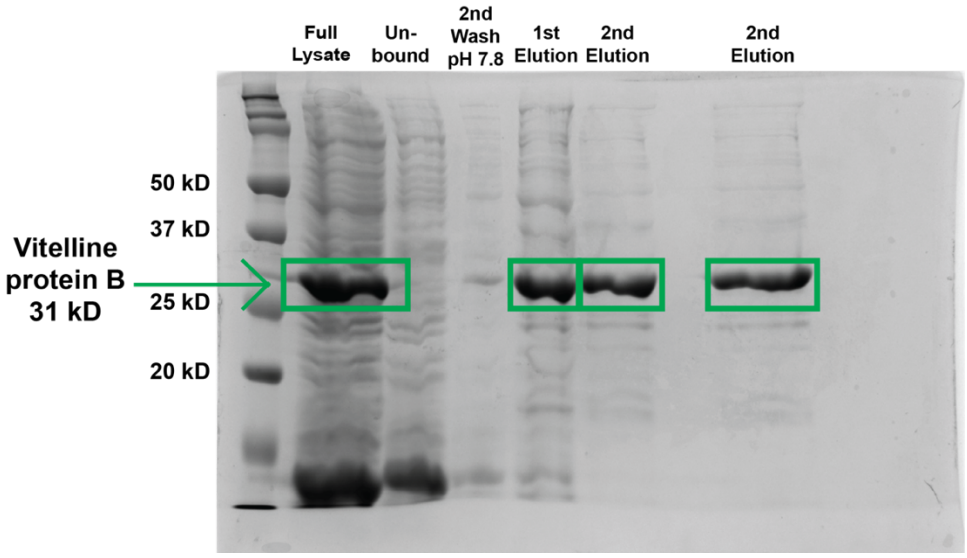

Figure 4.2. Purified VpB on SDS-PAGE at a molecular weight of ~31 kD. Washes from a his-tagged purification resin column and purified VpB were separated by molecular weight via SDS-PAGE for visual confirmation of purified VpB. Samples run on this gel are as follows: full bacterial lysate prior to resin binding (Full Lysate), unbound protein collected after initial binding to the resin column (Un-bound), supernatant from the second resin column wash with guanidine pH 7.8 (2^{nd} Wash pH 7.8), purified VpB in guanidine, pH 4 collected after a 2 hour incubation (1^{st} Elution) and two lanes of purified VpB collected in the second elution in guanidine pH 4 after an overnight incubation (2^{nd} Elution).

4.2.2. Preparation of Sterne spores

All Sterne spores used in this experiment were grown from a vial of the Anthrax Spore Vaccine (Colorado Serum Company, Denver, CO, USA) as described previously [62]. A small volume of LB broth was inoculated with 200 µl of the Anthrax Spore Vaccine and incubated overnight with shaking at 37°C. The culture was pelleted and resuspended in 1 ml of LB broth, then plated on LB agar and incubated for 6 days at 37°C. Full lawns of Sterne spores were scraped from the plates and washed repeatedly with sterile water. All vegetative cells were removed by heating at 68°C and filtering

through a 3.1 μm filter then final Sterne spore concentrations were determined by plating serial dilutions on LB agar.

4.2.3. Vaccine preparation

4.2.3.1. Sterne vaccine

A new vial of the Anthrax Spore Vaccine was used for these mouse experiments exactly as received from Colorado Serum Company with an approximate dose of $5x10^6$ spores/ml.

4.2.3.2. Production of microencapsulated *Bacillus anthracis* Sterne strain 34F2 spores

A variety of microencapsulated vaccine formulations were prepared for evaluation in this study as outlined in Table 4.1. A complete explanation of formulation origins can be found in Appendix B. In general, sodium alginate (NovaMatrix, Sandvika, Norway) was dissolved in MOPS buffer (10 mM MOPS, 0.85% NaCl [pH 7.4]) to a final concentration of 1.5% (w/v) alginate. The 6 ml microcapsule core solution consisted of 5 ml of 1.5% alginate mixed with 1 ml of Sterne spores at the desired concentration. This mixture was extruded through a 170 μm nozzle on a Nisco Encapsulator VARV1 unit (Nisco Engineering AG, Zurich, Switzerland) directly into 40 ml of cross-linking solution (100 mM $CaCl_2$, 10mM MOPS) and stirred for 30 minutes. Microcapsules were washed thoroughly with MOPS and then mixed for 30 minutes in 15 ml of the proteolysis resistant shell solution containing varying proportions of VpB and

poly-L-lysine (PLL) dissolved in either 100 mM $CaCl_2$, 10mM MOPS ($CaCl_2$) or MOPS. Capsules were washed with MOPS then coated with an outer shell of 0.03% (w/v) alginate by mixing for 5 minutes. Lastly, capsules were washed and resuspended in MOPS for storage at 4°C until use. VpB Core & Shell capsules were prepared in the same way, except 0.5 mg of VpB was added to the microcapsule core solution prior to extrusion through the encapsulator. The approximate dose of viable Sterne spores in each microcapsule vaccine was determined by dissolving and plating 1 ml of capsules in 50 mM sodium citrate, 0.45% NaCl, 10 mM MOPS prior to permanent cross-linking with the proteolysis resistant shell. Final microcapsule formulations were 1:1 solutions of prepared capsules in MOPS.

Microcapsule Formulation	Microcapsule Core Details	Microcapsule Shell Details								Purpose
		Vitelline protein B (VpB)			Poly-L-Lysine (PLL)			$\frac{PLL\ \%}{VpB\ \%}$	MOPS to 15 ml	
		VpB A_{280}	VpB (mg)	VpB %	PLL (mg)	PLL Solvent	PLL %			
VpB Shell	$5.3x10^{11}$Sterne spores 1.5% (w/v) alginate	1.71	0.48	0.0032	2.5	100 mM $CaCl_2$, 10 mM MOPS	0.017	5.3	9.46	Mouse Vaccinations Bacterial Release
VpB Core & Shell	$5.3x10^{11}$ Sterne spores 0.479 mg VpB 1.5% (w/v) alginate	1.71	0.48	0.0032	2.5	100 mM $CaCl_2$, 10 mM MOPS	0.017	5.3	9.46	Mouse Vaccinations Bacterial Release
Low VpB $CaCl_2$	$5x10^8$ Sterne spores 1.5% (w/v) alginate	0.87	0.37	0.0025	2.5	100 mM $CaCl_2$, 10 mM MOPS	0.017	6.8	9.08	pH Response
Low VpB MOPS	$5x10^8$ Sterne spores 1.5% (w/v) alginate	0.87	0.37	0.0025	2.5	10 mM MOPS, 0.85% NaCl	0.017	6.8	9.08	pH Response
Mid VpB $CaCl_2$	$5x10^8$ Sterne spores 1.5% (w/v) alginate	0.87	0.95	0.0064	2.5	100 mM $CaCl_2$, 10 mM MOPS	0.017	2.7	7.78	pH Response
Mid VpB MOPS	$5x10^8$ Sterne spores 1.5% (w/v) alginate	0.87	0.95	0.0064	2.5	10 mM MOPS, 0.85% NaCl	0.017	2.7	7.78	pH Response
High VpB $CaCl_2$	$4.3x10^{10}$ Sterne spores 1.5% (w/v) alginate	1.07	2.5	0.017	2.5	100 mM $CaCl_2$, 10 mM MOPS	0.017	1.0	-	pH Response
Spore Capsules*	$6x10^7$ Sterne spores 1.5% (w/v) alginate	1.76	1	0.0067	7	100 mM $CaCl_2$, 10 mM MOPS	0.047	7.0	-	Mouse Vaccinations
Microcapsule vaccine prepared in Chapter 3. Information provided here for comparison										

Table 4.1. Microcapsule formulations prepared to assess the microcapsule efficacy, release rate and stability in gastrointestinal environment. A complete explanation of why these formulations were used is included in Appendix B.

4.2.4. Mouse vaccination

The immunogenicity of two microcapsule formulations were compared to the immunogenicity of the Sterne vaccine. Four to six-week-old female BALBc/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were randomly distributed into four groups of five mice each (Table 4.2) and allowed to acclimate for at least a week prior to any handling. All animal care and experimental procedures were performed in compliance with the Texas A&M University Institutional Animal Care and Use Committee regulations.

Mice were inoculated subcutaneously or by oral gavage with 0.2 ml of one of three prepared vaccines: (i) Sterne vaccine, (ii) VpB Shell Capsules, (iii) VpB Core & Shell Capsules (Table 4.2). All mice inoculated with either the VpB Shell or VpB Core & Shell Capsules received approximately $8.8x10^9$ spores/mouse in prepared microcapsules while mice immunized with the Sterne vaccine received approximately $1x10^6$ spores/mouse in saponin. Oral vaccination groups were given a vaccine booster with approximately $4x10^6$ spores/ml at 28 days post vaccination. Antibody responses were evaluated in blood samples that were collected three to seven days prior to vaccination and then every seven to ten days for eight weeks.

Route	Group (n=5)	Inoculation Volume	Spores/ ml	Spores/ Mouse	Blood Collection (days post-vaccination)
SC	Sterne Vaccine	0.2 ml	$5x10^6$	$1x10^6$	0, 8, 15, 22, 31, 43, 55
	VpB Shell	0.2 ml	$4.4x10^{10}$	$8.8x10^9$	0, 8, 15, 22, 31, 43, 55
Oral	VpB Shell	0.2 ml	$4.4x10^{10}$	$8.8x10^9$	0, 8, 15, 22, 31, 43, 55
	VpB Core & Shell	0.2 ml	$4.4x10^{10}$	$8.8x10^9$	0, 8, 15, 22, 31, 43, 55

SC = subcutaneous
Sterne Vaccine = *B. anthracis* Sterne strain 34F2 spores in saponin
VpB Shell = *B. anthracis* Sterne strain 34F2 microcapsules with PLL and VpB shell
VpB Core & Shell = *B. anthracis* Sterne strain 34F2 microcapsules with VpB in the core and shell

Table 4.2. Vaccination groups testing two microcapsule formulations with higher Sterne spore doses.

4.2.5. Bacterial release from microcapsules

The bacterial release rates from the VpB Shell and VpB Core & Shell capsules were examined *in vitro* by suspending 1 ml of capsules in 9 ml of MOPS buffer and placing the tubes on a rocker at 37°C [89]. At each sampling time point, the capsules were allowed to settle out of the buffer and then as much of the supernatant as possible was collected without disturbing the capsule pellet. The supernatant was plated on LB agar to estimate the bacterial release since the last timepoint. Capsules were resuspended in the same volume of MOPS buffer that had been removed and returned to the rocker at 37°C. Samples were collected every day for 22 days, approximately every other day until day 38 and a final sample was collected at day 56 when the mouse study was terminated. Results are reported in terms of bacterial release per timepoint versus time.

4.2.6. Detection of anthrax-specific IgG and IgA

Anthrax specific antibody levels in serum and feces samples from BALBc/J mice were measured by ELISA similar to previous studies [62]. Anthrax protective antigen was dissolved in carbonate, bicarbonate buffer, pH 9.6 and coated on high-binding ELISA plates (100 ng/well). Plates were incubated for 1 hour at 37°C and then overnight at 4°C. The next day, plates were washed 3-5 times with phosphate buffered saline containing 0.5% Tween 20 (PBST) and blocked with 100 µl per well of 1% bovine serum albumin (w/v) in PBST (1% BSA) for 1 hour at 37°C. Plates were washed as above, loaded with 100 µl per well of serum samples serially diluted in 1% BSA and incubated at 37°C for 1 hour. Anti-Mouse IgG (H+L) (SeraCare, Milford, MA, USA) diluted 1:5000 in 1% BSA was loaded 100 µl to a well and incubated for 1 hour at 37°C. The TMB/E Substrate (Sigma-Aldrich, St. Louis, MO, USA) reaction was stopped after 12 minutes with 0.5 M H_2SO_4 and antibody levels were measured as the optical density at 450 nm on a Tecan Infinite F50 Plate Reader. Samples from each time point, at each dilution were run in duplicate and are reported as average absorbance values for the vaccination group.

Fecal samples were prepared by dissolving approximately 0.1 g of fecal pellets in 1 ml of PBS with 0.1% sodium azide and then centrifuged at 6400 rpm for 15 minutes. IgA levels in prepared fecal and serum samples were measured as described above except with anti-mouse IgA (SeraCare, Milford, MA, USA) diluted 1:500 in 1% BSA.

4.2.7. Toxin neutralization assays

The protective abilities of all experimental vaccines was estimated by lethal toxin (LeTx) neutralization assays [77,78,92]. J774A.1 macrophages at a concentration of $5x10^4$ cells/ml were loaded on to a 96-well flat-bottom tissue culture plate at 200 µl/well and incubated overnight at 37°C in 5% CO_2. Lethal factor (0.25 µg/ml, List Biological Laboratories Inc., Campbell, CA, USA) and protective antigen (0.1 µg/ml, List Biological Laboratories Inc., Campbell, CA, USA) were mixed with DMEM containing 10% FBS to make lethal toxin. Serial dilutions of mouse serum samples were made in the prepared LeTx mixture and incubated for 1 hour at 37°C, 5% CO_2. The media was removed from the prepared cells then replaced with 100 µl/well of the serum LeTx mixture for a 4-hour incubation at 37°C, 5% CO_2. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Roche, Basel, Switzerland) was added to each well (10 µl) and incubated at 37°C, 5% CO_2. After a 4-hour incubation, 100 µl of solubilization solution (Roche, Basel, Switzerland) was added to each well and plates were incubated overnight at 37°C, 5% CO_2. The optical density was read at 595 nm using a Tecan Infinite F50 Plate Reader. Cells exposed to only LeTx and no serum were used as a positive control. Cells that did not receive any LeTx or serum were used to determine 100% cell viability and 50% cell viability, also referred to as 50% protection. The LeTx neutralizing titer was defined as antibody titers resulting in 50% protection.

4.2.8. Characterization of microcapsules in simulated gastrointestinal environments

Microcapsule morphology and bacterial presence within the alginate capsules were visualized with brightfield microscopy. Capsule responses, with and without the PLL and VpB shell, and with varying formulations of the PLL and VpB shell (Table 4.1), to simulated gastrointestinal fluids (GI fluids) were examined by suspending an aliquot of each capsule formulation in separate vials of the GI fluids. Simulated gastric fluids to mimic the pH range of a non-fasted stomach consisted of 0.2% (w/v) NaCl adjusted to pH 2 and pH 5 with 1 M HCl [91]. Simulated intestinal fluids were 0.68% (w/v) KH_2PO_4 adjusted to pH 7 and pH 8 with 0.2 M NaOH [91]. The above GI fluids were used to evaluate all capsule formulations except for the High VpB $CaCl_2$ capsules which were suspended in PBS prepared at pH 2, 5, 7 and 8. Only the pH of PBS pH 2 required adjusting with 6 N HCl. Vials were placed on a tube rocker at 37°C and samples were collected at 30 and 90 minutes for imaging on an Olympus CKX41 microscope. Capsule diameters were measured in ImageJ.

4.2.9. Statistical analysis

For all experiments, results are expressed as mean values ± standard deviations for all replicates at each timepoint for each group. Statistical analysis was performed using ANOVA followed by the Tukey-Kramer HSD test with p-values <0.05 considered significant.

4.3. Results

4.3.1. Evidence of controlled release from microcapsules

An *in vitro* release experiment was conducted for 56 days to evaluate the timeframe of bacterial release from two microcapsule formulations. Results validated sustained release abilities of VpB Shell and VpB Core & Shell capsule formulations with no apparent differences observed between the release rates of each formulation (Figure 4.3). The release experiment was terminated at the same time as the mouse immunization experiment and yet capsules still contained viable Sterne spores and vegetative cells suggesting that capsules would have been able to continue releasing viable bacteria for at least 56 days (Figure 4.4). In contrast to previous microencapsulation batches, these capsules did not have a homogenous distribution of Sterne spores throughout the entire capsule. Instead, there are darkened areas within the capsules that are a mix of aggregated Sterne spores and vegetative cells. In fact, each aggregation resembles a single Sterne CFU on an LB agar plate with lighter borders and darker centers (Figure 4.4).

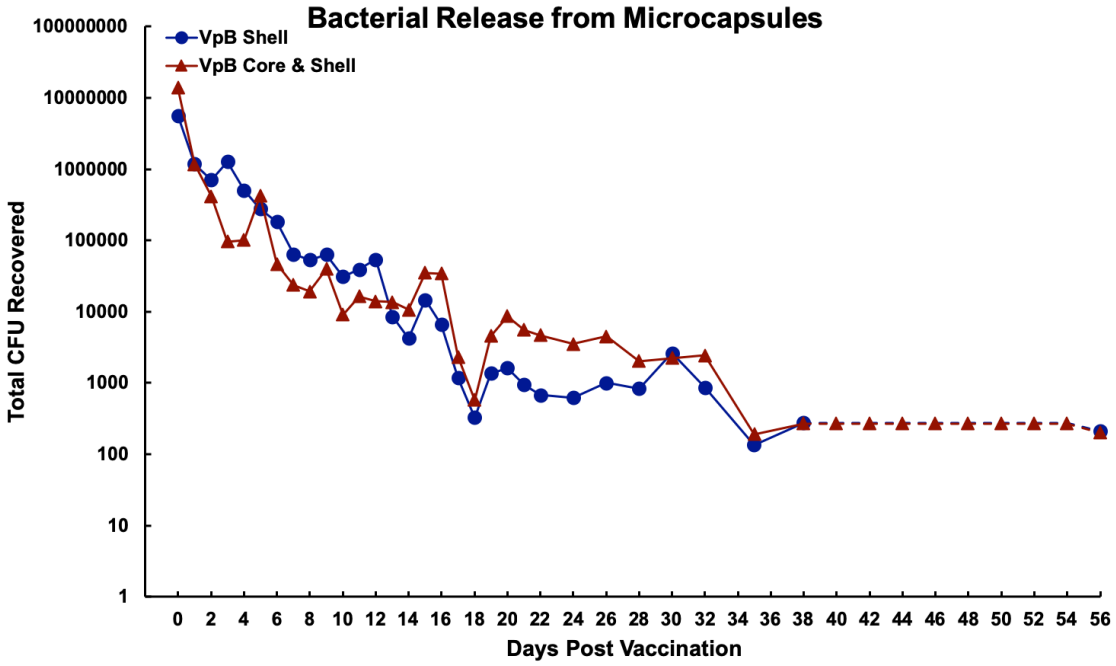

Figure 4.3. ***In vitro*** **release from VpB Shell and VpB Core & Shell capsules. Microcapsules were prepared with 10^9 Sterne spores and 1 ml of each capsule formulation was suspended in 10 ml MOPS. The MOPS buffer was replaced each day and the bacteria titer released into the MOPS from the capsules was serially diluted and plated onto LB agar. The dashed line represents missed sampling days with a final titer recorded on day 56.**

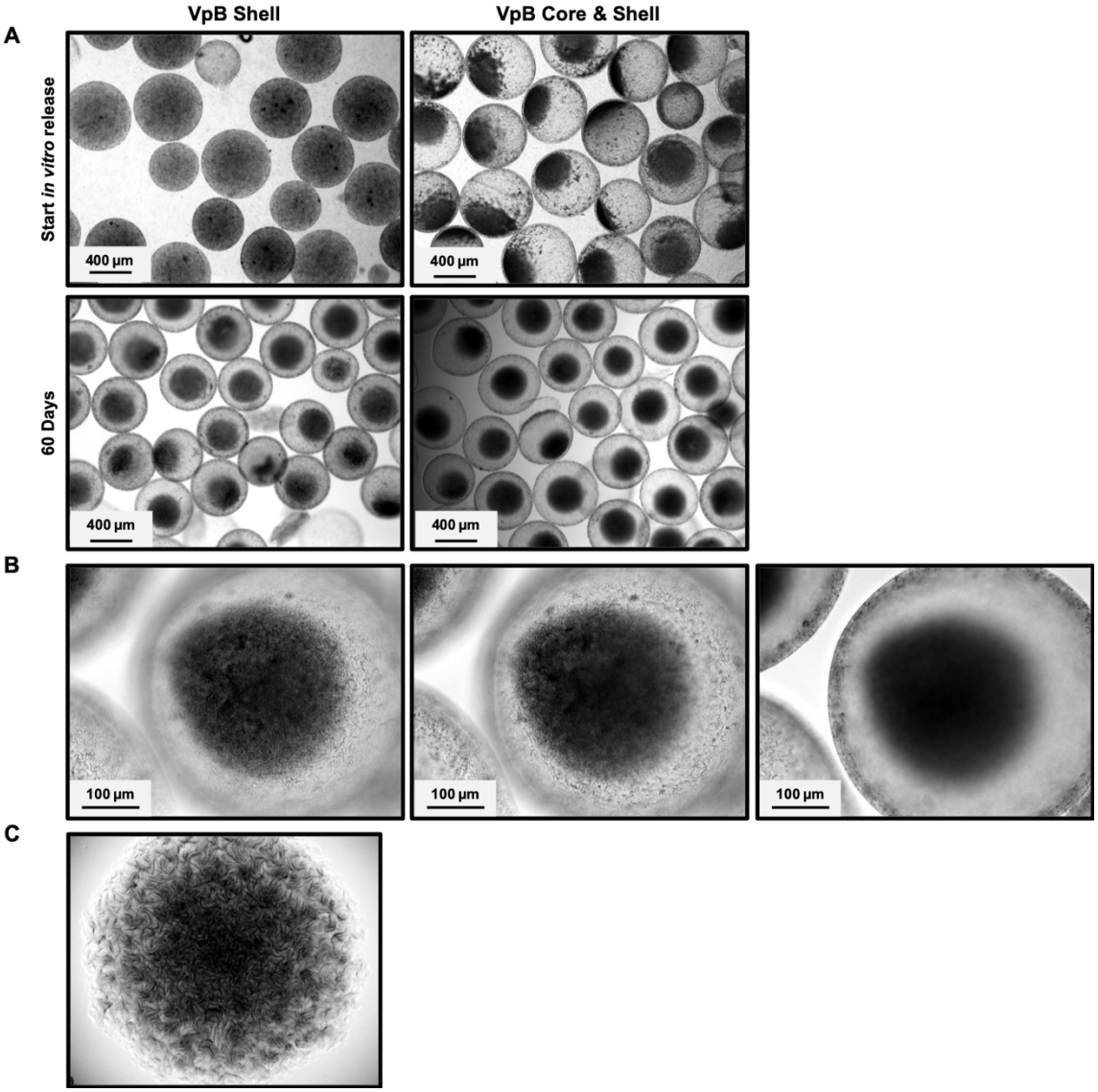

Figure 4.4. Sterne spore entrapment in VpB Shell and VpB Core & Shell capsules. (A) Two capsule formulations were assessed for bacterial release rates. Sterne strain spores and vegetative cells are visible in both formulations prior to beginning the *in vitro* release experiment (top). After 60 days, a considerable amount of Sterne cells is still entrapped within both capsule formulations (bottom). (B) Close up images of a single microcapsule at three depths containing both Sterne spores and vegetative cells as well as a concentration of bacteria contained within the PLL and VpB coating. (C) Brightfield image of a single Sterne spore colony on a LB agar plate.

4.3.2. Anti-anthrax protective antigen IgG and IgA

Anti-protective antigen titers were measured in collected serum and feces samples by ELISA. As expected, due to the greatly increased Sterne spore dose, subcutaneously injected VpB Shell capsules elicited incredibly high IgG levels compared to the Sterne vaccine at all measured timepoints (Figure 4.5). However corresponding results were not observed from oral administration of VpB Shell or VpB Core & Shell capsules despite the much higher dose of Sterne spores (Figure 4.6). There was a small increase in serum IgG observed from orally vaccinated mice after receiving a vaccine booster on day 28 with an immediate increase in titer from VpB Core & Shell capsules on day 31. Mice receiving the VpB Shell booster orally did not exhibit an antibody response to the booster until day 43.

IgA levels in mouse serum (Figure 4.7) and fecal samples (Figure 4.8) were also measured. Mice subcutaneously injected with the Sterne vaccine appeared to have elevated levels of serum IgA prior to vaccination and did not experience any significant increase over the course of the experiment. Serum IgA levels recorded from the three encapsulated vaccines were all lower than the subcutaneous Sterne vaccine levels but subcutaneously injected VpB Shell capsules did induce an increase in serum IgA starting at 15 days post vaccination. Orally administered capsules did not induce any significant increases in serum IgA but there may be some evidence of fecal IgA effects. VpB Shell capsules caused a small spike in fecal IgA levels at 8 days post vaccination and a smaller spike was observed in fecal samples from VpB Core & Shell capsules at 15 days post vaccination. Both IgA titers appeared to return to normal levels and were increased

again, though not significantly, after receiving the oral booster. Neither subcutaneous vaccine resulted in measurable fecal IgA titers.

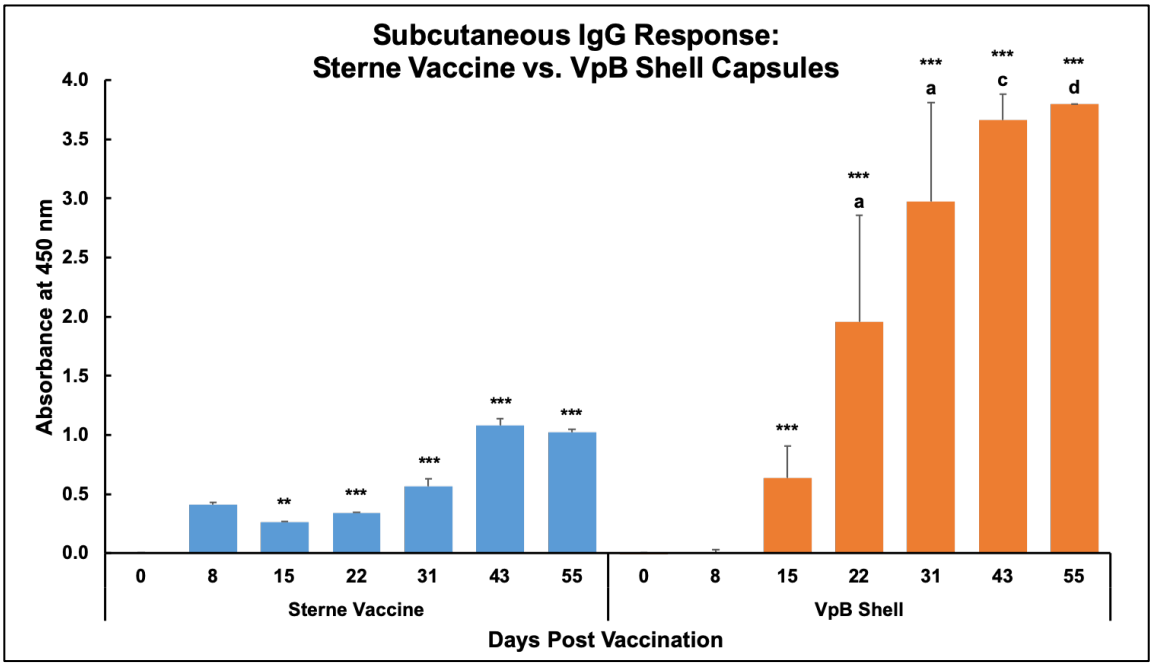

Figure 4.5. Serum IgG responses from subcutaneously injected VpB Shell capsules and Sterne vaccine. BALBc/J mice were inoculated with either 10^9 *B. anthracis* Sterne strain 34F2 spores encapsulated in VpB Shell capsules or 10^6 Sterne spores from the Sterne vaccine. Serum samples were collected at 0, 8, 15, 22, 31, 43- and 55-days post vaccination and analyzed by ELISA. Antibody titers are shown from the 1:4000 dilution as mean absorbance at 450 nm ± standard deviation. Significant differences from pre-vaccination (Day 0) within the same group are identified as **, p<0.01, and ***, p<0.001. Differences between the Sterne Vaccine and Spore Capsules titers at corresponding timepoints are identified with a, p<0.05; b, p<0.01, c, p<0.001; d, p<0.0001.

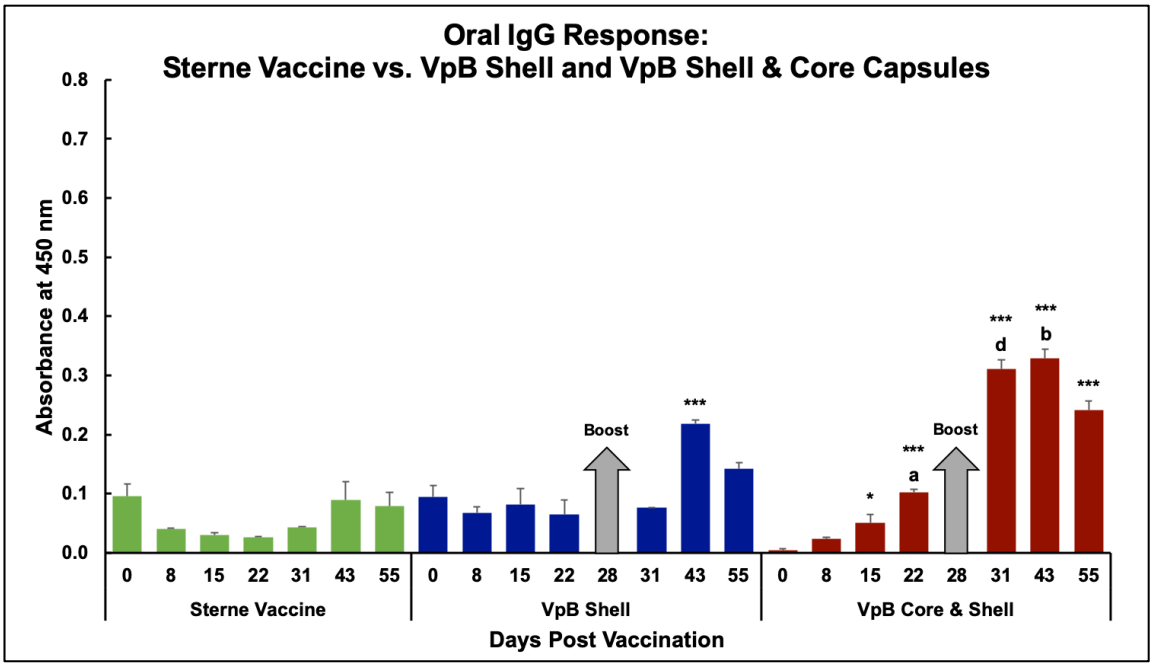

Figure 4.6. Serum IgG responses from orally inoculated VpB Shell or VpB Core & Shell capsules. BALBc/J mice were inoculated with either 10^9 *B. anthracis* Sterne strain 34F2 spores encapsulated in VpB Shell or VpB Core & Shell capsules. Serum results of mice orally inoculated with the Sterne vaccine in prior experiments are included as a reference. Serum samples were collected at 0, 8, 15, 22, 31, 43- and 55-days post vaccination and analyzed by ELISA. Antibody titers are shown from the 1:125 dilution as mean absorbance at 450 nm ± standard deviation. Significant differences from pre-vaccination (Day 0) within the same group are identified as *, p<0.05; **, p<0.01 and ***, p<0.001. Differences between the Sterne Vaccine and VpB Core & Shell titers at corresponding timepoints are identified with a, p<0.05; b, p<0.01, c, p<0.001; d, p<0.0001.

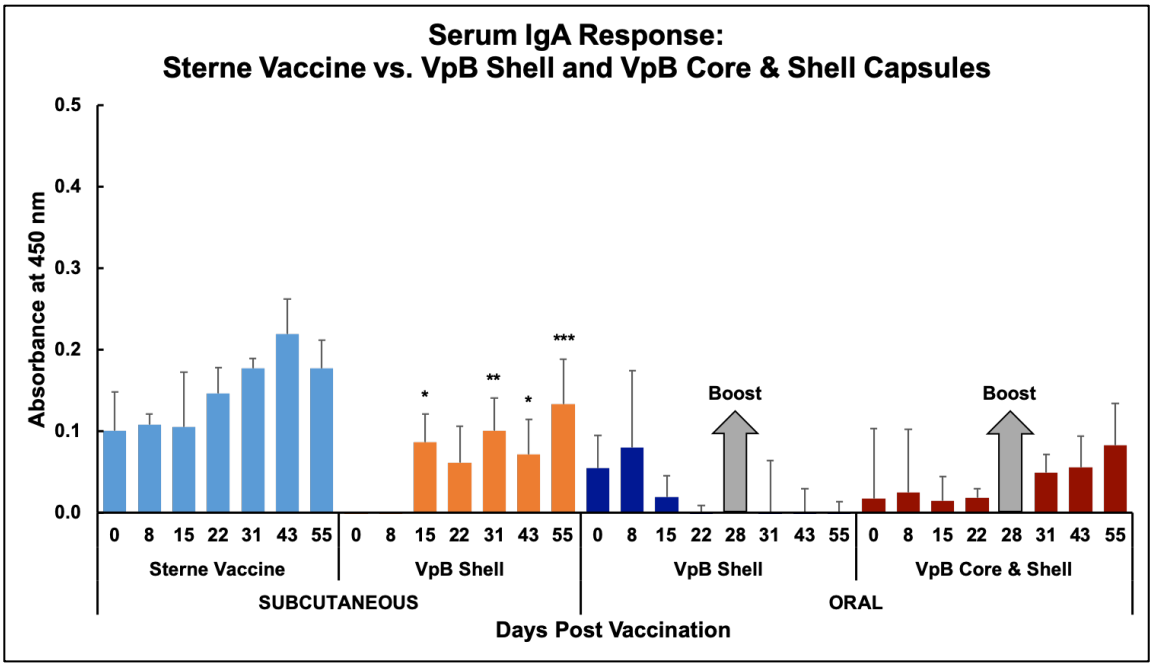

Figure 4.7. Serum IgA responses from subcutaneously and orally inoculated VpB Shell or VpB Core & Shell capsules. BALBc/J mice were inoculated with either 10^9 *B. anthracis* Sterne strain 34F2 spores encapsulated in VpB Shell or VpB Core & Shell capsules, or 10^6 Sterne spores from the Sterne vaccine. Serum samples were collected at 0, 8, 15, 22, 31, 43- and 55-days post vaccination and analyzed by ELISA. Antibody titers are shown from the 1:125 dilution as mean absorbance at 450 nm ± standard deviation. Significant differences from pre-vaccination (Day 0) within the same group are identified as *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.

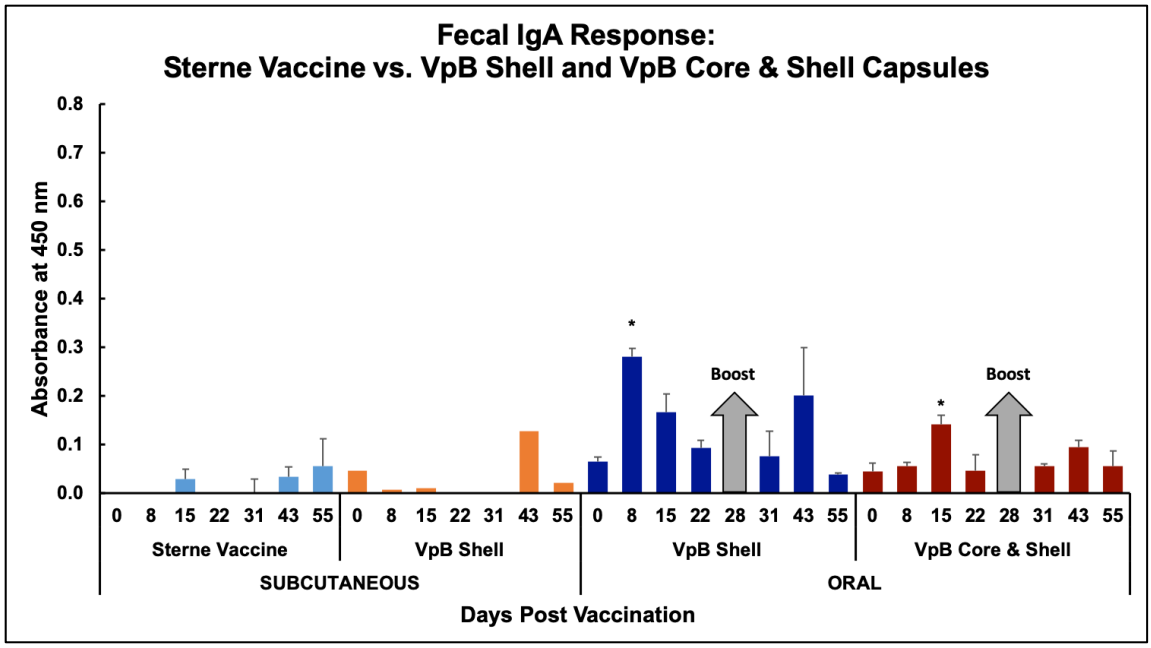

Figure 4.8. Fecal IgA responses from subcutaneously and orally inoculated VpB Shell or VpB Core & Shell capsules. BALBc/J mice were inoculated with either 10^9 *B. anthracis* Sterne strain 34F2 spores encapsulated in VpB Shell or VpB Core & Shell capsules, or 10^6 Sterne spores from the Sterne vaccine. Serum samples were collected at 0, 8, 15, 22, 31, 43- and 55-days post vaccination and analyzed by ELISA. Antibody titers are shown from the 1:200 dilution as mean absorbance at 450 nm ± standard deviation. Significant differences from pre-vaccination (Day 0) within the same group are identified as *, $p<0.05$.

4.3.3. LeTx neutralization assay

The protectiveness of subcutaneous and orally administered VpB Shell and VpB Core & Shell capsules was estimated by LeTx neutralization assays. Serum obtained from subcutaneously immunized mice was able to impart *in vitro* protection to cultured macrophages (Figure 4.9). Oral immunization with either VpB Shell or VpB Core & Shell capsules did not result in any neutralizing titers at any timepoint.

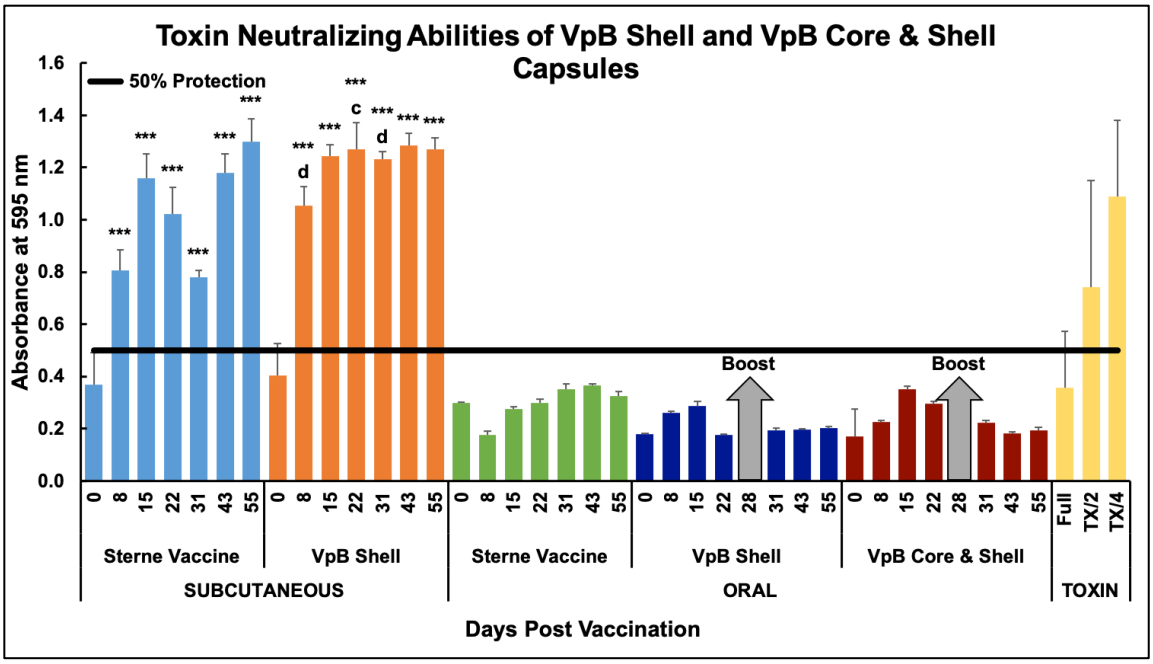

Figure 4.9. Toxin neutralizing abilities of VpB Shell and VpB Core & Shell capsules. Serum was collected from mice at 0, 8, 15, 22, 31, 43- and 55-days post vaccination with the Sterne vaccine or 10^9 *B. anthracis* Sterne strain 34F2 spores encapsulated in VpB Shell or VpB Core & Shell capsules. Diluted serum samples were added to J774A.1 cells pre-incubated with LeTx and resulting cell viability was assessed with MTT dye. Data presented here represents the average OD + standard deviations for each group at each timepoint at a 1:50 dilution. LeTx assay results of mice orally inoculated with the Sterne vaccine in prior experiments are included as a reference, as are serial dilutions of LeTx. Significant differences from pre-vaccination (Day 0) within the same group are identified as ***, $p<0.001$. Differences between VpB Shell capsules and the Sterne Vaccine at corresponding timepoints are identified with c, $p<0.001$ and d, $p<0.0001$.

4.3.4. Comparison of microcapsule formulations in gastrointestinal environments

Various microcapsule formulations were exposed to GI fluids to discern which formulation would result in the most stable capsules for oral vaccination. Formulations used in this experiment specifically revealed changes in the capsule response to GI fluids due to fluctuations in the PLL to VpB ratio and whether PLL was dissolved in $CaCl_2$ or

MOPS. At pH 2, capsules that were not coated with the PLL and VpB shell were shown to decrease in diameter compared to neutral storage conditions in MOPS (Pre-Exp.), whereas at pH 5 uncoated capsules experienced significant swelling (Figure 4.10). The most striking advantage of the PLL and VpB shell, of any formulation, was its capsule stabilization abilities at pH 7 and 8. Without the addition of this proteolysis resistant coating, the capsules completely disintegrated at neutral pHs (Figure 4.10). These patterns were also observed in uncoated capsules after 90 minutes in GI fluids, simply to a higher degree as a result of the extended exposure.

In comparison, all capsule formulations with the PLL and VpB shell exhibited overall enhanced stability in all GI fluids (Figures 4.11 - 4.18). Both Low VpB capsule formulations prevented shrinking at pH 2 and complete capsule dissolution at pH 7 and 8 (Figure 4.11, Figure 4.14, Figure 4.15). Low VpB MOPS capsules appeared to be less stable as shown by greater fluctuations in the capsule diameters compared to Low VpB $CaCl_2$ capsules (Figure 4.11). While the diameter change isn't significant, Low VpB MOPS capsules appeared to swell upon exposure to pH 2 as opposed to remaining constant in size like Low VpB $CaCl_2$ capsules.

When more VpB was added to the microcapsule formulations the pre-exposure capsule diameters increased by about 150 μm (Figure 4.12). In contrast to the stability observed in Low VpB capsules, Mid VpB capsules experienced some degradation upon exposure to pH 2 (Figure 4.16, Figure 4.17). Mid VpB $CaCl_2$ capsules maintained a relatively constant size in pH 5, 7 and 8 while the size of Mid VpB MOPS capsules fluctuated at all three pHs after 30 and 90 minutes (Figure 4.12).

These degradation and swelling patterns were also observed in High VpB $CaCl_2$ capsules when submerged in PBS pH 2, 5, 7 and 8, although not as much degradation of the capsules was observed at pH 2 (Figure 4.13, Figure 4.18). Interestingly, uncoated capsules continued to shrink and swell at PBS pH 2 and 5, respectively, but when exposed to PBS pH 7 and 8, uncoated capsules did not disintegrate as seen with 0.68% KH_2PO_4 at pH 7 and 8 (Figure 4.18).

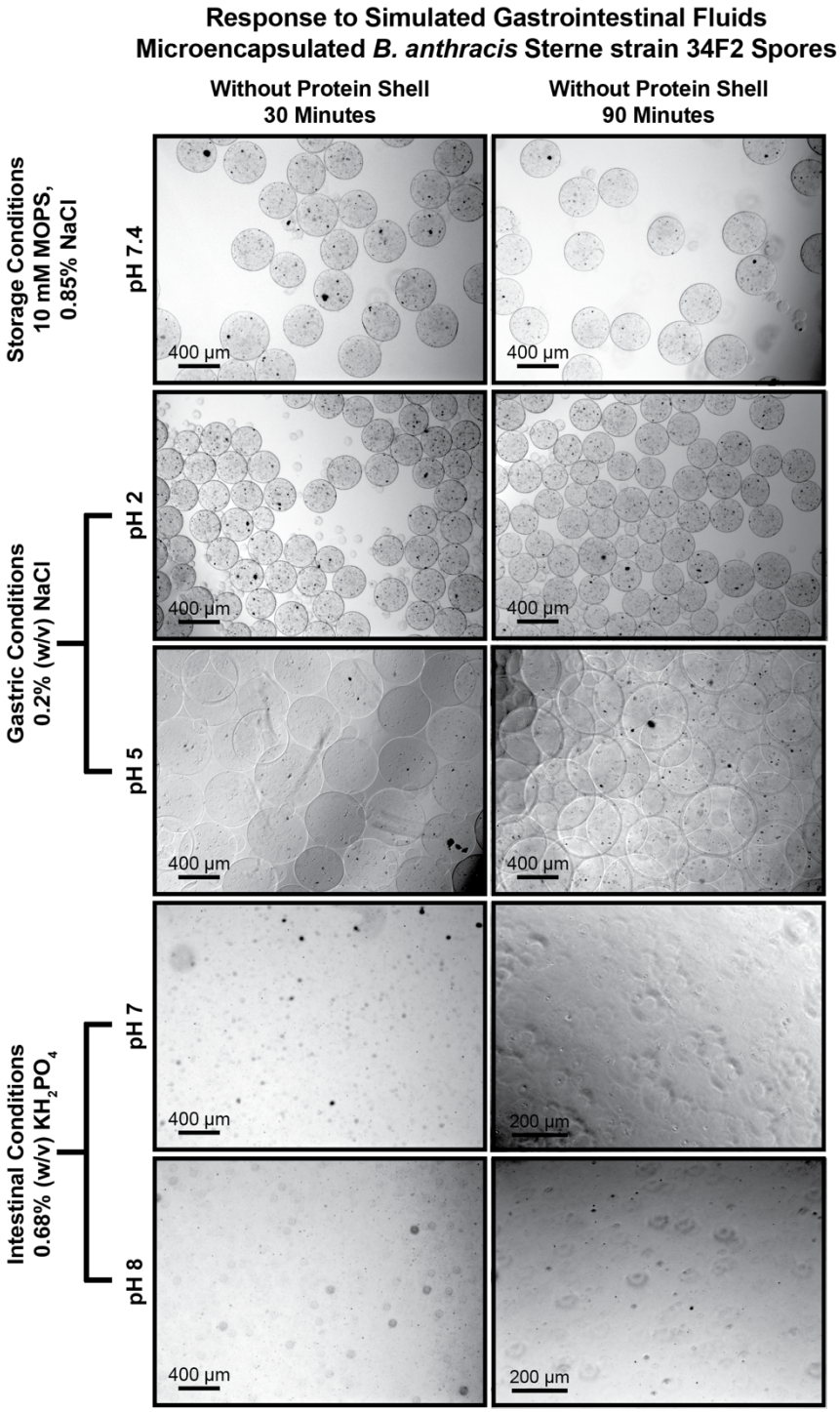

Figure 4.10. Uncoated microcapsule response to simulated gastrointestinal environments. Representative brightfield images of microcapsules without the PLL and VpB shell following exposure to simulated gastric and intestinal fluids for 30 minutes (left) and 90 minutes (right). The bottom two images of each column portray the dissolution of uncoated capsules at pH 7 and pH 8.

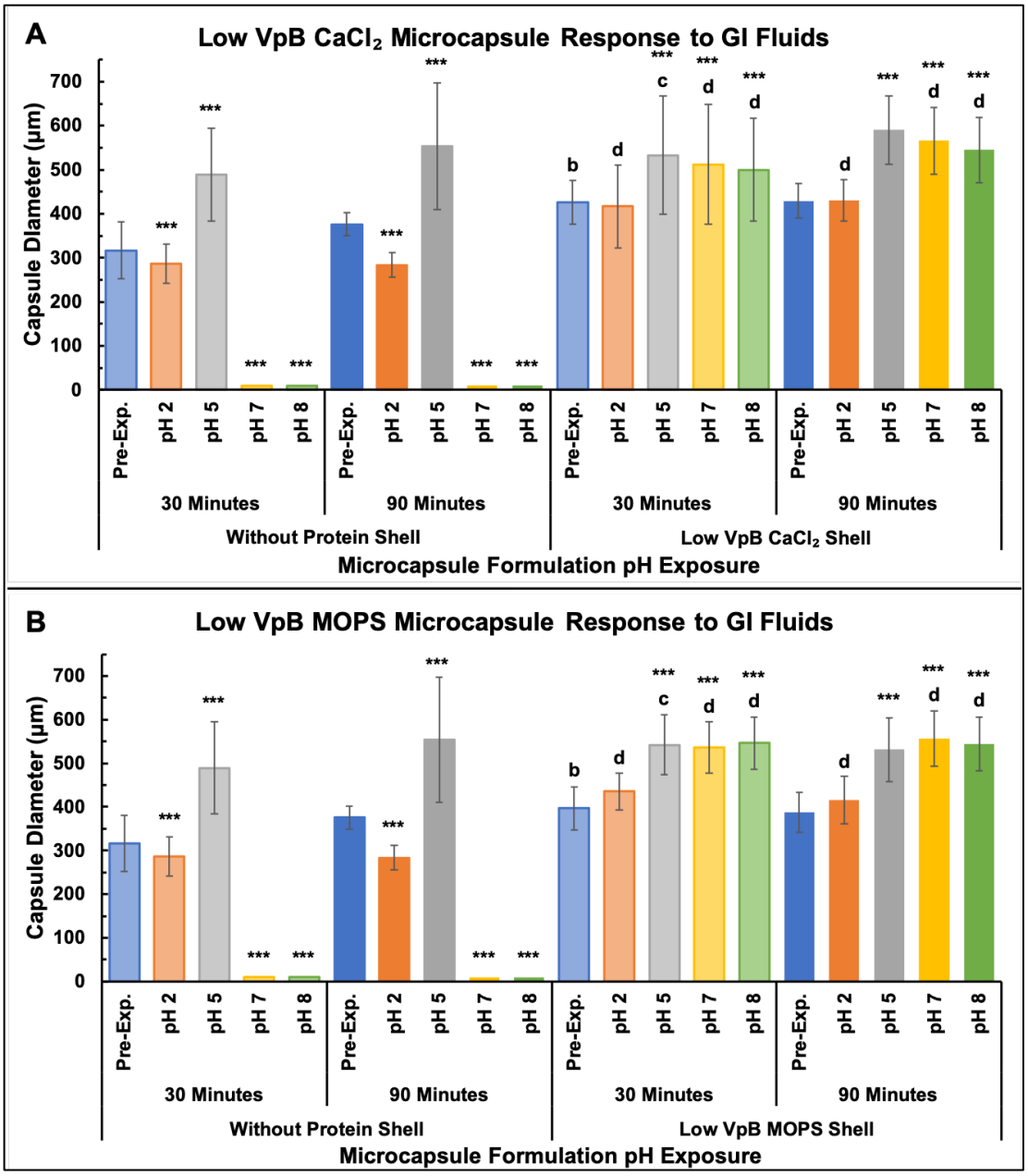

Figure 4.11. Low VpB $CaCl_2$ and Low VpB MOPS capsule diameter changes to simulated gastrointestinal environments. Low VpB capsules (0.017% PLL with 0.0025% VpB) were suspended in simulated GI fluids for 30 and 90 minutes at 37°C with shaking. Data is reported as the average capsule diameter for the group in µm ± the standard deviation. Significant differences from pre-exposure diameters in MOPS (Pre-Exp.) within the same group are identified as ***, p<0.001. Differences between (A) Low VpB $CaCl_2$ and (B) Low VpB MOPS capsules at the same pH and timepoint are identified with a, p<0.05; b, p<0.01; c, p<0.001; d, p<0.0001.

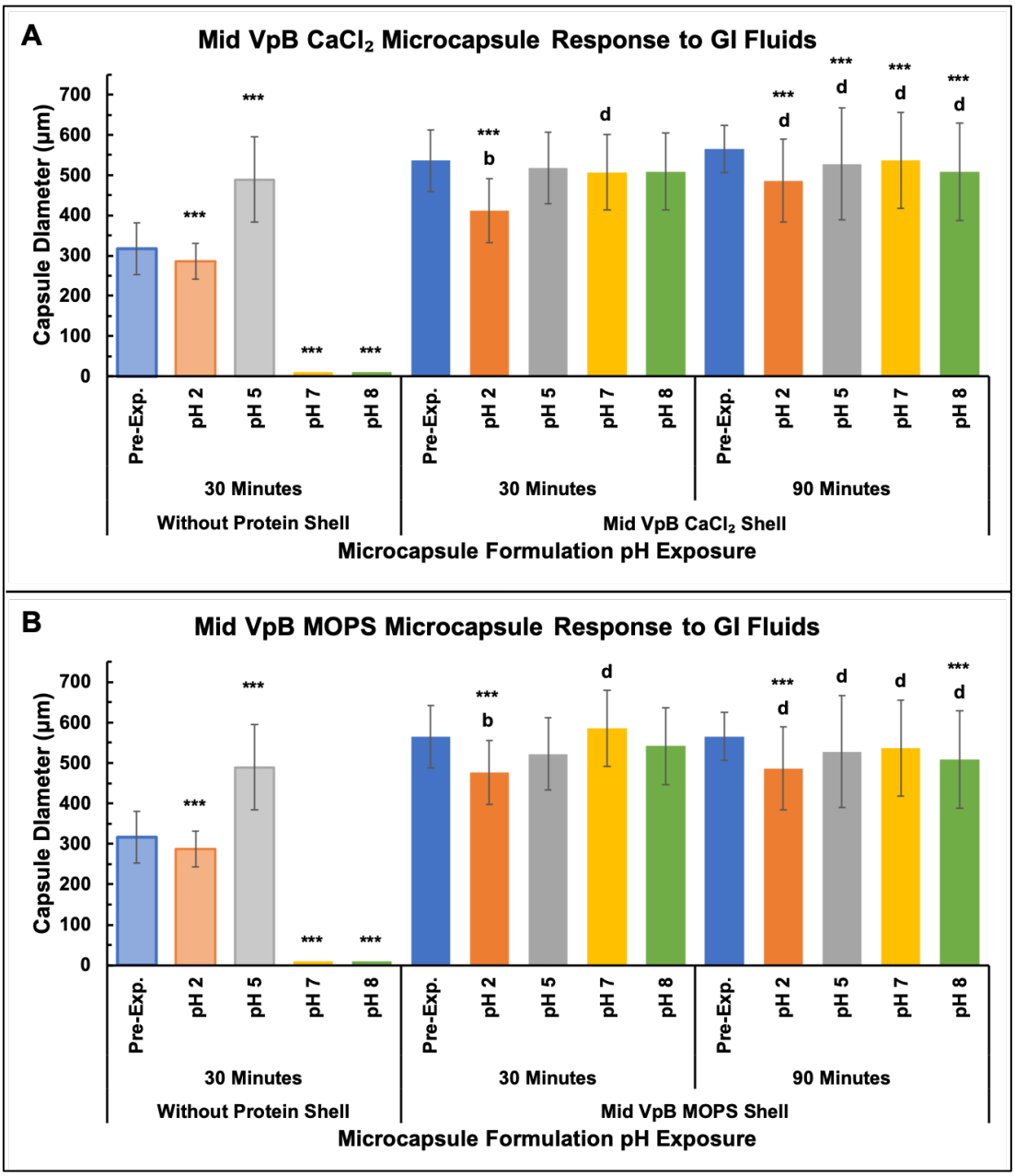

Figure 4.12. Mid VpB $CaCl_2$ and Mid VpB MOPS capsule diameter changes to simulated gastrointestinal environments. Mid VpB capsules (0.017% PLL with 0.0064% VpB) were suspended in simulated GI fluids for 30 and 90 minutes at 37°C with shaking. Data is reported as the average capsule diameter for the group in µm ± the standard deviation. Significant differences from pre-exposure diameters in MOPS (Pre-Exp.) within the same group are identified as ***, $p<0.001$. Differences between (A) Mid VpB $CaCl_2$ and (B) Mid VpB MOPS capsules at the same pH and timepoint are identified with a, $p<0.05$; b, $p<0.01$; c, $p<0.001$; d, $p<0.0001$.

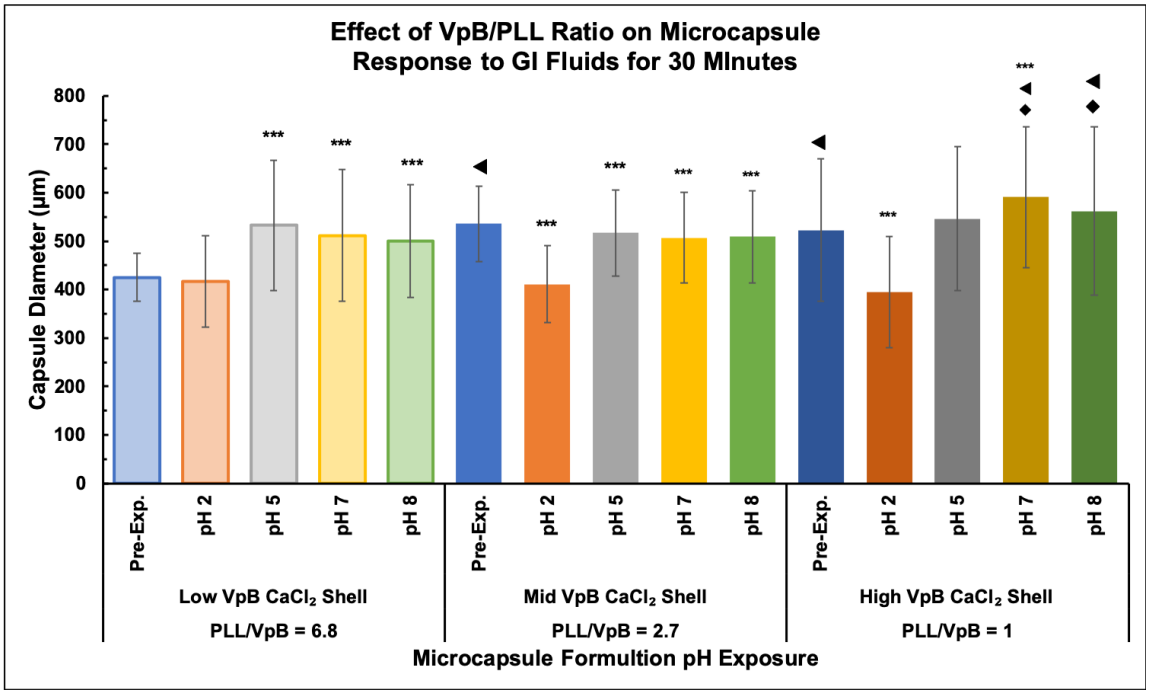

Figure 4.13. Low, Mid and High VpB $CaCl_2$ capsule diameter changes to simulated gastrointestinal environments. Low VpB (0.017% PLL with 0.0025% VpB), Mid VpB (0.017% PLL with 0.0064% VpB) and High VpB (0.017% PLL with 0.017% VpB) $CaCl_2$ capsules were suspended in simulated GI fluids for 30 minutes at 37°C with shaking. Capsule formulations are also labeled with the corresponding PLL%/VpB% ratios. Data is reported as the average capsule diameter for the group in µm ± the standard deviation. Significant differences from pre-exposure diameters in MOPS (Pre-Exp.) within the same group are identified as ***, $p<0.001$. Differences from Low VpB $CaCl_2$ are identified as ◀ $p<0.0001$ and differences from Mid VpB $CaCl_2$ are identified with ◆ $p<0.0001$.

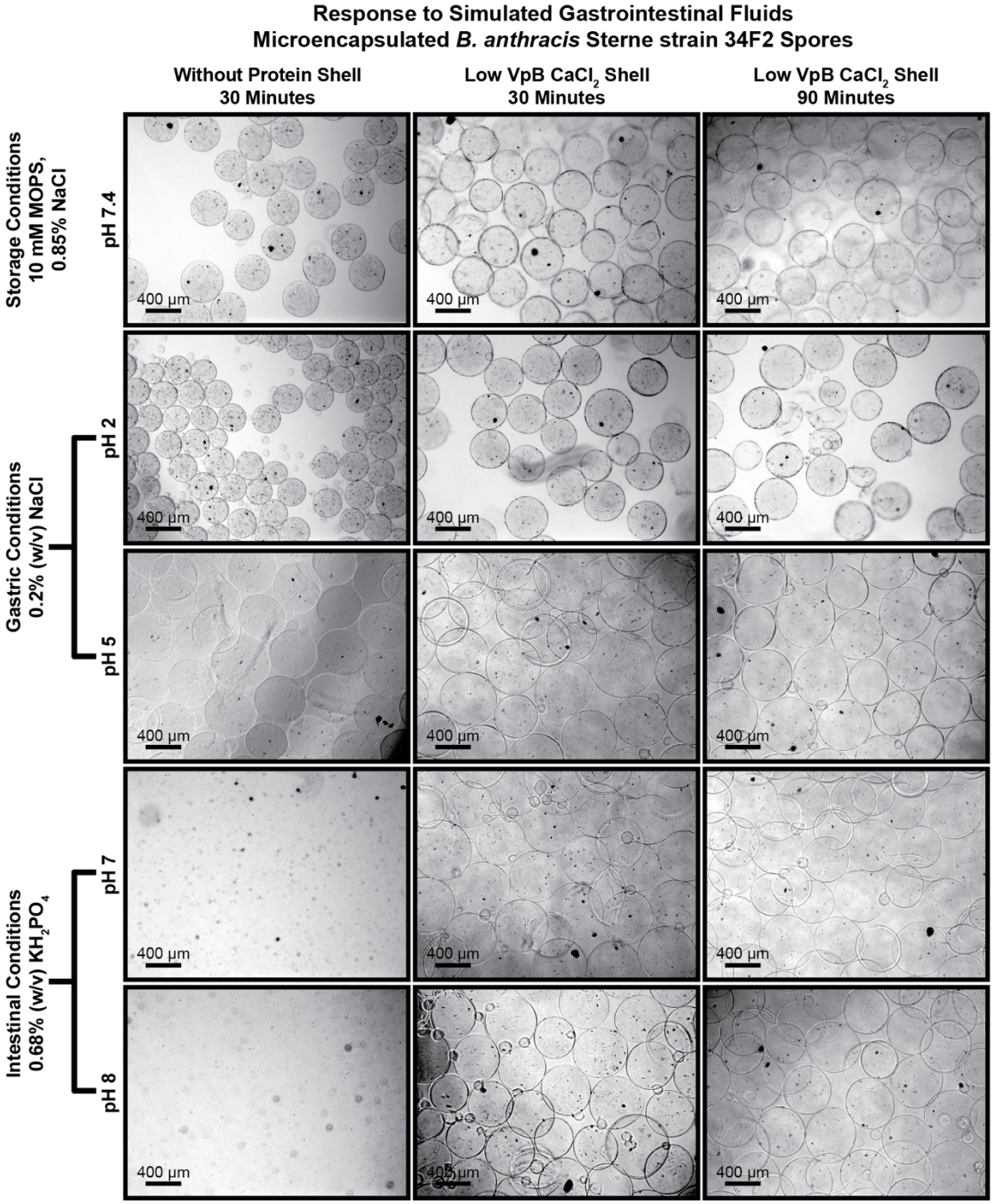

Figure 4.14. Microscope images of the Low VpB $CaCl_2$ capsule response to simulated gastrointestinal environments. Representative brightfield images of Low VpB $CaCl_2$ (0.017% PLL with 0.0025% VpB) capsules following exposure to simulated gastric and intestinal fluids for 30 minutes (middle) and 90 minutes (right). The uncoated capsule response after 30 minutes is also included for reference (left) with the bottom two images portraying the dissolution of uncoated capsules at pH 7 and pH 8.

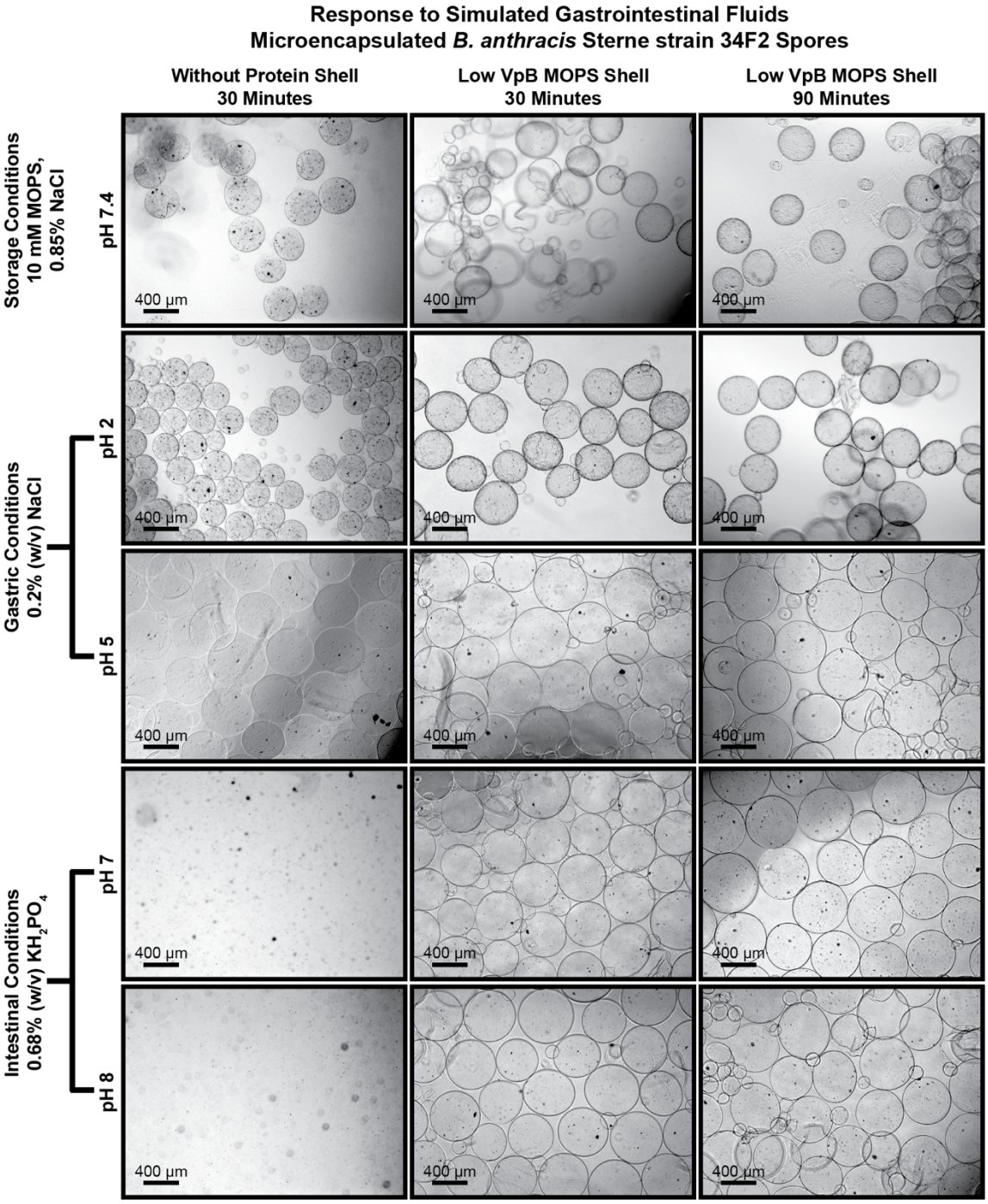

Figure 4.15. Microscope images of the Low VpB MOPS capsule response to simulated gastrointestinal environments. Representative brightfield images of Low VpB MOPS (0.017% PLL with 0.0025% VpB) capsules following exposure to simulated gastric and intestinal fluids for 30 minutes (middle) and 90 minutes (right). The uncoated capsule response after 30 minutes is also included for reference (left) with the bottom two images portraying the dissolution of uncoated capsules at pH 7 and pH 8.

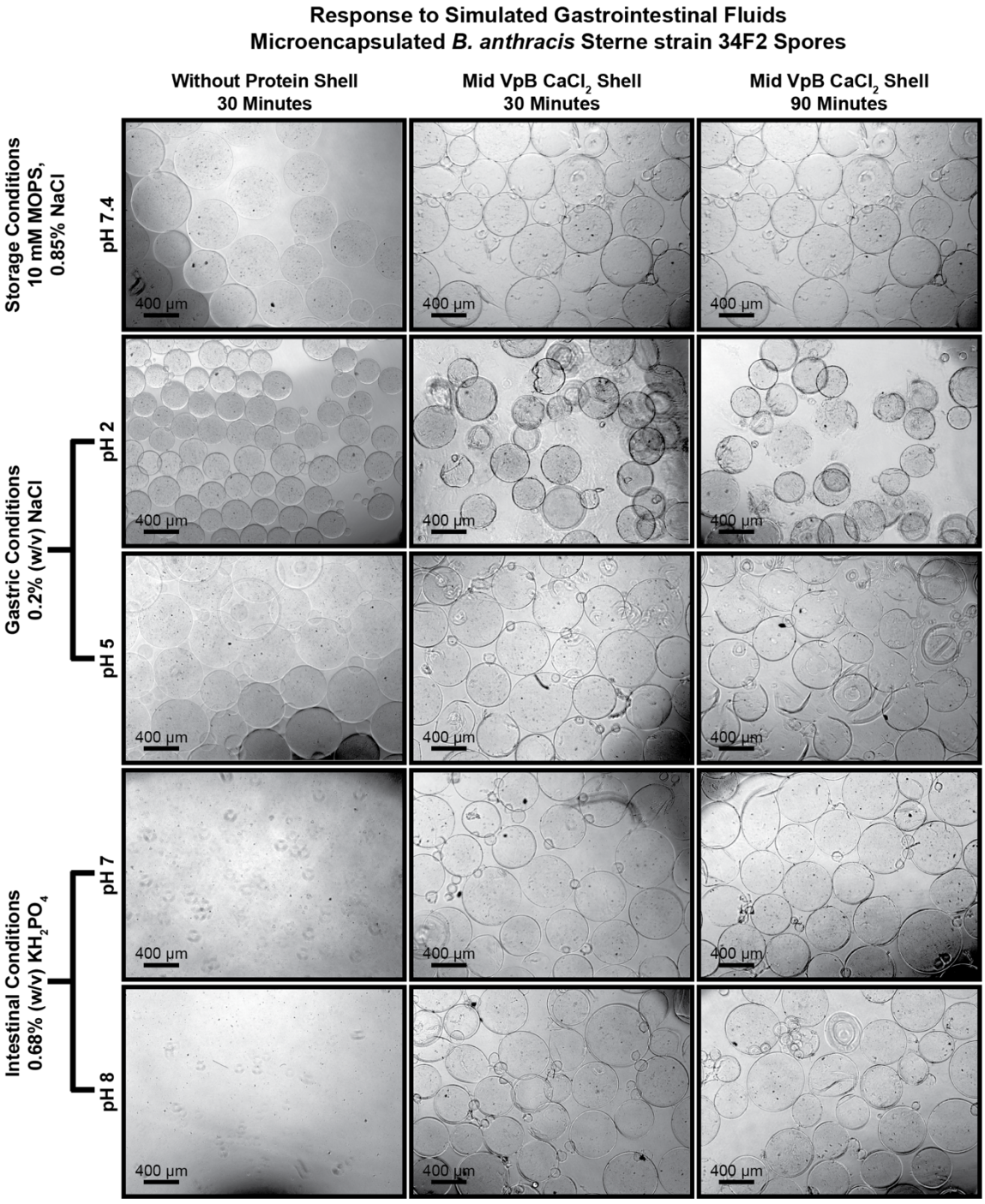

Figure 4.16. Microscope images of the Mid VpB $CaCl_2$ capsule response to simulated gastrointestinal environments. Representative brightfield images of Mid VpB $CaCl_2$ (0.017% PLL with 0.0064% VpB) capsules following exposure to simulated gastric and intestinal fluids for 30 minutes (middle) and 90 minutes (right). The uncoated capsule response after 30 minutes is also included for reference (left) with the bottom two images portraying the dissolution of uncoated capsules at pH 7 and pH 8.

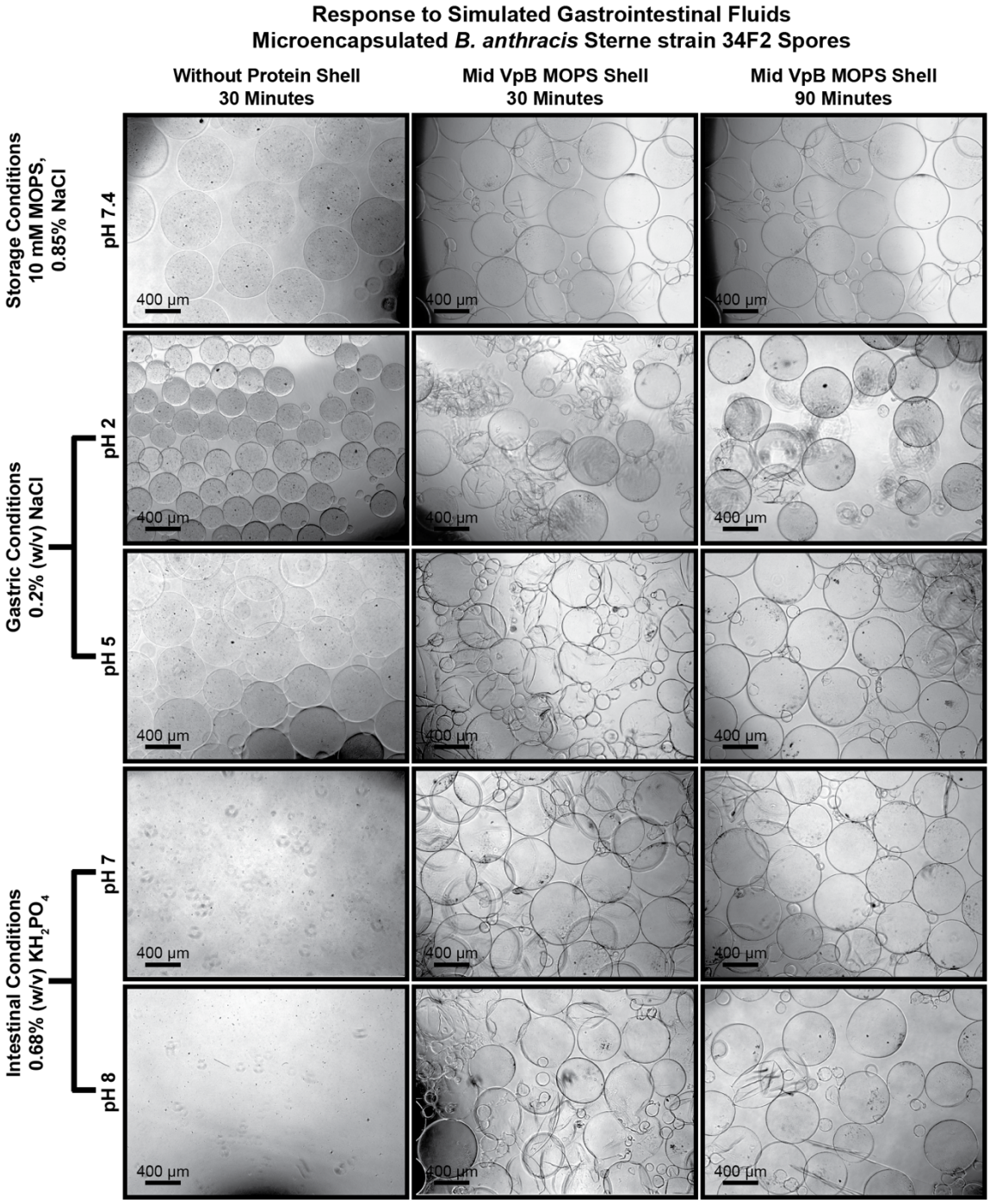

Figure 4.17. Microscope images of the Mid VpB MOPS capsule response to simulated gastrointestinal environments. Representative brightfield images of Mid VpB MOPS (0.017% PLL with 0.0064% VpB) capsules following exposure to simulated gastric and intestinal fluids for 30 minutes (middle) and 90 minutes (right). The uncoated capsule response after 30 minutes is also included for reference (left) with the bottom two images portraying the dissolution of uncoated capsules at pH 7 and pH 8.

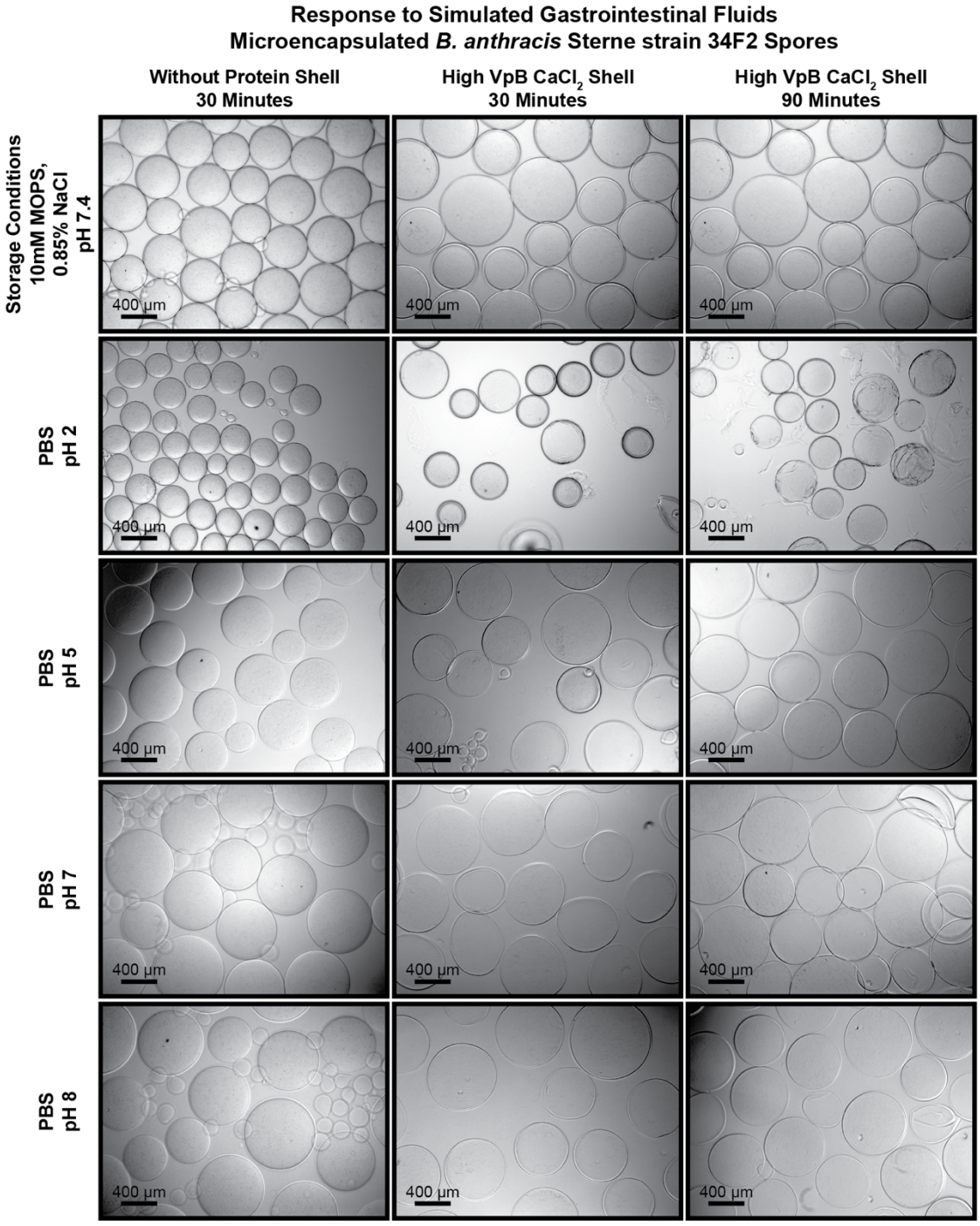

Figure 4.18. Microscope images of the High VpB $CaCl_2$ capsule response to simulated gastrointestinal environments. Representative brightfield images of High VpB $CaCl_2$ (0.017% PLL with 0.017% VpB) capsules following exposure to PBS at pH 2, 5, 7 and 8 for 30 minutes (middle) and 90 minutes (right). The uncoated capsule response after 30 minutes (left) portrays the lack of capsule dissolution in PBS pH 7 and 8.

4.4. Discussion

The benefits of oral delivery cannot be overstated, particularly when it comes to protecting free-ranging livestock and wildlife from current and emerging infectious diseases such as anthrax. Development of oral vaccines can allow for easy, wide-spread vaccination policies without needing to deal with the labor-intensive programs and painful injections associated with the majority of today's human and animal vaccines. It is also possible that effective oral vaccines may be intrinsically more stable and have longer shelf-lives as a collateral benefit of the stability required for transit through the gastrointestinal tract. Furthermore, oral vaccines can lead to enhanced efficacy with less adverse effects due to mucosal immunity and oral delivery. Experiments conducted in the current study evaluated alternate formulations of a microencapsulated vaccine to identify the optimal microcapsule formulation for vaccine efficacy and microcapsule stability for oral administration.

Previous studies from our laboratory have reported enhanced protection against wild type challenge following vaccination with microencapsulated *Brucella spp*.[87–90]. We recently applied the same encapsulation method to *B. anthracis* Sterne strain 34F2 spores by coating spore-laden capsules with a mixture of PLL and VpB. Resulting antibody titers from mice subcutaneously and orally vaccinated with similar microcapsules were already shown to be protective against *in vitro* LeTx challenge, but we administered the vaccine with a higher bacterial load in an effort to provide better protection. Subcutaneous injection with approximately $8.8x10^9$ Sterne spores per mouse in VpB Shell capsules resulted in extraordinarily high serum IgG responses (Figure 4.5)

that were fully protective within eight days post vaccination (Figure 4.9). This antibody response also may not yet have reached its peak prior to the end of the experiment. The *in vitro* release experiment demonstrated that VpB Shell capsules were still releasing Sterne spores 56 days after vaccination (Figure 4.3) and that there was an excessive amount of Sterne spores and vegetative cells still entrapped within the VpB Shell capsules suggesting that the controlled release could have continued for much longer (Figure 4.4).

According to previous work on mouse susceptibility to *B. anthracis* strains, the LD_{50} for BALB/cJ mice subcutaneously injected with the Sterne strain was $6.8x10^7$ spores [25]. In this study, BALBc/J mice were subcutaneously injected with over 100-fold times more Sterne spores with only one death, implying that this microencapsulation method can allow for enhanced protection with higher Sterne spore doses and less reactogenicity. Inoculation with a higher dose of Sterne spores could also be critical for successful oral vaccination. Sterne spore exposure to acidic environments greatly reduces the viable spore titer (Figure 3.3), so vaccinating with a higher dose of microencapsulated Sterne spores may account for any titer loss due to the gastric environment [82]. Unfortunately orally vaccinating mice with $8.8x10^9$ spores/mouse in VpB Shell capsules and VpB Core & Shell capsules did not support this theory (Figure 4.6), though the low serum IgG response from both orally inoculated capsules could be a consequence of Sterne spore auto-germination within the capsules (Figure 4.4) [110]. Sterne vegetative cells are not as resilient as Sterne spores, so it's plausible that the viable Sterne spore titer in the VpB Shell and VpB Core & Shell capsules was severely

reduced before the capsules reached the small intestine [1,8,110]. To account for the reduced spore titer, orally vaccinated mice were given a vaccine booster 28 post vaccination. This booster dose also did not induce the same level of serum IgG observed in our previous studies (Figure 3.5), but it did induce a significant antibody response following oral vaccination that was improved by incorporating VpB into the capsule core. Although the auto-germination was not intended and negatively affected the overall success of these oral vaccines, it confirmed that the mild encapsulation process maintained the bacterial viability so much so that the Sterne spores were able to germinate within the alginate microcapsules. Another explanation for the decreased antibody response observed here as opposed to the previous study could be the altered ratio of PLL to VpB. In the previous study, capsules were coated with 0.047% PLL and 0.0067% VpB whereas in this study capsules were coated with 0.0017% PLL and 0.0032%. Both PLL and VpB are reduced by approximately half and the ratio of PLL to VpB decreased from 7 to 5.3. It is likely that both modifications factored into the reduced success and further experiments must be conducted to elucidate whether it was the amount of each component or the ratio between them that had the greatest affect. While none of the serum IgG responses from orally vaccinated mice were protective against LeTx *in vitro* (Figure 4.9), the minor responses still imply that further research to prevent auto-germination, such as by incorporating the anti-germinator D-alanine into the microcapsule, and optimizing the capsule formulation could lead to a protective oral vaccine.

Anthrax infections in wildlife usually develop when anthrax spores gain access to mucosal surfaces following ingestion or inhalation by a grazing animal. Ideally, successful oral vaccination would neutralize the pathogen at the site of entry by stimulating the mucosal immune response, generally marked by the induction of secretory IgA [102,111]. To estimate the level of mucosal stimulation, both serum and fecal IgA were measured following subcutaneous and oral vaccination. Serum IgA only appeared to be induced by subcutaneously injected VpB Shell capsules implying that this microcapsule formulation may be stimulating the immune response differently than the Sterne vaccine (Figure 4.7). Conversely, small amounts of fecal IgA were induced by both oral capsule vaccines before the booster dose suggesting that, despite losing titer due to auto-germination, the capsules provided some protection for Sterne spores in transit to the intestines, and potentially assisted in Sterne spore uptake and processing through mucoadhesion (Figure 4.8) [82]. Interestingly, the Sterne vaccine did not induce IgA in serum or feces, yet it is still generally protective for animals exposed to anthrax via mucosal surfaces. While other pathogens may require IgA for neutralization at mucosal surfaces, these contradicting results suggest that serum IgG may be enough for successful oral vaccination against anthrax. However, given the ambiguity of both the serum and fecal IgA results, and the relatively low levels we've measured compared to other studies, the data presented here may not be accurate and will need to be clarified through future studies [76,77].

The remaining microcapsule formulations evaluated in this study specifically investigated how modifications in the PLL and VpB coating affected the capsule

stability in simulated gastrointestinal environments. Sodium alginate polymers are composed of linear copolymer blocks of α-L-guluronic acid and β (1→4) linked D-mannuronic acids residues that are converted to guluronate (G) and mannuronate (M) when extracted from natural sources of brown algae, such as *Laminaria hyperborean* and *Macrocystis pyrifera* [83,84,104]. When alginate capsules are extruded through an encapsulator nozzle and released into 100 mM $CaCl_2$, 10 mM MOPS ($CaCl_2$), G residues on opposing polymer chains will cooperatively bind Ca^{2+} ions from the solution, thus cross-linking the alginate polymers to the "pre-gel" state [83,109]. Exposure of a calcium cross-linked pre-gel to nongelling cations, such as Na^+, will reduce the mechanical stability of the alginate gel and possibly disintegrate the entire polymer matrix, as exhibited in Figure 4.10 [83,112]. This can be prevented by adding additional cross-linked layers to the microcapsules, thus resulting in more stable capsules [113]. PLL is a polycation that cross-links preferentially with the negatively charged M residues in alginate to form an electrostatic complex [107,112]. In this way, PLL stabilizes the alginate gel and helps control its permeability [107,109]. PLL used in previous capsule formulations has been dissolved in $CaCl_2$, but it's possible that the presence of divalent cations may negatively affect PLL cross-linking. Therefore, we assessed the changes to capsule stability when PLL was dissolved in $CaCl_2$ or MOPS, and when varying amounts of VpB were added to the capsules.

Microcapsule shrinking, swelling and overall morphology can be used as markers of capsule stability in different environments because changes in the alginate polymer network such as these can greatly affect the rate of diffusion through and the erosion of

the network, thereby altering the antigen release rate [84,114,115]. Capsule shrinkage, as observed in uncoated capsules at pH 2 (Figure 4.10), is indicative of increased acid-gel strength under higher proton concentrations [91]. Conversely, suspending uncoated capsules in pH 5 caused excessive swelling which implies increased pore size and therefore release of bacteria, whereas pH 7 and pH 8 resulted in complete disintegration of uncoated alginate capsules in the presence of nongelling ions (Figure 4.10) [83,112]. Regardless of what PLL was dissolved in or how much VpB was incorporated, results of this study demonstrate the efficacy of using both PLL and VpB in the microcapsule formulation because the PLL and VpB shell prevented most of the destabilizing affects observed in uncoated capsules. Specifically, the PLL and VpB shell reduced the degree of swelling experienced by the capsules at pH 5, thereby avoiding drastic changes in the polymer network that could have led to premature bacterial release. Of most importance was that the PLL and VpB shell maintained the capsule integrity at pH 7 and 8, whereas other studies have observed alginate disintegration at pH 7 and 8 [116,117]. By preventing complete capsule dissolution at pH 7 and 8, the PLL and VpB shell ensures that the capsule is stable enough to serve its controlled release purpose by stimulating mucosal immunity and uptake in the intestines. While it would have also been valuable to determine the surviving encapsulated spore titer following uncoated and coated capsule exposure to gastrointestinal pHs, this would have involved dissolving the PLL and VpB coated capsules in trypsin which likely would have had its own effect on the viable Sterne spore recovery, therefore confounding any results that may have been acquired from the experiment.

When comparing VpB MOPS capsules to VpB $CaCl_2$ capsules, both versions of the VpB MOPS capsules are less stable. Under storage conditions (10 mM MOPS, 0.85% NaCl) and upon exposure to pH 2, Low VpB MOPS capsules were wrinkly (Figure 4.15) implying that the integrity of the capsules were threatened and on the verge of collapse [118]. Low VpB MOPS capsules also appeared to swell slightly at pH 2 (Figure 4.11). While this swelling was insignificant, it can be assumed that any swelling at such a low pH may have released bacteria into the gastric environment. The instability of the Low VpB MOPS capsules was exacerbated in the Mid VpB MOPS capsules. Mid VpB MOPS capsules did undergo some shrinking at pH2 again, suggesting increased acid-gel strength, but the shrunken capsules appeared to be degraded, as evidenced by the alginate fragments in the microscope image (Figure 4.17). At pH 5, 7 and 8 there was a lot of variability in the average capsule diameter (Figure 4.12). There was also a lot of Mid VpB MOPS capsule degradation at pH 5 and even a small degree of degradation at pH 7 and 8 suggesting that the combination of MOPS as the PLL solvent and VpB counteracted the stabilization abilities of a PLL cross-linked layer. In comparison, the relative stability observed from all VpB $CaCl_2$ capsule formulations corroborates that the presence of divalent cations in the PLL cross-linking solution doesn't inhibit PLL cross-linking but likely enhances it by promoting more G to G residue cross-linking by Ca^{2+} concurrently with PLL cross-linking the M residues [107,112].

After confirming that VpB $CaCl_2$ capsules demonstrated greater stability in simulated gastrointestinal environments, we examined the effect of varying amounts of VpB on capsule stability. VpB is resistant to chemical and enzymatic breakdown, thus it

was incorporated into these microcapsule formulations to prolong the bacterial release rate from the capsules once they had arrived in the small intestine [85,86,89]. When visually comparing Low VpB $CaCl_2$ capsules to Mid and High VpB $CaCl_2$ capsules, we observed decreased capsule diameters at pH 2 along with increased capsule degradation as the amount of VpB increased. According to the average capsule diameters, Low VpB $CaCl_2$ capsules maintained a constant size between storage conditions and pH 2 while Mid and High VpB $CaCl_2$ capsules initially had larger diameters that were significantly reduced by exposure to pH 2. As before, this suggests that the Mid and High VpB capsules may have been more protective because capsule shrinking insinuates increased acid-gel strength and stability at pH 2, but the observed degradation also suggests that the acid-gel state has been destabilized [91]. Comprehensive studies on the alginate and PLL relationship have suggested that there is a very fine balance between the microcapsule size, the amount of PLL and the binding time of PLL [118]. Small modifications to this balance, such as the addition of VpB in a slightly different ratio, can cause destabilized microcapsules at pH 2, as exhibited by the collapsed and eroded capsules in Figure 4.16 and Figure 4.18 [118]. This capsule degradation was only observed following capsule exposure to acidic conditions because all VpB $CaCl_2$ capsules appeared very stable at pH 5, 7 and 8 (Figure 4.14, Figure 4.16, Figure 4.18), however another consideration when assessing capsule stability is the number of shrinking and swelling phases a capsule has to endure. Mid and High VpB $CaCl_2$ capsules have an initial diameter of approximately 500 µm, then shrink at pH 2 and swell again at pH 5, 7 and 8, while Low VpB $CaCl_2$ capsules would likely only endure

one swelling phase in transit through the gastrointestinal tract (Figure 4.13). Encapsulated Sterne spore titers would likely need to be adjusted to account for more than one shrinking or swelling phase and ensure that the necessary bacterial load reaches the small intestine. These comparisons do contain some ambiguity as the pH testing buffers were different for the High VpB $CaCl_2$ capsules, nevertheless these results indicate that coating microcapsules with PLL and VpB ensures stability at pH 5, 7 and 8 to allow for sustained release once arriving in the small intestine. It is also evident that the PLL and VpB ratio must be optimized to ensure microcapsule stability at pH 2 as well as at the higher pHs because, as evidenced by the minimal antibody response to orally administered capsules in this study, sustained release in pH 7 and 8 will not be effective if the bacterial load does not survive the stomach.

In summary, the findings of this study suggest that microencapsulation of *Bacillus anthracis* Sterne strain 34F2 spores can enhance the immune response following subcutaneous injection by inoculating a higher bacterial dose with limited adverse effects. We have also demonstrated the controlled release aspects of our microcapsule formulation containing PLL and VpB. A corresponding immune response was not detected following oral vaccination but other experiments in this study have revealed approaches to continue optimizing this encapsulated vaccine for oral delivery. By observing the changes in capsule stability that result from minor changes in the PLL and VpB coating mixture, we've confirmed that PLL should be dissolved in 100 mM $CaCl_2$, 10 mM MOPS and that by incorporating different amounts of VpB, we can tailor the microcapsule formulation to the desired range of stability. Further studies can

continue defining the exact parameters of the PLL and VpB ratio for ideal capsule stability in a variety of gastrointestinal environments, eventually resulting in a stable and controlled release vehicle for oral administration of an anthrax vaccine and many other antigens in the future.

5. CONCLUSION

5.1. Summary

Anthrax is a zoonotic disease with worldwide distribution. Caused by the spore-forming bacterium, *Bacillus anthracis*, anthrax spores are capable of surviving for decades in nature's harshest environments. The unparalleled resilience of anthrax spores ensures that there will be always be anthrax outbreaks in animals, and inevitably humans, making anthrax a one health disease of global economic, ecological and conservational importance. There is no realistic method for global eradication of anthrax spores from the environment, thus the best method of prevention is vaccination.

The Sterne vaccine is the current vaccine formulation produced worldwide for anthrax prevention in domesticated livestock. It consists of a suspension of live attenuated *B. anthracis* Sterne strain 34F2 spores in saponin and is distributed as a subcutaneously injected vaccine. Free-ranging livestock and wildlife cannot practically be vaccinated by individual injections, so an oral vaccine formulation is required to permit realistic and effective vaccination for all animals at risk of exposure. To clearly demonstrate that the Sterne vaccine is not effective when orally administered, mice were vaccinated subcutaneously and orally with three doses of the Sterne vaccine. Strong antibody responses from all three doses were only detected following subcutaneous injection. Oral vaccination with any dose of the Sterne strain did not induce any detectable antibody response.

To begin development of an oral anthrax vaccine formulation, we encapsulated Sterne strain 34F2 spores in alginate microcapsules that were coated with a mixture of

poly-L-lysine and vitelline protein B, a non-immunogenic eggshell precursor protein isolated from the parasite *Fasciola hepatica*. Since viable Sterne spore titers are greatly reduced after exposure to acidic environments, we assessed whether the stability of alginate at acidic pHs would make oral vaccination possible by preserving Sterne spore viability while in transit through the gastric environment. We used the mouse model to test the immunogenicity of a microencapsulated vaccine formulation with subcutaneous and oral administration. In comparison to the unencapsulated Sterne vaccine, the microcapsule vaccine stimulated an improved antibody response following subcutaneous injection. Oral vaccination with the microcapsule vaccine also resulted in a detectable antibody response. Moreover, the resulting immune responses from both subcutaneous and oral vaccination with the microcapsule vaccine induced protective antibody titers against anthrax lethal toxin challenge *in vitro*. Also, white-tailed deer serum samples from outside of the enzootic anthrax zone in Texas were screened for seroprevalence against anthrax protective antigen and exhibited over 80% seroprevalence for anthrax specific antibodies implying that anthrax exposure may be more common than previously thought.

We also investigated if making small modifications to the microcapsule formulations could result in improved immune responses and stability from the microcapsule vaccine. Increasing the dose of Sterne spores stimulated much stronger antibody responses with limited adverse effects after subcutaneous injection. The same enhancement was not observed after oral vaccination, possibly because of reduced Sterne spore titers from auto-germination or the modified microcapsule formulations did

not work as effectively as the first one. We were able to demonstrate controlled release of Sterne spores from poly-L-lysine and vitelline protein B coated capsules over at least 56 days with the potential to continue for much longer. Additionally, we evaluated how minor changes in the microcapsule formulation affected the stability of capsules exposed to simulated gastrointestinal environments. Results of these experiments exhibited better stability in capsules with poly-L-lysine dissolved in 100 mM $CaCl_2$, 10 mM MOPS. We also observed an apparent dose response of microcapsule stability at gastric pHs to the amount of vitelline protein B incorporated into the capsule.

5.2. Future Directions

A protective immune response against *in vitro* toxin challenge was induced by oral vaccination with microencapsulated *B. anthracis* Sterne strain 34F2 spores. These capsules were coated with a mixture of 0.047% poly-L-lysine and 0.0067% vitelline protein B. Future work can continue to assess changes in the microcapsule vaccine stability and efficacy from alternative ratios of poly-L-lysine and vitelline protein B. Additional studies on this microcapsule formulation should examine the efficacy in various animal models, particularly ruminants, as well as the shelf life, stability at ambient temperatures and incorporation into wildlife bait. The research of this dissertation established a novel vaccine candidate for oral vaccination against anthrax that, with further development, has the potential to provide practical and effective vaccination to wildlife worldwide.

REFERENCES

[1] Bergman NH. Bacillus anthracis and Anthrax. 2010. doi:10.1002/9780470891193.

[2] KLEMM DM, KLEMM WR. A history of anthrax. J Am Vet Med Assoc 1959.

[3] Sterne M. Distribution and economic importance of anthrax. Fed Proc 1967;26:1493–5.

[4] Hugh-Jones ME, de Vos V. Anthrax and wildlife. Rev Sci Tech 2002;21:359–83.

[5] Scorpio A, Blank TE, Day WA, Chabot DJ. Anthrax vaccines: Pasteur to the present. Cell Mol Life Sci 2006;63:2237–48. doi:10.1007/s00018-006-6312-3.

[6] Sterne M. Variation in Bacillus anthracis. Onderstepoort J Vet Sci Anim Ind 1937;8:271–348.

[7] Manchee RJ, Broster MG, Stagg AJ, Hibbs SE. Formaldehyde solution effectively inactivates spores of Bacillus anthracis on the Scottish Island of Gruinard. Appl Environ Microbiol 1994;60:4167–71.

[8] Fasanella A, Galante D, Garofolo G, Jones MH. Anthrax undervalued zoonosis. Vet Microbiol 2010;140:318–31. doi:10.1016/j.vetmic.2009.08.016.

[9] Munyua P, Bitek A, Osoro E, Pieracci EG, Muema J, Mwatondo A, et al. Prioritization of zoonotic diseases in Kenya, 2015. PLoS One 2016;11:e0161576. doi:10.1371/journal.pone.0161576.

[10] Smith KL, DeVos V, Bryden H, Price LB, Hugh-Jones ME, Keim P. Bacillus anthracis diversity in Kruger National Park. J Clin Microbiol 2000.

[11] Carlson CJ, Kracalik IT, Ross N, Alexander KA, Hugh-Jones ME, Fegan M, et al.

The global distribution of Bacillus anthracis and associated anthrax risk to humans, livestock and wildlife. Nat Microbiol 2019;4:1337–43. doi:10.1038/s41564-019-0435-4.

[12] Turnbull P, editor. Anthrax in Humans and Animals. 4th ed., World Health Organization and International Office of Epizootics; 2008.

[13] Morris LR, Proffitt KM, Asher V, Blackburn JK. Elk resource selection and implications for anthrax management in Montana. J Wildl Manage 2016;80:235–44. doi:10.1002/jwmg.1016.

[14] Blackburn JK, Asher V, Stokke S, Hunter DL, Alexander K a. Dances with anthrax: wolves (Canis lupus) kill anthrax bacteremic plains bison (Bison bison bison) in southwestern Montana. J Wildl Dis 2014;50:393–6. doi:10.7589/2013-08-204.

[15] Morris LR, Blackburn JK. Predicting Disease Risk, Identifying Stakeholders, and Informing Control Strategies: A Case Study of Anthrax in Montana. Ecohealth 2016;13:262–73. doi:10.1007/s10393-016-1119-7.

[16] Dragon DC, Elkin BT. An overview of early anthrax outbreaks in northern Canada: Field reports of the health of animals branch, agriculture Canada, 1962-71. Arctic 2001. doi:10.14430/arctic761.

[17] Dragon DC, Rennie RP, Elkin BT. Detection of anthrax spores in endemic regions of northern Canada. J Appl Microbiol 2001. doi:10.1046/j.1365-2672.2001.01389.x.

[18] Blackburn JK, Curtis A, Hadfield TL, O'Shea B, Mitchell MA, Hugh-Jones ME.

Confirmation of Bacillus anthracis from flesh-eating flies collected during a West Texas anthrax season. J Wildl Dis 2010;46:918–22. doi:10.7589/0090-3558-46.3.918.

[19] Blackburn JK, Goodin DG. Differentiation of springtime vegetation indices associated with summer anthrax epizootics in west Texas, USA, deer. J Wildl Dis 2013;49:699–703. doi:10.7589/2012-10-253.

[20] Gates CC, Elkin BT, Dragon DC. Investigation, control and epizootiology of anthrax in a geographically isolated, free-roaming bison population in northern Canada. Can J Vet Res 1995.

[21] Hugh-Jones M, Blackburn J. The ecology of Bacillus anthracis. Mol Aspects Med 2009;30:356–67. doi:10.1016/j.mam.2009.08.003.

[22] Lincoln RE, Walker JS, Klein F, Rosenwald AJ, Jones WI. Value of field data for extrapolation in anthrax. Fed Proc 1967.

[23] Sterne M. The use of Anthrax Vaccines Prepared from Avirulent (Uncapsulated) Variants of Bacillus anthracis. Onderstepoort J Vet Sci Anim Ind 1939;13:307–12.

[24] Welkos SL, Friedlander AM. Comparative safety and efficacy against Bacillus anthracis of protective antigen and live vaccines in mice. Microb Pathog 1988;5:127–39.

[25] Welkos SL, Keener TJ, Gibbs PH. Differences in Susceptibility of Inbred Mice to Bacillus-Anthracis. Infect Immun 1986;51:795–800.

[26] Blackburn JK, Hadfield TL, Curtis AJ, Hugh-Jones ME. Spatial and Temporal

Patterns of Anthrax in White-Tailed Deer, Odocoileus virginianus, and Hematophagous Flies in West Texas during the Summertime Anthrax Risk Period. Ann Assoc Am Geogr 2014;104:939–58. doi:10.1080/00045608.2014.914834.

[27] Gainer RS, Saunders JR. Aspects of the epidemiology of anthrax in Wood Buffalo National Park and environs. Can Vet J = La Rev Vet Can 1989.

[28] Lindeque PM, Turnbull PCB. Ecology and Epidemiology of Anthrax in the Etosha-National-Park, Namibia. Onderstepoort J Vet Res 1994;61:71–83.

[29] Braack LE, De Vos V. Feeding habits and flight range of blow-flies (Chrysomyia spp.) in relation to anthrax transmission in the Kruger National Park, South Africa. Onderstepoort J Vet Res 1990.

[30] Blackburn JK, Van Ert M, Mullins JC, Hadfield TL, Hugh-Jones ME. The necrophagous fly anthrax transmission pathway: Empirical and genetic evidence from wildlife epizootics. Vector-Borne Zoonotic Dis 2014;14:576–83. doi:10.1089/vbz.2013.1538.

[31] Caroid T, Argueta Y, Briolat ES, Bruggink J, Kasprowsky M, Lake J, et al. Benefits of zebra stripes: Behaviour of tabanid flies around zebras and horses 2019. doi:10.1371/journal.pone.0210831.

[32] Pilo P, Frey J. Bacillus anthracis: Molecular taxonomy, population genetics, phylogeny and patho-evolution. Infect Genet Evol 2011;11:1218–24. doi:10.1016/j.meegid.2011.05.013.

[33] Takken W, Verhulst NO. Host Preferences of Blood-Feeding Mosquitoes. Annu

Rev Entomol 2013. doi:10.1146/annurev-ento-120811-153618.

[34] Service MW. The daytime distribution of mosquitoes resting in vegetation. J Med Entomol 1971. doi:10.1093/jmedent/8.3.271.

[35] Blitz M. The Best Places to See Wild Horses in North America 2015. https://www.smithsonianmag.com/travel/best-places-see-wild-horses-north-america-180956363/ (accessed June 30, 2018).

[36] Beever E. Management implications of the 2019;31:887–95.

[37] Wolfe ML. POPULATION DYNAMICS OF FERAL HORSES IN WESTERN NORTH AMERICA. n.d.

[38] Lembo T, Hampson K, Auty H, Beesley CA, Bessell P, Packer C, et al. Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009. Emerg Infect Dis 2011;17:387–94. doi:10.3201/eid1703.101290.

[39] Berry HH. Surveillance and control of anthrax and rabies in wild herbivores and carnivores in Namibia. Rev Sci Tech 1993;12:137–46.

[40] Hampson K, Lembo T, Bessell P, Auty H, Packer C, Halliday J, et al. Predictability of anthrax infection in the Serengeti, Tanzania. J Appl Ecol 2011;48:1333–44. doi:10.1111/j.1365-2664.2011.02030.x.

[41] Bagamian KH, Skrypnyk A, Rodina Y, Bezymennyi M, Nevolko O, Skrypnyk V, et al. Serological anthrax surveillance in wild boar (Sus scrofa) in Ukraine. Vector Borne Zoonotic Dis 2014;14:618–20. doi:10.1089/vbz.2013.1521.

[42] Lyon DG. An outbreak of anthrax at the Chester Zoological Gardens. Vet Rec 1973. doi:10.1136/vr.92.13.334.

[43] Turnbull PCB, Doganay M, Aygen B, Lindeque PM, Mclaughlin J. Serology and anthrax in humans, livestock and Etosha National Park wildlife. Epidemiol Infect 1992;108:299–313. doi:10.1017/S0950268800049773.

[44] Miller RM. A case of anthrax in an American mountain lion. Vet Med 1968;63:952–3.

[45] Shury TK, Frandsen D, O'Brodovich L. Anthrax in free-ranging bison in the prince albert national park area of saskatchewan in 2008. Can Vet J 2009.

[46] Lindeque PM, Nowell K, Preisser T, Brian C, Turnbull PCB. Anthrax in wild cheetahs in the Etosha NP, Namibia. ARC- Onderstepoort OIE Int. Congr. 9-15 August 1998., 1998.

[47] Leendertz FH, Ellerbrok H, Boesch C, Couacy-Hymann E, Mätz-Rensing K, Hakenbeck R, et al. Anthrax kills wild chimpanzees in a tropical rainforest. Nature 2004;430:451–2. doi:10.1038/nature02722.

[48] Hoffmann C, Zimmermann F, Biek R, Kuehl H, Nowak K, Mundry R, et al. Persistent anthrax as a major driver of wildlife mortality in a tropical rainforest. Nature 2017;548:82–5. doi:10.1038/nature23309.

[49] Leendertz FH, Yumlu S, Pauli G, Boesch C, Couacy-Hymann E, Vigilant L, et al. A new Bacillus anthracis found in wild chimpanzees and a gorilla from west and central Africa. PLoS Pathog 2006. doi:10.1371/journal.ppat.0020008.

[50] Blackburn JK, Skrypnyk A, Bagamian KH, Nikolich MP, Bezymennyi M, Skrypnyk V. Anthrax in a Backyard Domestic Dog in Ukraine: A Case Report. Vector-Borne Zoonotic Dis 2014;14:615–7. doi:10.1089/vbz.2013.1519.

[51] Turnbull PC, Bell RH, Saigawa K, Munyenyembe FE, Mulenga CK, Makala LH. Anthrax in wildlife in the Luangwa Valley, Zambia. Vet Rec 1991. doi:10.1136/vr.128.17.399.

[52] Ebedes H. Anthrax Epizootics in Wildlife in the Etosha National Park, South west Africa. Wildl. Dis., 1976. doi:10.1007/978-1-4757-1656-6_58.

[53] Anthrax in South Africa. J Comp Pathol Ther 1920. doi:10.1016/s0368-1742(20)80025-3.

[54] Pienaar UD. Epidemiology of anthrax in wild animals and the control of anthrax epizootics in the Kruger National Park, South Africa. Fed Proc 1967.

[55] Carlson CJ, Getz WM, Kausrud KL, Cizauskas CA, Blackburn JK, Bustos Carrillo FA, et al. Spores and soil from six sides: interdisciplinarity and the environmental biology of anthrax (Bacillus anthracis). Biol Rev 2018;93:1813–31. doi:10.1111/brv.12420.

[56] Ganz HH, Turner WC, Brodie EL, Kusters M, Shi Y, Sibanda H, et al. Interactions between Bacillus anthracis and Plants May Promote Anthrax Transmission. PLoS Negl Trop Dis 2014;8:e2903. doi:10.1371/journal.pntd.0002903.

[57] Havarua Z, Turner WC, Mfune JK. Seasonal variation in zebra foraging behaviour may alter contact with the anthrax bacterium. Can J Zool 2014;337:140227143805006. doi:10.1139/cjz-2013-0186.

[58] Schwartz A, Director E. NEWS RELEASE Texas Animal Health Commission Texas Anthrax Situational Update. n.d.

[59] Pienaar EF, Rubino EC, Saayman M, Van Der Merwe P. Attaining sustainable use on private game ranching lands in South Africa. Land Use Policy 2017;65:176–85. doi:10.1016/j.landusepol.2017.04.005.

[60] Bulte EH, Horan RD. Does Human Population Growth Increase Wildlife Harvesting? An Economic Assessment. J Wildl Manage 2002;66:574. doi:10.2307/3803125.

[61] Traweek M, Welch R. Exotics in Texas. Texas Park Wildl Dep 1992;PWD-BK-W70.

[62] Benn Felix J, Chaki SP, Ficht TA, Rice-Ficht AC, Cook W. Bacillus anthracis Sterne Strain 34F2 Vaccine Antibody Dose Response by Subcutaneous and Oral Administration. Poultry, Fish Wildl Sci 2019;7:1–6. doi:10.35248/2375-446X.19.7.206.

[63] Chitlaru T, Altboum Z, Reuveny S, Shafferman A. Progress and novel strategies in vaccine development and treatment of anthrax. Immunol Rev 2011;239:221–36. doi:10.1111/j.1600-065X.2010.00969.x.

[64] Mongoh MN, Dyer NW, Stoltenow CL, Khaitsa ML. Risk factors associated with anthrax outbreak in animals in North Dakota, 2005: A retrospective case-control study. Public Health Rep 2008;123:352–9. doi:10.1177/003335490812300315.

[65] Food and Drug Administration. Veterinary Feed Directive; Final Rule. 2015.

[66] Ndumnego OC, Köhler SM, Crafford J, van Heerden H, Beyer W. Comparative analysis of the immunologic response induced by the Sterne 34F2 live spore Bacillus anthracis vaccine in a ruminant model. Vet Immunol Immunopathol

2016;178:14–21. doi:10.1016/j.vetimm.2016.06.005.

[67] Shakya KP, Hugh-Jones ME, Elzer PH. Evaluation of immune response to orally administered Sterne strain 34F2 anthrax vaccine. Vaccine 2007;25:5374–7. doi:10.1016/j.vaccine.2007.05.003.

[68] Turnbull PC., Tindall BW, Coetzee JD, Conradie CM, Bull RL, Lindeque PM, et al. Vaccine-induced protection against anthrax in cheetah (Acinonyx jubatus) and black rhinoceros (Diceros bicornis). Vaccine 2004;22:3340–7. doi:10.1016/j.vaccine.2004.02.037.

[69] Creel S, Creel NM. Six ecological factors that may limit African wild dogs, Lycaon pictus. Anim Conserv 1998;1:1–9. doi:10.1111/j.1469-1795.1998.tb00220.x.

[70] Brossier F, Mock M, Sirard JC. Antigen delivery by attenuated Bacillus anthracis: New prospects in veterinary vaccines. J Appl Microbiol 1999;87:298–302. doi:10.1046/j.1365-2672.1999.00895.x.

[71] Turnbull PCB. Anthrax vaccines: past, present and future. Vaccine 1991;9:533–9. doi:10.1016/0264-410X(91)90237-Z.

[72] Hassell JM, Begon M, Ward MJ, Fèvre EM. Urbanization and Disease Emergence: Dynamics at the Wildlife–Livestock–Human Interface. Trends Ecol Evol 2017;32:55–67. doi:10.1016/j.tree.2016.09.012.

[73] Wiethoelter AK, Beltran-Alcrudo D, Kock R, Mor. Siobhan M. Global trends in infectious diseases at the wildlife-livestock interface. Proc Natl Acad Sci 2015;112. doi:10.1073/pnas.1422741112.

[74] Kracalik I, Malania L, Broladze M, Navdarashvili A, Imnadze P, Ryan SJ, et al. Changing livestock vaccination policy alters the epidemiology of human anthrax, Georgia, 2000–2013. Vaccine 2017;35:6283–9. doi:10.1016/j.vaccine.2017.09.081.

[75] Hudson MJ, Beyer W, Böhm R, Fasanella A, Garofolo G, Golinski R, et al. Bacillus anthracis: Balancing innocent research with dual-use potential. Int J Med Microbiol 2008;298:345–64. doi:10.1016/j.ijmm.2007.09.007.

[76] Aloni-Grinstein R, Gat O, Altboum Z, Velan B, Cohen S, Shafferman A. Oral spore vaccine based on live attenuated nontoxinogenic Bacillus anthracis expressing recombinant mutant protective antigen. Infect Immun 2005;73:4043–53. doi:10.1128/IAI.73.7.4043-4053.2005.

[77] Gorantala J, Grover S, Goel D, Rahi A, Jayadev Magani SK, Chandra S, et al. A plant based protective antigen [PA(dIV)] vaccine expressed in chloroplasts demonstrates protective immunity in mice against anthrax. Vaccine 2011;29:4521–33. doi:10.1016/j.vaccine.2011.03.082.

[78] Gorantala J, Grover S, Rahi A, Chaudhary P, Rajwanshi R, Sarin NB, et al. Generation of protective immune response against anthrax by oral immunization with protective antigen plant-based vaccine. J Biotechnol 2014;176:1–10. doi:10.1016/j.jbiotec.2014.01.033.

[79] Sim BKL, Li M, Osorio M, Wu Y, Wai TT, Peterson JW, et al. Protection against inhalation anthrax by immunization with Salmonella enterica serovar Typhi Ty21a stably producing protective antigen of Bacillus anthracis. Npj Vaccines

2017. doi:10.1038/s41541-017-0018-4.

[80] Brossier F, Levy M, Mock M. Anthrax spores make an essential contribution to vaccine efficacy. Infect Immun 2002;70:661–4. doi:10.1128/IAI.70.2.661.

[81] Rice-Ficht AC, Arenas-Gamboa AM, Kahl-McDonagh MM, Ficht TA. Polymeric particles in vaccine delivery. Curr Opin Microbiol 2010;13:106–12. doi:10.1016/j.mib.2009.12.001.

[82] Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy V V. Microencapsulation of probiotics for gastrointestinal delivery. J Control Release 2012;162:56–67. doi:10.1016/j.jconrel.2012.06.003.

[83] Simó G, Fernández-Fernández E, Vila-Crespo J, Ruipérez V, Rodríguez-Nogales JM. Research progress in coating techniques of alginate gel polymer for cell encapsulation. Carbohydr Polym 2017. doi:10.1016/j.carbpol.2017.04.013.

[84] Gombotz WR, Wee SF. Protein release from alginate matrices. Adv Drug Deliv Rev 2012;64:194–205. doi:10.1016/j.addr.2012.09.007.

[85] Rice-Ficht AC, Dusek KA, John Kochevar G, Herbert Waite J. Eggshell precursor proteins of Fasciola hepatica, I. Structure and expression of vitelline protein B. Mol Biochem Parasitol 1992;54:129–41. doi:10.1016/0166-6851(92)90106-T.

[86] Herbert Waite J, Rice-Ficht AC. Eggshell precursor proteins of Fasciola hepatica, II. Microheterogeneity in vitelline protein B. Mol Biochem Parasitol 1992;54:143–51. doi:10.1016/0166-6851(92)90107-U.

[87] Arenas-Gamboa AM, Ficht TA, Davis DS, Elzer PH, Kahl-McDonagh M, Wong-Gonzalez A, et al. Oral vaccination with microencapsuled Strain 19 vaccine

confers enhanced protection against Brucella abortus strain 2308 challenge in Red deer (Cervus elaphus elaphus). J Wildl Dis 2009;45:1021–9. doi:10.7589/0090-3558-45.4.1021.

[88] Arenas-Gamboa AM, Ficht TA, Kahl-McDonagh MM, Gomez G, Rice-Ficht AC. The Brucella abortus S19 δvjbR live vaccine candidate is safer than S19 and confers protection against wild-type challenge in BALB/c mice when delivered in a sustained-release vehicle. Infect Immun 2009;77:877–84. doi:10.1128/IAI.01017-08.

[89] Arenas-Gamboa AM, Ficht TA, Kahl-McDonagh MM, Rice-Ficht AC. Immunization with a single dose of a microencapsulated Brucella melitensis mutant enhances protection against wild-type challenge. Infect Immun 2008;76:2448–55. doi:10.1128/IAI.00767-07.

[90] Arenas-Gamboa AM, Ficht TA, Davis DS, Elzer PH, Wong-gonzalez A, Rice-ficht AC. Enhanced Immune Response of Red Deer (Cervus elaphus) to Live RB51 Vaccine Strain Using Composite Microspheres 2009;45:165–73.

[91] Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy V V. Production and evaluation of dry alginate-chitosan microcapsules as an enteric delivery vehicle for probiotic bacteria. Biomacromolecules 2011;12:2834–40. doi:10.1021/bm200576h.

[92] Hanson JF, Taft SC, Weiss AA. Neutralizing antibodies and persistence of immunity following anthrax vaccination. Clin Vaccine Immunol 2006;13:208–13. doi:10.1128/CVI.13.2.208-213.2006.

[93] Our Land | East Foundation n.d. https://www.eastfoundation.net/our-land/ (accessed September 21, 2019).

[94] Meeusen ENT, Walker J, Peters A, Pastoret PP, Jungersen G. Current status of veterinary vaccines. Clin Microbiol Rev 2007;20:489–510. doi:10.1128/CMR.00005-07.

[95] CDC, Ncird. U.S. Vaccines: Table 1 and Table 2. n.d.

[96] Vela Ramirez JE, Sharpe LA, Peppas NA. Current state and challenges in developing oral vaccines. Adv Drug Deliv Rev 2017. doi:10.1016/j.addr.2017.04.008.

[97] Yer Y, Kail K, Ail A, And CI, Kailasapathy K. Effect of Co-encapsulation of Probiotics with Prebiotics on Increasing the Viability of Encapsulated Bacteria under In Vitro Acidic and Bile Salt Conditions and in Yogurt. J Food Sci 2005;70. doi:10.1111/j.1365-2621.2005.tb09041.x.

[98] Li XY, Kong XY, Shi S, Zheng XL, Guo G, Wei YQ, et al. Preparation of alginate coated chitosan microparticles for vaccine delivery. BMC Biotechnol 2008;8:1–11. doi:10.1186/1472-6750-8-89.

[99] Storni T, Kündig TM, Senti G, Johansen P. Immunity in response to particulate antigen-delivery systems. Adv Drug Deliv Rev 2005. doi:10.1016/j.addr.2004.09.008.

[100] Csaba N, Kumar A, Bhatnagar R, Malik A, Gupta M, Mani R, et al. Trimethyl chitosan nanoparticles encapsulated protective antigen Protects the mice against anthrax. Front Immunol 2018. doi:10.3389/fimmu.2018.00562.

[101] Jang S-F, Goins BA, Phillips WT, Santoyo C, Rice-Ficht A, McConville JT. Size discrimination in rat and mouse gastric emptying. Biopharm Drug Dispos 2013;34:107–24. doi:10.1002/bdd.1828.

[102] McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 1992;10:75–88. doi:10.1016/0264-410X(92)90021-B.

[103] Kronhardt A, Rolando M, Beitzinger C, Stefani C, Leuber M, Flatau G, et al. Cross-reactivity of anthrax and C2 toxin: Protective antigen promotes the uptake of botulinum C2I toxin into human endothelial cells. PLoS One 2011. doi:10.1371/journal.pone.0023133.

[104] Lee KY, Mooney DJ. Alginate: Properties and biomedical applications. Prog Polym Sci 2012;37:106–26. doi:10.1016/j.progpolymsci.2011.06.003.

[105] Sundar S, Kundu J, Kundu SC. Biopolymeric nanoparticles. Sci Technol Adv Mater 2010;11:014104. doi:10.1088/1468-6996/11/1/014104.

[106] Akagi T, Wang X, Uto T, Baba M, Akashi M. Protein direct delivery to dendritic cells using nanoparticles based on amphiphilic poly(amino acid) derivatives. Biomaterials 2007;28:3427–36. doi:10.1016/J.BIOMATERIALS.2007.04.023.

[107] Thu B, Bruheim P, Espevik T, Smidsrød O, Soon-Shiong P, Skjåk-Bræk G. Alginate polycation microcapsules: I. Interaction between alginate and polycation. Biomaterials 1996;17:1031–40. doi:10.1016/0142-9612(96)84680-1.

[108] Gugerli R, Cantana E, Heinzen C, Von Stockar U, Marison IW, Stockar U Von. Quantitative study of the production and properties of alginate/poly-L-lysine

microcapsules 2008. doi:10.1080/02652040210140490.

[109] De S, Robinson D. Polymer relationships during preparation of chitosan–alginate and poly-l-lysine–alginate nanospheres. J Control Release 2003;89:101–12. doi:10.1016/S0168-3659(03)00098-1.

[110] Brahmbhatt TN, Janes BK, Stibitz ES, Darnell SC, Sanz P, Rasmussen SB, et al. Bacillus anthracis exosporium protein BclA affects spore germination, interaction with extracellular matrix proteins, and hydrophobicity. Infect Immun 2007;75:5233–9. doi:10.1128/IAI.00660-07.

[111] Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005. doi:10.1038/nm1213.

[112] Martín MJ, Lara-Villoslada F, Ruiz MA, Morales ME. Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. Innov Food Sci Emerg Technol 2015;27:15–25. doi:10.1016/j.ifset.2014.09.010.

[113] Gåserød O, Sannes A, Skjåk-Bræk G. Microcapsules of alginate-chitosan. II. A study of capsule stability and permeability. Biomaterials 1999;20:773–83. doi:10.1016/S0142-9612(98)00230-0.

[114] Thu B, Bruheim P, Espevik T, Smidsrød O, Soon-Shiong P, Skjåk-Bræk G. Alginate polycation microcapsules: II. Some functional properties. Biomaterials 1996;17:1069–79. doi:10.1016/0142-9612(96)85907-2.

[115] Kuo CK, Ma PX. Maintaining dimensions and mechanical properties of ionically crosslinked alginate hydrogel scaffolds in vitro. J Biomed Mater Res - Part A

2008. doi:10.1002/jbm.a.31375.

[116] Chuang JJ, Huang YY, Lo SH, Hsu TF, Huang WY, Huang SL, et al. Effects of pH on the Shape of Alginate Particles and Its Release Behavior. Int J Polym Sci 2017;2017. doi:10.1155/2017/3902704.

[117] Rasel MAT, Hasan M. Formulation and evaluation of floating alginate beads of diclofenac sodium. Dhaka Univ J Pharm Sci 2012. doi:10.3329/dujps.v11i1.12484.

[118] Strand BL, Ê SERéD OG, Kulseng B, Espevik T, SKJA Ê K-BRáK G. Alginate-polylysine-alginate microcapsules: effect of size reduction on capsule properties. J Microencapsul 2002;19:615–30. doi:10.1080/02652040210144243.

APPENDIX A

NOMENCLATURE

ASV	Anthrax Spore Vaccine
$CaCl_2$	100 mM $CaCl_2$, 10 mM MOPS
Capsules	alginate microcapsules, used interchangeably with microcapsules
GI Fluids	Gastrointestinal fluids
LeTx	Lethal toxin
Microcapsules	alginate microcapsules, used interchangeably with capsules
MOPS	Also, MOPS buffer, 10 mM MOPS, 0.85% NaCl
PLL	Poly-L-lysine
SC	Subcutaneous
Sterne	*Bacillus anthracis* Sterne strain 34F2
Sterne spores	*Bacillus anthracis* Sterne strain 34F2 spores
VpB	Vitelline protein B
VpB $CaCl_2$	Capsules with PLL dissolved in 100 mM $CaCl_2$, 10 mm MOPS
VpB MOPS	Capsules with PLL dissolved in 10 mM MOPS, 0.85% NaCl

APPENDIX B

VPB MICROCAPSULE FORMULATION ORIGINS

The following explanation is meant to clarify the preparation of the poly-L-lysine (PLL) and vitelline protein B (VpB) coating solutions used for the microcapsule formulations in this dissertation. VpB concentration was measured using Bradford assays, BCA assays, the extinction coefficient and A280 nm, and by measuring protein bands on SDS-PAGE against a BSA standard. For simplicity and consistency all concentrations throughout the studies described in this dissertation were calculated using the extinction coefficient and the absorbance at 280 nm.

- Spore Capsules (Chapter 3)
    - Began *in vivo* experiments with a single formulation of microencapsulated *B. anthracis* Sterne strain 34F2 spores.
    - Prepared the 15 ml solution of PLL and VpB as follows:
        - 1 mg of VpB in the 15 ml coating solution.
        - Filled up the remaining volume to 15 ml with 0.05% PLL dissolved in 100 mM $CaCl_2$, 10 mM MOPS
    - **Resulting microcapsule formulation:**
        - **0.047% PLL** (dissolved in 100 mM $CaCl_2$, 10 mM MOPS) **with 0.0067% VpB**
        - Ratio of PLL% to VpB% = 7

- High VpB $CaCl_2$ capsules (Chapter 4)
    - Microcapsule formulations prepared as described in previous publications
        - PMID: 18362129, 19047401, 19901378
    - PLL and VpB solution contained 0.017% PLL and 0.017% VpB which corresponds to 2.5 mg of each in 15 ml
    - Remaining volume up to 15 ml was filled with 100 mM $CaCl_2$, 10 mM MOPS
    - **Resulting microcapsule formulation:**
        - **0.017% PLL** (dissolved in 100 mM $CaCl_2$, 10 mM MOPS) **with 0.017% VpB**
        - Ratio of PLL% to VpB% = 1

- Low and Mid VpB $CaCl_2$ capsules and Low and Mid VpB MOPS Capsules (Chapter 4)
    - PLL was dissolved in 10 mM MOPS, 0.85% NaCl and the remaining volume was made up to 15 ml with 10 mM MOPS, 0.85% NaCl
    - Revisited the best method of quantifying the VpB concentration due to consistently low purification yields.
    - A group decision was made to use a microcapsule formulation containing 2.5 mg of PLL with 1 mg of VpB according to the concentration estimated by the Bradford assay.

        - General PLL and VpB solution contains:
            - 5.1 ml of 0.05% PLL
            - X ml of VpB to reach 1 mg
            - 15 ml – (5.1 ml of PLL + X ml of VpB) = Y ml of MOPS to reach 15 ml coating solution
    - Two batches of microcapsules were prepared with different amounts of VpB.
    - **Resulting microcapsule formulations:**
        - **Low VpB $CaCl_2$ and MOPS capsules (Chapter 4) → 0.017% PLL** (dissolved in 100 mM $CaCl_2$, 10 mM MOPS) **with 0.0025% VpB**
            - Ratio of PLL% to VpB% = 6.8
        - **Low and Mid VpB MOPS capsules (Chapter 4) → 0.017% PLL** (dissolved in 10 mM MOPS, 0.85% NaCl) **with 0.0064% VpB**
            - Ratio of PLL% to VpB% = 2.7

- VpB Shell and VpB Core & Shell Capsules (Chapter 4)
    - Following a review of microscope images, a group decision was made to use 0.05% PLL dissolved in 100 mM $CaCl_2$, 10 mM MOPS.
    - **Resulting microcapsule formulations:**

        - **0.017% PLL** (dissolved in 100 mM $CaCl_2$, 10 mM MOPS) **with 0.0032% VpB**
            - Ratio of PLL% to VpB% = 5.3
            - VpB Core & Shell capsules also contained 1 mg of VpB in the core

- Summary
    - PLL was used in the range of 0.017% and 0.047%
    - VpB was used in the range of 0.0025% and 0.017%