IMMUNOGENICITY AND EFFECTS ON FECAL MICROBIOME OF AN ELECTRON-BEAM INACTIVATED *RHODOCOCCUS EQUI* VACCINE IN NEONATAL FOALS

A Dissertation

by

ANGELA ILHA BORDIN

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,	Noah D. Cohen
Committee Members,	Waithaka Mwangi
	David McMurray
	Charles C. Love
Head of Department,	Allen Roussel

May 2014

Major Subject: Biomedical Sciences

ABSTRACT

Rhodococcus equi is a bacterium commonly isolated from soil that primarily causes pneumonia in foals and immunocompromised adult horses. Many vaccines were designed and tested to protect foals from developing pneumonia; however, to date, there is no vaccine that will protect foals from intrabronchial challenge with *R. equi*, except live, virulent *R. equi*. To evaluate electron-beam (e-beam) irradiation as a method of inactivation of *R. equi*, 2 concentrations (Concentration 1, 1×10^8 colony-forming units/ml [CFU/ml] or Concentration 2, 1×10^9 CFU/ml) of *R. equi* were submitted to a range of e-beam radiation doses, ranging from 0 to 7 kGy. All microorganisms of Concentrations 1 and 2 were adequately inactivated by 4 and 5 kGy, respectively, and the bacterial cell wall remained intact, whereas heat-inactivated samples indicated a compromised cell wall. Both concentrations were tested for immunogenicity and effects on fecal microbiome in neonatal foals. Mucosal and serum antibody responses were studied, as well as cell-mediated immune responses. Enteral administration of e-beam inactivated *R. equi* increased IFN−γ production and generated naso-pharyngeal *R. equi*-specific IgA in newborn foals. The inactivated vaccine appeared safe and immunogenic in neonatal foals in the presence of maternal antibody. No impact of treatment on fecal microbiome composition or diversity was observed among vaccinated foals; however, marked and significant differences in microbial communities and diversity were observed between foals at 32 days of age relative to 2 days of age regardless of treatment.

In conclusion, electron-beam irradiation is an appropriate method for inactivation of *R. equi*, and e-beam irradiated *R. equi* vaccine is immunogenic in neonatal foals. Also, age-related changes in immune responses and the fecal microbial population occurred in healthy foals vaccinated enterally with e-beam inactivated *R. equi.* Mucosal vaccination does not result in major changes of the fecal microbiome in foals.

DEDICATION

I dedicate this work to my husband Fabiano, my son Samuel, and our cats. If I could choose one of all families in the world, I would still choose you. Always.

ACKNOWLEDGEMENTS

I would like to thanks my committee chair, Dr. Noah Cohen, and my committee members, Drs. Waithaka Mwangi, David McMurray and Charles Love, for their guidance and motivation during my graduate studies.

I would like to thank Dr. Noah Cohen for his patience, humor, friendship, and especially for showing me how to lead by example.

I would like to thank everybody I had the pleasure to work with at the Equine Infectious Diseases Laboratory, Ms. Courtney Brake, Mrs. Kaytee Bagley, Mrs. Kristin Knabe, Mr. Kyle Kuskie, Dr. Jessica Bourquin, Dr. Michelle Coleman, and all the student workers, technicians, and postdocs, for their assistance with my projects and for their friendship.

I would also like to thank Mrs. Krissy Schoeder, Mr. Dave Golden, and all the students at the Horse Center for their willingness to help me every sampling day.

I would like to extend a special thanks to Drs. William Moyer and Allen Roussel, for their support while Department Head of the VLCS.

I would like to thank Drs. Julia Felippe and Sara Lawhon for their contributions to make me a better scientist.

I would like to thank Drs. Julia Felippe, Steeve Giguère, Suresh Pillai, Robert Alaniz, Roger Smith, Hugh Townsend, Philip Griebel, Volker Gerdts for helping with the design of experiments and vaccine expertise.

Finally, I would like to thank my family for their unconditional love and support, and for understanding why I was an absent wife/daughter/sister for a long time. Love you with all my heart.

NOMENCLATURE

BAL	Broncho-alveolar lavage
BHI	Brain-heart infusion
BSA	Bovine serum albumin
CBC	Complete blood count
CFU	Colony-forming units
CMI	Cell-mediated immunity
ConA	Concanavalin A
CTB	Cholera toxin B
e-beam	Electron-beam
EBRE 1	Electron-beam inactivated *R. equi* vaccine dose 1
EBRE 2	Electron-beam inactivated *R. equi* vaccine dose 2
ELISA	Enzyme-linked immunosorbent assay
FET	Fisher's exact test
IFN-γ	Interferon gamma
Ig	Immunoglobulin
LDA	Linear discriminant analysis
LEfSe	linear discriminant analysis effect size
LVRE	Live *R. equi* group
MOI	Multiplicity of infection
NA	Nuclear area

NP	Naso-pharyngeal
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
RT	Room temperature
TBS	Tris-buffered Saline
TBST	Tris-buffered Saline 0.005% Tween® 20
TEM	Transmission electron microscopy
VapA	Virulence-associated protein A

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

NOMENCLATURE vii

TABLE OF CONTENTS ix

LIST OF FIGURES xii

LIST OF TABLES xiv

INTRODUCTION AND LITERATURE REVIEW 1

R. equi pneumonia 1
Epidemiological considerations 4
Prevention of *R. equi* pneumonia 10
Electron-beam inactivation of bacteria 12
Cholera toxin B as mucosal adjuvant 13
Microbiome 14

IMMUNOGENICITY OF AN ELECTRON-BEAM IRRADIATED

RHODOCOCCUS EQUI VACCINE 16

Introduction 16
Material and methods 17
Results 27
Discussion 42

Page

EFFECTS OF ADMINISTRATION OF LIVE OR INACTIVATED VIRULENT *RHODOCOCCUS EQUI* AND AGE ON THE FECAL MICROBIOME OF NEONATAL FOALS 50

Introduction 50
Material and methods 52
Results 58
Discussion 66

SUMMARY AND CONCLUSIONS 71

Summary 71
Conclusions 73

REFERENCES 75

APPENDIX A 104

APPENDIX B 105

APPENDIX C 110

APPENDIX D 112

APPENDIX E 115

APPENDIX F 117

APPENDIX G 119

APPENDIX H 121

APPENDIX I 123

APPENDIX J 125

APPENDIX K 126

APPENDIX L 129

Page

APPENDIX M 130

APPENDIX N 133

APPENDIX O 144

APPENDIX P 154

APPENDIX Q 169

APPENDIX R 175

APPENDIX S 181

LIST OF FIGURES

FIGURE		Page
1	Survival curves of electron-beam irradiated *R. equi*	28
2	Ratio of green/red fluorescence of e-beam irradiated *R. equi*	29
3	Ultrastructure of live, heat-inactivated, and e-beam irradiated *R. equi*	30
4	Effects of stimulus on IFN-γ production in cultured PBMCs of foals	32
5	Mean ratio of IFN-γ concentration in supernatant of cultured PBMCs	33
6	Mean ratio of total IgA and IgG isotypes concentration from foal serum	34
7	Mean ratio of *R. equi*-specific IgA and IgG isotypes in serum samples from foals	35
8	Mean ratios of total and *R. equi*-specific IgA in naso-pharyngeal samples	37
9	Mean ratio of total and *R. equi*-specific IgA and IgG isotypes on BAL fluid from foals	38
10	Association between mean ratio BAL fluid *R. equi*-specific IgA concentration from foal NP swab eluates and BAL fluid	39
11	Association between milk and foal serum samples on day 2 for *R. equi*-specific immunoglobulins	41
12	Rarefaction analysis of 16 S rRNA gene sequences obtained from fecal swabs from foals from different vaccination groups	59
13	Rarefaction analysis of 16 S rRNA gene sequences obtained from fecal swabs from foals at different ages.	60
14	Principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16 S rRNA genes for foals from different vaccination groups at all ages	61

FIGURE Page

15 Principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16 S rRNA genes for foals from different vaccination groups at 32 days of age..... 62

16 Principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16 S rRNA genes for foals at different ages 63

17 LEfSe results on foal microbiome..... 66

LIST OF TABLES

TABLE		Page
1	Oligonucleotide primers/probes used for this study	57
2	Summary of alpha diversity measures	64

INTRODUCTION AND LITERATURE REVIEW

R. equi pneumonia

Infectious diseases are leading causes of disease and death in foals, and pneumonia is a principal infectious disease of foals. Respiratory disease was the most common cause of disease and death in foals in Texas in 1991, and ranked 3rd as a cause of morbidity and 2nd (after a combined category of trauma, injury, and wounds) as a cause of mortality in U.S. foals 1 to 6 months of age [1]. Although many organisms cause pneumonia in foals, *Rhodococcus equi* is considered the most common cause of severe pneumonia [2-5]. Pneumonia caused by *R. equi* occurs among foals worldwide [5-8]. *Rhodococcus equi* pneumonia is important to the equine industry for several reasons. At affected farms, prevalence and case-fatality rates may be high [9, 10], and treatment is generally prolonged, expensive, associated with adverse effects, and not uniformly successful [3-5]. Further, currently recommended methods of screening for early detection are expensive, labor-intensive, and imperfect [4, 5, 11-14]. Most importantly, a highly effective method for prevention, including a vaccine, is lacking for this disease despite its global importance.

Passive immunization of foals with administration of *R. equi* hyperimmune plasma not only has conflicting results regarding efficacy [12, 13, 15-17], but it is an expensive and laborious strategy. Ideally, a vaccine would be used to prevent foals from

developing pneumonia, but, to date, there is no approved vaccine effective against *R. equi* in spite of the efforts of several laboratories [17-27].

The lack of an effective vaccine is likely attributable to the complexity of immunity to *R. equi* [28-30], and the finding that foals appear to be infected very early in life [31, 32], when immune responses are naïve or deficient. It is generally accepted that a vaccine will have to be able to provide foals with protection against infection with *R. equi* during early life [33]. Despite all the research done since the first report of *R. equi* causing pneumonia in foals in 1923 [34], fundamental questions of why some foals are susceptible, and how to prevent them from developing clinical pneumonia, remain unanswered. The only approach that has been demonstrated repeatedly to protect foals from intra-bronchial challenge is the administration of live, virulent *R. equi* [35, 36]. This strategy, however, is not acceptable because of possible environmental contamination with virulent bacteria, potential to cause disease in the host, and restrictions and regulations for distributing live vaccines.

The objectives of the studies in this dissertation were to characterize mucosal and serum antibody and cell-mediated immune (CMI) responses, and the effects on fecal microbiome of foals receiving intragastrically either: 1) live, virulent *R. equi* (positive control); 2) electron-beam (e-beam) inactivated virulent *R. equi* with a mucosal adjuvant*;* or, 3) saline solution with a mucosal adjuvant (negative control). The specific aims of the studies were:

Aim 1) to describe an inactivation method of *R. equi* using e-beam irradiation;

Hypothesis 1) *R. equi* can be inactivated by e-beam irradiation without losing membrane integrity;

Aim 2) to compare respiratory mucosal and serum antibody responses among foals receiving intragastrically either live, virulent *R. equi* (1×10^{10} CFUs; positive control group), e-beam inactivated *R. equi* at 2 doses (2×10^{10} [lower-dose vaccine group; EBRE1] or 1×10^{11} CFUs [higher-dose vaccine group; EBRE2] combined with a mucosal adjuvant), or saline with the mucosal adjuvant (negative control group);

Hypothesis 2a) enteral administration of live virulent *R. equi* and e-beam inactivated *R. equi* will produce detectable mucosal and serum antibody responses that are significantly greater than saline control foals;

Hypothesis 2b) mucosal and serum antibody responses will not differ significantly between the 2 e-beam vaccine groups or between either vaccine group and the foals administered live, virulent *R. equi*;

Aim 3) to compare the effects of age and administration of live, virulent *R. equi* and e-beam inactivated *R. equi* on the fecal microbiome of foals;

Hypothesis 3) intragastric administration of either dose of e-beam inactivated virulent *R. equi* or live *R. equi* to foals will not substantively alter the microbiome of foals; however, significant age-related changes in the fecal microbiome composition and diversity will be detected during the first month of life in foals.

Epidemiological considerations

Virtually all foals are exposed to *R. equi* early in life, but not all are susceptible to *R. equi* pneumonia [37]. Susceptibility to disease cannot be explained by a single factor; there must be a balance among 3 key aspects: environment, bacteria and host [38]. Each of these elements will be considered in this review of the literature.

Agent

Rhodococcus equi is a facultative intracellular bacterium. It is found in grazing animals and their environment [5], and the presence of serum antibody is evidence that *R. equi* is widespread among horses [36, 39-41]. *Rhodococcus equi* can, however, be genotypically diverse, and numerous strains of both virulent and avirulent *R. equi* exist [42, 43]. Multiple virulent strains can be associated with infection in foals [44], in fact, a few reports show that more than one strain can be found simultaneously causing infection in foals [44-46]. In one report, identical strains were found in multiple anatomic sites in one infected foal [46].

The major route of pulmonary infection reported is the inhalation of dust containing *R. equi* [47]. Once *R. equi* reaches the lungs, it is phagocytosed, and survives and replicates inside the macrophages [6]. Its virulence is related to the ability of the organism to resist intracellular killing by macrophages [48], mainly by inhibiting phagosome-lysosome fusion [49]. After infecting macrophages, intracellular numbers of *R. equi* increase, reaching macrophage-compromising levels by 48 h [50]. *Rhodococcus equi* isolates that are virulent in foals bear an 85- to 90-kilobase plasmid that encodes for

a pathogenicity island, which includes the gene for the virulence-associated protein A (vapA); vapA is necessary but not sufficient to cause disease [33, 51-54]. It was demonstrated that avirulent, non-plasmid-containing strain failed to replicate in macrophages [53], confirming the importance of vapA in disease occurrence. Another factor that may influence *R. equi* fate inside the cell is the way it is taken up by macrophages. This process is receptor-mediated, where *R. equi* binds to macrophages primarily via the macrophage complement receptor type 3, Mac-1 (CD11b/CD18), and fixes complement by activating the alternative complement pathway [55]. When bacteria are opsonized with specific antibodies, however, phagocytosis occurs via Fc receptor [56], which enhances *R. equi* killing by equine macrophages [57].

Environment

Rhodococcus equi is commonly found in soil [58] and in equine feces [59-63]. Mares shed both avirulent and virulent *R. equi* in their feces, and therefore, become a source of bacteria for the environment of their foal [64]. Even though *R. equi* is found in soil, according to one study, there is not a strong correlation between the presence of virulent *R. equi* in farm soils and the *R. equi* disease status of those farms [43]. Other farm-level risk factors appear to include density of mares and foals, and perhaps foaling at pasture [9, 10, 65]. Studies showed that incidence of foals with *R. equi* pneumonia can be expected to be higher at farms with large acreage and greater density of mare and foals [9, 10, 65].

Exposure of foals to *R. equi* in farms is usually high [63-69]. Foals kept in stalls were more likely to be exposed to airborne virulent *R. equi* than those housed in paddocks [69]; moreover, foals kept in stalls with dirt floors may be at higher risk for developing *R. equi* pneumonia [70]. This is contributing evidence that the main route of pulmonary infection is via inhalation of dust containing *R. equi* [47]. The lack of attention to preventative health practices, however, does not appear to be associated with occurrence of *R. equi* pneumonia [70]. Desirable management practices were significantly associated with increased risk of *R. equi* pneumonia, possibly because of closer monitoring of foals for sign of disease [9]. All these observed environmental associations are an important source of information and contribute to the knowledge about the rhodococcal infection. However, there are both resistant and susceptible foals on those farms studied, which reinforces the fact that host-associated factors may be more important than environment factors in preventing or controlling *R. equi* foal pneumonia.

Host

Susceptibility to pneumonia development may also be related to individual inability to cope with this organism [40]. Host factors that influence the development of pneumonia or clearance of *R. equi* from lungs are related to the development of an appropriate immune response [37]. Foals appear to become naturally infected within the first 2 weeks of life [31, 32], when both their innate and adaptive immune systems are still developing. Some studies show impairment of bactericidal activity and serum

opsonic capacity in foals relative to adult horses [71-73]. Neutrophils from adult horses when stimulated with *R. equi* are a source of proinflammatory cytokines, and this is more strongly induced by virulent *R. equi* than avirulent *R. equi* [74]. Foal neutrophils also increase mRNA expression of many pro-inflammatory cytokines, but this function increases with age [75]. In another study, no age-dependent maturation of phagocyte function in foals was found, with phagocytic and oxidative burst activity comparable to adult horses [76]; however, lower expression of major histocompatibility complex class II (MHC-II) has been reported in macrophages and PBMCs from foals compared to adult horses [76-78].

Age-related increases in maturation of adaptive immune responses of foals have also been reported in the literature, and include changes in many components of the immune system, such as immunoglobulin concentration [76, 79], cytokine production [75, 80-82], T-cell subsets [76, 83, 84], as well as ability to respond to inactivated vaccines [85].

Antibodies alone do not adequately protect foals against experimental infection with *R. equi* [18]. There is evidence, however, that antibodies may influence the immune responses to *R. equi*. Antibodies could block the initial stages of cellular infection, alter the route by which bacteria enters the macrophage, and decrease the bacteria's ability to inhibit phagosome-lysosome fusion [37, 42]. Reported associations of specific IgG isotypes with protection against *R. equi* are inconclusive and conflicting. While there is evidence that $IgG_{4/7}$ is the most important isotype in protection against intracellular pathogens [86-88], it was suggested that IgG_1 (formerly known as IgGa) was expected to

be protective because it represented a Th1 isotype, whereas and $IgG_{3/5}$ and $IgG_{4/7}$ (formerly known as IgGb and IgGT, respectively) were Th2 isotypes [89]. Both IgG_1 and $IgG_{4/7}$ interact with Fc receptors on effector cells and activate complement [88], and opsonization with anti-VapA IgG_1 significantly enhanced uptake by murine macrophages [90]. Subsequent studies, however, have indicated that IgG isotype dominance is not indicative of protection against *R. equi* in foals [35], and may vary with breed, age, sex and herd management [79]. IgA is another class of immunoglobulins that has been studied in horses, and it is especially important regarding mucosal immunity [91-94]. At mucosal surfaces, it functions primarily as a neutralizing antibody, but can also opsonize and activate complement [95]. Evidence suggests that equine IgA mediated killing through Fc receptors on phagocytes is the strong opsonophagocytic activity against *S. equi* [96], so induction of IgA response in mucosal surfaces may be important for adequate opsonization of *R. equi* for subsequent killing by macrophages. Another evidence of the importance of antibodies is the favorable results some studies have shown with immunoprophylaxis with hyperimmune plasma [15, 97]. In one study, administration of hyperimmune plasma decreased the severity of radiographic lesions and prolonged time to increased respiratory effort due to *R. equi*-induced pneumonia [16]. Another study demonstrated that immune plasma conferred protection against challenge if administered prior to exposure [17]. The mechanisms of protection remain unclear, and theories include the presence of neutralizing antibodies or other non-specific factors such as complement components and cytokines.[15]. There is controversy over the efficacy of hyperimmune plasma administration. A few studies

show conflicting results, and failed to demonstrate significant protection against challenge [12, 13].

Cell-mediated immune responses are of irrefutable importance to immunity to intracellular pathogens, including *R. equi* [47, 98]. The 2 major mechanisms by which T lymphocytes mediate clearance of intracellular pathogens are secretion of cytokines and direct cytotoxicity (usually mediated by MHC class I-restricted CD8+ T lymphocytes) [98]. In adult horses, increased numbers of IFN-γ producing CD4+ and CD8+ T cells was associated with clearance of virulent *R. equi* from the lungs after experimental infection [99]. The role of IFN-γ in clearance of *R. equi* was shown in mice with the use of anti-IFN-γ monoclonal antibody, where treated mice generated a Th2 response and failed to clear the infection and developed pulmonary granulomas [100]. Studies in mice have shown that experimental infection with virulent *R. equi* in immunocompetent BALB/c mice generated a Th1 cytokine response and progressively cleared the infection [101]. Both CD4+ and CD8+ T cells are important in pulmonary defense against *R. equi*, but clearance is dependent on CD4+ T cells [102]. Not only are foals known to have age-related impaired ability to produce IFN-γ, [81] [82], essential for activating macrophages to kill intracellular *R. equi* [103], but their CD4+ and CD8+ T cells counts also increase with age [76, 84]. Neonatal foals are also deficient in producing other cytokines such as IL-4 in response to mitogen stimulation [80, 104, 105].

Some reports suggest that foals are born immunocompetent. In one study, no age-dependent maturation of phagocyte function was observed, and foal cells responded with similar phagocytic and oxidative burst activity compared to adult horses [76]. In the

same study, the number of immune cells, rather than function, was the limiting factor in the immune system of neonatal foals. In one report on the immune responses of foals to virulence-associated proteins of *R. equi*, it was demonstrated that foals are immunocompetent, and able to induce recall lymphoproliferation with the same magnitude and cytokine phenotype as adult horses, with a strong IFN- γ response [105]. Similarly, it has been demonstrated that foals can mount a robust humoral and CMI responses to *Mycobacterium bovis* BCG [106]. The subject of equine neonate immunocompetence is controversial, however, there is evidence that their immune system can be modulated at an early age, which is of paramount importance in vaccine design.

Prevention of *R. equi* pneumonia

Preventing infectious diseases is generally a more effective approach for control than treating affected cases. Currently, the only methods proven to reduce the incidence of *R. equi* pneumonia at farms are chemoprophylaxis and administration of hyperimmune plasma [15, 17, 32]. However, chemoprophylaxis with macrolides is not desirable because of concerns of development of microbial resistance [107, 108], and also because the approach is not always effective [109]. Transfusion of hyperimmune plasma for prevention is expensive, labor-intensive, and not uniformly effective, and carries some risk for transfused foals [12, 13, 15, 17, 97].

To date, there is no approved vaccine effective against *R. equi*. A variety of vaccine strategies has been evaluated, including immunization of mares [17-21], inactivated *R. equi* administered parenterally to foals or mice [19, 22], sub-unit vaccines [20, 21, 23], DNA vaccines [24, 25], and live, mutant vaccines [26, 27]. Vaccines that may be protective against experimental infection in mice are not effective in foals [23-25, 90, 110]. These discrepant results may be explained in part because there is no mouse (or other laboratory animal) model of pneumonia caused by *R. equi*: mouse models of *R. equi* infection measure concentrations in tissues (primarily liver and spleen) following intravenous or intraperitoneal infection with *R. equi*. Hence, the lack of an effective vaccine is attributable to the complexity of immunity to *R. equi* [28-30], and also that foals appear to be infected early in life [31, 32], at an age when immune responses are not fully developed.

Despite the success of oral administration of live organisms to protect foals against experimental challenge [35, 36], very limited information is available regarding immune and other biological responses to the enteral route of vaccination. Oral vaccination of foals with a live attenuated mutant strain of *R. equi* protected 2 out of 4 foals from developing pyogranulomatous pneumonia after intratracheal challenge [111]. Traditional approaches of parenteral vaccination generally do not provide strong protection against mucosal infections [112, 113]. Protective responses induced by oral vaccination against other respiratory pathogens have also been documented in mice [114-116]. One major limitation of the cited studies of oral vaccination of foals is that evaluation of immune responses to *R. equi* antigens has been limited to serum antibody

responses; mucosal antibodies and CMI responses have not been systematically evaluated.

Despite demonstrated efficacy, the use of live organisms for mucosal vaccines has important drawbacks. The use of live, virulent *R. equi* in foals is not generally acceptable because of the potential to cause disease in the host, even when reduced-virulence mutant strains are used [27]. Moreover, there are societal and regulatory concerns for environmental contamination with virulent bacteria or genetically modified bacteria. The manufacturing and distribution of live vaccines also can be restrictive. To circumvent limitations of use of live, replicating virulent bacteria as a vaccine, we propose to use e-beam irradiation to inactivate *R. equi* while causing minimal structural damage in hopes of eliciting immune responses in neonatal foals following enteral administration similar to those generated by live organisms.

Electron-beam inactivation of bacteria

Electron-beam irradiation is a technology for microbial inactivation that currently is used for sterilization and pasteurization [117-120], and at appropriate doses can be used to inactivate large volumes of microbial cultures or to sterilize materials [121-123]. Bacterial survival is inversely related to e-beam irradiation dose, as reported for *Escherichia coli* K-12 [124], *Bacillus atrophaeus* [125], and avian influenza virus [123]. Ionizing radiation inactivates microorganisms by directly causing breaks in DNA strands or indirectly by the generation of radiolytic byproducts that interact with DNA causing

breakages. At doses that are used to inactivate microorganisms, the numbers of such DNA strand-breaks are so numerous and extensive that the cell's DNA repair mechanisms cannot cope with the extent of damage such that the cells become inactivated [126].

Electron-beam inactivation has advantages relative to inactivating bacteria using heat or formalin. Heat inactivation is known to denature proteins, including immunogenic epitopes on the cell surface [127]. Formalin also alters immunogenic epitopes [128]. More importantly, formalin is widely recognized as resulting in incomplete inactivation of organisms, and has been associated with vaccine-associated disease resulting from inadequate inactivation [129]. In contrast, e-beam inactivation is highly reliable. Moreover, e-beam inactivation can be titrated such that there is no structural damage to cell walls and membranes, including surface antigens: the bacteria are rendered replication-incompetent and metabolically inactive, but the cell structure is maintained (Pillai, 2012, personal communication). Preservation of the cell wall integrity after e-beam irradiation has also been shown for *Paracoccidioides brasiliensis* [130], and membrane damage of *Bacillus* spp. spores was observed only after high doses of e-beam irradiation [131].

Cholera toxin B as mucosal adjuvant

Inactivated vaccines generally require an adjuvant to enhance immune responses [132]. Cholera toxin B (CTB) produced by *Vibrio cholerae* is a strong mucosal

adjuvant. It binds avidly to receptors on the intestinal mucosal surface, is resistant to degradation by intestinal proteolytic enzymes [133], and stimulates antigen-specific mucosal immunity and dendritic cell maturation with synthesis of costimulatory factors and secretion of inflammatory cytokines and chemokines [134]. In mice vaccinated against H5N1 influenza, CTB has been shown to induce serum IgG, mucosal IgA, and CMI responses [135], and to protect against lethal infection [136]. Both A and B subunits of CTB may promote capture of pathogens by dendritic cells in Peyer's patches [137].

Microbiome

An important safety consideration for administration of live or inactivated bacteria for enteral vaccination is the impact that it might have on the intestinal microbiome. Although there are a few reports of intestinal microbiome in adult horses [138-143], the intestinal microbiome has not been systematically evaluated in neonatal foals [144]. The resident intestinal or fecal microbiota has been described for neonates of other species, such as cats [145, 146], dogs [147], and humans [148-151], and evidence exists that the intestinal microbiome of neonates develops with age [148-150], and is linked to the functional development of the gut and gut immunity [148-150, 152, 153]. In humans, the initial microbes colonizing infants are facultative anaerobic bacteria, such as *E. coli* and *Streptococcus* spp. [154]. In puppies and kittens, the sterile gastro-intestinal tract is presumably colonized by bacteria present in the birth canal and from

the environment [155], and human neonates appear to become colonized by these sources as well as through the intestinal microbiota of the mother [148, 154]. One of the challenges of studying the microbiome in horses is the complexity of the gastro-intestinal tract. The microbial population of adult horse fecal samples is likely to represent that of the right dorsal colon, but not that of the cecum [156]. In one study, rectal samples were not entirely representative of intestinal compartments in the small or large intestine [143]. This represents one limitation in the study of microbiome in horses, where most samples are fecal samples. To our knowledge, the impact of enteral vaccination on the intestinal or fecal microbiome of neonates of domesticated animals has not been evaluated.

In summary, there is a lot of controversy regarding foal's immune responses to *R. equi*. The correlates of protection against *R. equi* pneumonia are also unknown, which imposes difficulty to design vaccines that will generate the appropriate immune responses necessary to protect foals from naturally or induced exposure to *R. equi*. We aim, with the studies presented in this dissertation, to characterize mucosal and serum antibody and cell-mediated immune responses to enterally administrated both live and e-beam irradiated *R. equi*, and their effects on fecal microbiome of foals. The results presented here will help to elucidate more aspects of the immune response of foals to this bacterium, as well as generate a candidate vaccine for potential use in foals in affected farms.

IMMUNOGENICITY OF AN ELECTRON-BEAM IRRADIATED *RHODOCOCCUS EQUI* VACCINE

Introduction

Rhodococcus equi is a facultative intracellular pathogen recognized clinically as a leading cause of severe pneumonia in foals [5, 7, 8, 98]. To date, an efficacious vaccine against *R. equi* for foals is lacking and there is no approved vaccine for foals against *R. equi* in North America. Although a variety of strategies have been evaluated for vaccination against *R. equi* (including immunization of mares [17-21], inactivated *R. equi* administered parenterally to foals or mice [19, 22], sub-unit vaccines [20, 21, 23], DNA vaccines [24, 25], and live, mutant vaccines [26, 27]), oral administration of live, virulent *R. equi* is the only vaccination strategy that has been demonstrated repeatedly to protect foals against experimental intrabronchial challenge with virulent *R. equi* [35, 36, 157]. However, the administration of live, virulent organisms is not considered an acceptable strategy for vaccination of foals at horse breeding farms because of concerns for environmental dissemination and the potential to cause disease in some foals.

Inactivated bacteria and viruses can elicit protective immune responses against systemic infections, including those of the respiratory tract [158-161]. Electron-beam irradiation is a technology for microbial inactivation that is currently used for sterilization and pasteurization [117-120]. Electron-beam irradiation at appropriate doses

can be used to inactivate large volumes of microbial cultures or to sterilize materials such as medical devices [121-123]. Electron-beam inactivation has advantages relative to inactivating bacteria using heat or formalin. Inactivation with either heat or formalin is known to denature proteins, including immunogenic epitopes on the cell surface [127, 128]. More importantly, formalin is widely recognized as resulting in incomplete inactivation of organisms, and has been associated with vaccine-associated disease resulting from inadequate inactivation [129]. Thus, there is need for a reliable method of microbial inactivation that will retain the bacterial cell structure as similar as possible to a live organism for use in producing vaccines. We therefore identified a dose of e-beam irradiation that would inhibit bacterial replication while maintaining outer membrane integrity of *R. equi*, and examined the immunogenicity of *R. equi* inactivated accordingly when administered enterally to newborn foals.

Material and methods

Ethics statement

All procedures for this study were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee (protocol number AUP# 2011-124) and the Texas A&M University Institutional Biosafety Committee (permit number 20110183-Cohen). The foals used in this study are owned by Texas A&M University, and permission for their use was provided in compliance with the Institutional Animal Care and Use Committee procedures.

Preparation of bacteria and electron-beam irradiation

Rhodococcus equi strain EIDL 5-331 (a virulent isolate from a Texas foal) was used for this study. One colony-forming unit (CFU) was inoculated into 50 ml of brain-heart infusion (BHI) broth and shaken for 24 h at 37°C, sub-cultured in 1000 ml of BHI broth and shaken for 24 h at 37°C. The bacterial suspension was centrifuged at 3400 × g (5810R, Eppendorf AG, Hamburg, Germany) for 20 min at 4°C, the supernatant discarded, and the pellets washed with 100 ml of phosphate-buffered saline (PBS), using the same centrifugation protocol (described in Appendix A). The supernatant was discarded, the bacteria were resuspended in sterile 0.9% NaCl solution, and the concentration of bacteria was determined spectrophotometrically (Genesys™ 20, Thermo Scientific, Waltham, MA, USA). For the e-beam dose identification experiment, 25 ml of bacterial suspensions of either approximately 1×10^8 (concentration 1) or 1×10^9 CFU/ml (concentration 2) were double-bagged in heat-sealed sacs with no headspace, sealed inside a 95-kPa transport bag (Therapak, Duarte, CA, USA; standard operating procedure in Appendix B, package pictures on Appendix C), and exposed to irradiation doses ranging from 0 to 7 kGy (in integer-unit doses) using a 10-MeV, 18-kW linear accelerator. Alanine dosimeters were used to verify the delivered e-beam dose. The interaction of ionizing radiation with alanine releases free radicals [162], which were detected by an alanine dosimeter reader (E-scan, Bruker BioSpin, Corp., Billerica, MA, USA). Twenty-five ml of non-irradiated bacteria were inactivated for 30 min in a water bath at 85°C, and were used as the heat-inactivated negative control. After irradiation, quantitative culture was performed to determine concentration of replicating

R. equi in each irradiated sample, and to calculate the D_{10}-value. Experiments were conducted in triplicates, performed on 3 different days. For vaccine preparations administered to foals, e-beam irradiated *R. equi* were cultured on days 1, 3, 5, 7, and 14 post-irradiation to confirm absence of bacterial replication.

Cell wall integrity of irradiated R. equi

The immunogenic proteins of *R. equi* are expressed in the surface of the bacterium [163], therefore, keeping the cell wall integrity is important for maintaining the immunogenicity of the vaccine. Bacteria were grown as described above, and e-beam irradiated at the minimum dose that effectively inactivated all microorganisms for each concentration; live and heat-inactivated *R. equi* were prepared as positive and negative controls, respectively. Samples were kept at 4^{o}C for 12 h, and 1, 2, and 4 weeks after either irradiation or heat-inactivation. Two methods were used to determine whether the bacterial cell wall was intact. The first was a fluorescence-based assay (LIVE/DEAD® BacLight™ bacterial viability kit, Molecular Probes, Inc., Eugene, OR, USA; see Appendix D for protocol), which utilizes a mixture of SYTO® 9 green-fluorescent nucleic acid stain (which stains all bacteria) and propidium iodide (which only penetrates damaged membranes) [164], which was used according to the manufacturer's instructions using a microplate reader (Synergy 2, Biotek, Winooski, VT, USA). The second method was transmission electron microscopy (TEM) of irradiated samples, heat-inactivated, and live *R. equi* at 12 h, and 1, 2, or 4 weeks after processing. Bacterial cells were fixed with 2% glutaraldehyde, 3% formaldehyde in 0.1 M sodium cacodylate

buffer, then post-fixed with 1% osmium tetroxide and 0.5% potassium ferrocyanide, dehydrated in an ascending alcohol series, and embedded in epoxy resin. Ultrathin sections of the cells were examined with an FEI Morgagni 268 transmission electron microscope at an accelerating voltage of 80 kV. Digital images were acquired with a MegaView III camera operated with iTEM software (Olympus Soft Imaging Systems, Germany), then post-processed with Adobe Photoshop (see Appendix S for a full paper on development of an *R. equi* vaccine using e-beam irradiation).

Study animals

Thirty-four healthy Quarter Horse foals were used for this study. All foals had age-appropriate results of complete blood count (CBC) on day 2 of life, and had adequate transfer of passive immunity as assessed by a commercially-available qualitative immunoassay for serum concentration of total immunoglobulin G (IgG; SNAP test; IDEXX, Inc., Westbrook, ME, USA). All foals were monitored daily by technical staff and twice weekly by a veterinarian and remained in good health without clinical signs of disease throughout the study. Individual foals were randomly assigned to the following groups: 1) **EBRE 1** group (n=9) which received 2×10^{10} CFUs of *R. equi* inactivated by 4 kGy of e-beam radiation in 100 ml of saline adjuvanted with 100 µg of cholera toxin B subunit (CTB, List Biological Laboratories, Campbell, CA, USA); 2) **EBRE 2** group (n=10) which received 1×10^{11} CFUs of *R. equi* inactivated by 5 kGy of e-beam radiation in 100 ml of saline adjuvanted with 100 µg of CTB; 3) **Saline** (negative) control group (n=9) which received 100 ml of saline adjuvanted with 100 µg

of CTB; and, 4) **LVRE** (positive) control group (n=6) which received 1×10^{10} CFUs of live *R. equi* in 100 ml of saline. All treatments (including live *R. equi*) were administered enterally with a nasogastric tube on days 2, 9, 16, and 23 of life, administration route, perinatal period and frequency that have been demonstrated to protect foals against intrabronchial challenge when using live *R. equi* [35, 36]. Furthermore, intragastric route is better tolerated by foals than intranasal route, and it is more accepted by foals' owners. Physiological saline (NaCl 0.9%) was used as a diluent for e-beam vaccines, live bacteria, and the negative control. The doses for irradiating both preparations of vaccine were identified on previous experiments (see section "Preparation of bacteria and electron-beam irradiation" and "Cell wall integrity of irradiated *R. equi"*). The dose of 100 µg of CTB was has been shown previously to be safe and to induce local antibody production in horses [165].

Serum and milk samples from the respective mares were collected on day 2 of foals' life for assessment of maternal antibodies. All mares and foals are naturally exposed to *R. equi* from the environment [63], therefore, a considerable level of antibody response is expected from the tested mares and foals, and likely represents natural exposure.

Sample collection

Samples were collected on day 2 (prior to vaccination; birth day was considered day 1) and on day 32 of life. For the bronchoalveolar lavage (BAL) procedure, a 3-meter endoscope disinfected with glutaraldehyde prior to use was passed via the nose into the

lungs, until the tube became gently lodged in a bronchus. Sterile saline (30 ml) was instilled into the lung via the scope's infusion channel, followed by 20 ml of air to flush, and immediately aspirated to recover a minimum volume of 15 ml of fluid.

Naso-pharyngeal samples were collected by inserting a 16-inch cotton swab pre-moistened with 3 ml of sterile saline in the nasal ventral meatus. The naso-pharyngeal area was swabbed, the liquid was manually squeezed from the swab using a 60-ml syringe into a tube, and samples were frozen at -80^{o}C until assayed.

Blood was collected from a jugular vein into tubes; 5 ml of blood was collected into a tube without anticoagulant and centrifuged at 3000 ×g for 5 min to harvest serum which was separated and frozen at -80^{o}C until assayed, and 16 ml of blood was collected into tubes with sodium heparin as an anticoagulant for isolation of peripheral blood mononuclear cells (PBMCs; protocol in Appendix E), constituted mostly of T lymphocytes (CD4+ and CD8+ T cells), followed by B lymphocytes and a smaller number of monocytes [76, 166].

Cell-mediated immune response

The CMI response to vaccination was assessed by IFN-γ production by PBMCs following specific stimulation with e-beam inactivated *R. equi*. Peripheral blood mononuclear cells were isolated using Ficoll-Paque™ (GE Healthcare, Piscataway, NJ, USA) gradient separation, and carbonyl iron (Sigma-Aldrich, St. Louis, MO, USA) magnetic separation was performed to completely remove neutrophils from the PBMC suspension [167]. Cells were resuspended in RPMI-1640 media (Gibco®, Life

Technologies, Grand Island, NY, USA) with 15% fetal bovine serum (Gibco®, Life Technologies, Grand Island, NY, USA) and 1.5% penicillin-streptomycin (Gibco®, Life Technologies, Grand Island, NY, USA). Each well of a 24-well plate received 1 ml containing 1×10^6 cells, which were cultured for 48 h at 37°C with 5% CO_2 with either media only, the mitogen ConA (positive control; 5 μg/ml, Sigma-Aldrich, St. Louis, MO, USA), or e-beam inactivated *R. equi* (multiplicity of infection of 1:10). The interval of 48 h was chosen to provide enough time for antigen-presentation to T cells in culture.

The supernatants from each group were harvested, centrifuged at 300 ×g, and frozen at -80°C until examined for IFN-γ production using an equine IFN-γ ELISA kit (Mabtech Inc., Mariemont, Ohio, USA) according to manufacturer's instructions (protocol on Appendix F). Optical densities were determined using a microplate reader, standard curves were generated, and IFN-γ concentrations in each sample were calculated using Gen 5 software.

Mucosal and serum antibody responses

Mucosal humoral immune responses were assessed by quantifying total and *R. equi*-specific IgA and IgG isotypes IgG_1, $IgG_{3/5}$, and $IgG_{4/7}$ in BAL fluid, and total and *R. equi*-specific IgA in naso-pharyngeal swab eluates. Serum antibody response was assessed by quantifying total and *R. equi*-specific IgA and IgG isotypes (IgG_1, $IgG_{4/7}$, $IgG_{3/5}$) concentrations in serum of vaccinated foals.

Concentrations of total IgA and IgG isotypes (IgG_1, $IgG_{3/5}$, and $IgG_{4/7}$) were determined by ELISA using a commercial kit (Bethyl Laboratories, Montgomery, TX,

USA) according to manufacturer's instructions (protocol on Appendix G). Reference serum (Bethyl Laboratories, Montgomery, TX, USA) was added for the positive controls, and to establish standard curves, and dilution buffer was used as blank. Optical densities (ODs) were determined by using a microplate reader Synergy 2 (Biotek, Winooski, VT, USA). Standard curves were generated and immunoglobulin concentrations in each sample were calculated for each isotype using Gen 5 software (Biotek, Winooski, VT, USA).

For determination of *R. equi*-specific IgA and IgG isotypes, we used a protocol described previously [86] (Appendix H). Briefly, ELISA plates (Maxisorp, Nalge Nunc International, Rochester, NY) were coated with 2.5 µg/ml of *R. equi* antigen diluted in coating buffer (Carbonate-bicarbonate buffer, Sigma-Aldrich, St. Louis, MO) overnight at 4^{o}C. The protocol for preparation of *R. equi* antigen has been described previously [87] (Appendix I), except *R. equi* virulent field strain 5-331 was used in this study. The antigen was produced by inoculating blood agar plates with *R. equi* strain 5-331 and incubating at 37oC for 48 hours. One colony from this pure culture was selected and used to inoculate 500 ml of BHI broth. The flask with inoculated broth will be placed on a shaker for 24 h at 37oC, and then centrifuged at 3400 × g for 10 min to pellet the bacteria. The supernatant was discarded and the pellet resuspended in 200 ml of PBS, then centrifuged again at 3400 × g for 10 min and the pellet resuspended in 10 ml of PBS. Three freeze/thaw cycles were done by freezing at -80^{o}C and thawing in 37^{o}C. After the last thawing cycle, the suspension was centrifuged at 12,000 × g for 15 min at 4oC, the pellet discarded and the supernatant saved and centrifuged again at 14000 × g

for 35 min at 4oC. The suspension was tested for protein concentration using a BCA kit according to manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL).Plates were washed as described above, blocked with 200 µl TBS 1% BSA for 30 min at room temperature (RT), and washed again. Two-fold serial dilutions of serum samples from study foals, positive control *R. equi* hyperimmune plasma (Mg Biologics, Ames, IA), and negative control fetal horse serum (Biowest, Miami, FL, USA) were added in duplicates to the wells and incubated for 60 min at 22^{o}C. Both BAL fluid and NP swab eluates were used undiluted. After another washing, goat anti-horse IgA, IgG_1, and $IgG_{3/5}$ peroxidase conjugated, and sheep anti-Horse $IgG_{4/7}$ peroxidase conjugated (Bethyl Laboratories, Montgomery, TX) were added to the wells and incubated for 60 min at RT. Plates were washed again, and TMB One Component HRP Microwell Substrate (Bethyl Laboratories, Montgomery, TX) was added to the wells and incubated for 15 min at RT in the dark. The reaction was stopped by adding stop solution to the wells. Optical densities were determined by using a microplate reader. Relative quantities for day 2 and day 32 samples were obtained by using the following formula:

$$\text{Relative quantities} = \frac{\text{OD sample - OD negative control}}{\text{OD positive control - OD negative control}}$$

The ratio of the relative quantities on day 32 to the relative quantities on day 2 was used to describe the relative increase/decrease of antibodies following vaccination.

When determining concentration of total immunoglobulins in BAL fluid, a urea-dilution method [168] was used to estimate the volume of pulmonary epithelial lining fluid:concentrations of BAL fluid IgG and IgA titers and to account for differences in recovery of BAL fluid.

Data analysis

All analyses were conducted using S-PLUS (Version 8.0; Insightful, Inc.) and R (Version 2.12.1; R Statistical Project) and a significance level of $P < 0.05$. Analysis of growth curves and cell wall integrity fluorescence data were performed using linear mixed-effects models with experimental replicates modeled as random effects [169]. The D_{10}-value was calculated from the negative inverse of the slope from the linear mixed-effects regression of the irradiation dose on the logarithm_{10} of the microbial population [170]. Transmission electron microscopy data were descriptive only.

Foal data were analyzed using the ratio of relative quantities on day 32 to relative quantities on day 2 of life (baseline) of immunoglobulins or IFN-γ concentrations, or using the proportion of foals that had an increase in these relative quantities. This approach was used because of considerable inter-individual variation among animals irrespective of treatment group. Even though the ratio of day 32 relative to day 2 was used for statistical analysis, the primary data from both days 2 and 32 of life are presented in the Appendices J (IFN-γ), K (serum antibody), L (NP antibodies) and M (BAL fluid antibodies). To adjust for the skewness of the data, $\log_{10}$-transformation was used to ensure they met the assumptions underlying the modeling strategy. Ratio data

were analyzed using a generalized linear model with concentrations as the outcome variable and study group as the independent variable of interest. Model fit was assessed by examining diagnostic plots of residuals. Post-hoc pair-wise comparisons among groups were made using the method of Sidak [171]. Proportions of foals with increased immunoglobulin isotypes were compared among groups using Fisher's exact test.

Association (correlation) between immunoglobulin concentration in mare serum and milk immunoglobulins, and mare milk and NP swab eluates IgA were made using linear regression analysis. Comparisons of immunoglobulin concentrations among treatment groups were performed using Kruskal-Wallis testing.

Results

Effects of e-beam irradiation on R. equi

The doses required to prevent replication of *R. equi* at concentrations of 1×10^8 and 1×10^9 CFU/ml were 4 and 5 kGy, respectively (Fig. 1). The D_{10}-values estimated for *R. equi* strain 5-331 in 0.9% NaCl exposed to 10-MeV, 18-kW e-beam irradiation for the 2 concentrations were similar (0.48 [0.37 to 0.69] and 0.53 [0.47 to 0.61]), approximately 0.505 kGy) and did not differ significantly ($P>0.05$).

The green/red fluorescence ratio increased significantly ($P < 0.05$) with the addition of intact bacteria in all groups except the heat-inactivated group (Fig. 2A, B, C and D), indicating that e-beam irradiation did not damage bacterial membrane integrity

but that heat-inactivation did. Using TEM, the overall integrity of the outer bacterial cell wall of all treated groups was preserved (Fig. 3A, B, C, and D).

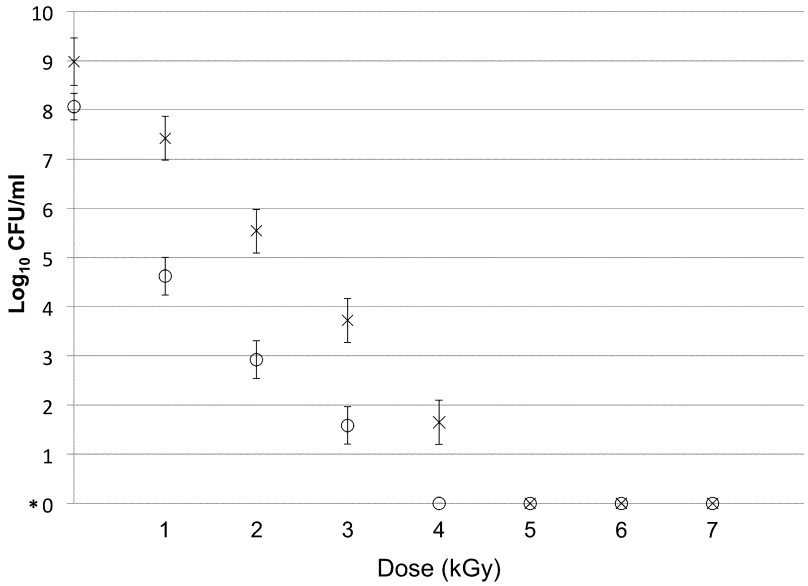


Fig. 1. Survival curves of electron-beam irradiated *R. equi*. Survival curves for *R. equi* samples resuspended in 0.9% NaCl irradiated with e-beam doses ranging from 0 to 7 kGy. Survival curve for Concentration 1 (1×10^8 CFU/ml) is indicated by the symbol ○; Survival curve for Concentration 2 (1×10^9 CFU/ml) is indicated by the symbol ×; *0 represents true 0 and not $10^0 = 1$.

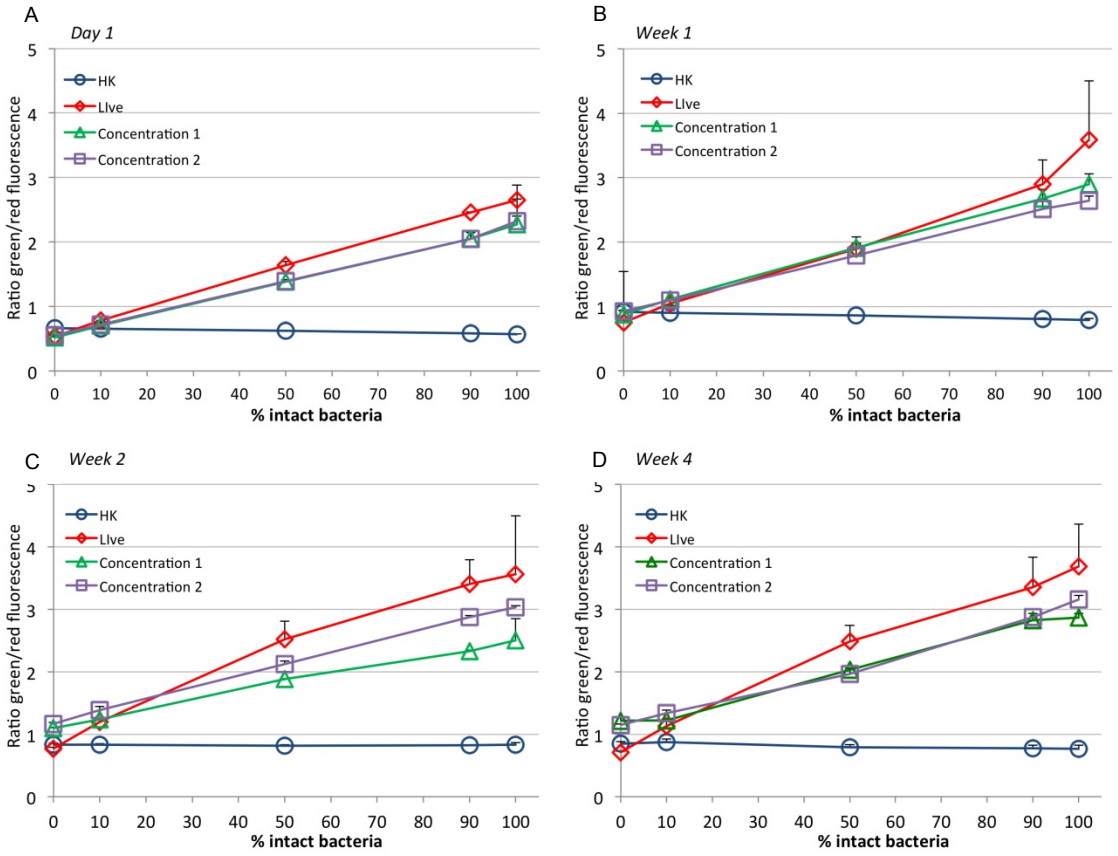


Fig. 2. Ratio of green/red fluorescence of e-beam irradiated *R. equi*. Fluorescence-based LIVE/DEAD BacLight bacterial viability kit for Concentration 1 (approximately 1×10^8 colony-forming CFU/ml; square) and Concentration 2 (approximately 1×10^9 CFU/ml; triangle) e-beam irradiated, live (diamond shape) and heat-inactivated samples (circle). A) Day 1, B) Week 1, C) Week 2, and D) Week 4 of storage at 4°C. Error bars are symmetric, but only plus bar is shown.

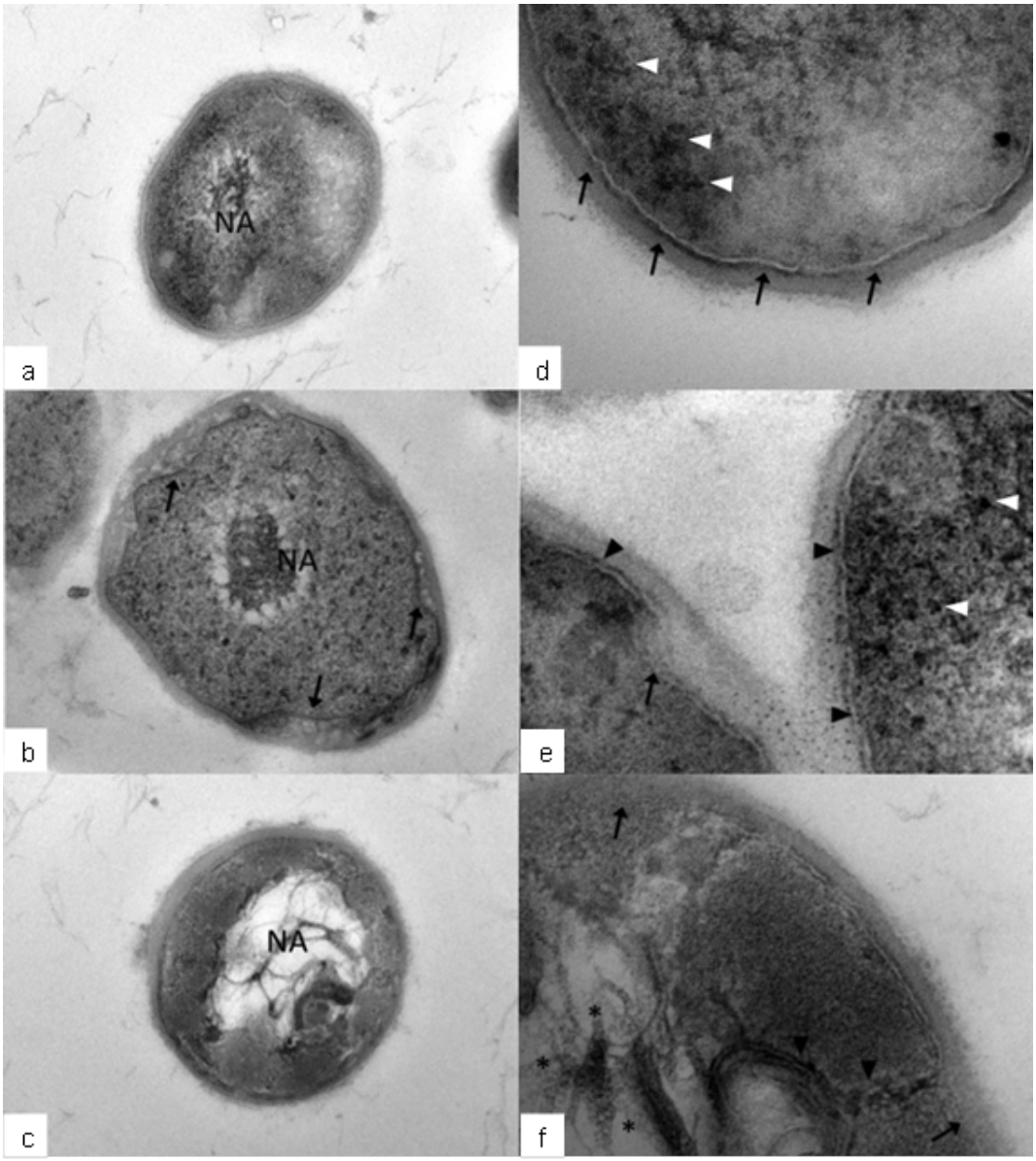


Fig. 3 Ultrastructure of live, heat-inactivated, and e-beam irradiated *R. equi*. a) Live group, week 4. Normal morphologic appearance of the nuclear area (NA). b) Concentration 2 e-beam irradiated, week 4. Similar morphologic appearance of the NA compared to the live bacterium of image "a". The arrows are indicating invaginations of the layered cell wall. c) Heat-inactivated group, day 1. Nuclear area (NA) markedly vacuolated and has increased electron lucency. d) Live group, day 1. Closer magnification of a live bacterium depicting the localization of the layered cell wall (black arrows) and of glycogen-like material (white arrowheads). e) Concentration 2 e-beam irradiated with 5 kGy, day 1. Closer view of radiated bacteria demonstrating intact layered cell walls (black arrowheads), invaginations of the layered wall (arrow), and preservation of glycogen-like material (white arrowheads). f) Heat-inactivated, day 1. Closer magnification of a heat-killed bacterium that presents large areas where the layered cell wall is either not present (arrows) or presents marked invagination/coiling (arrowheads). Note the vacuolated nuclear area (*), and inconspicuous glycogen-like material. Concentration 2 (b) and Live bacteria (a) remains intact after 4 weeks of refrigeration, whereas heat-inactivated (c) bacteria denote changes after 12 h of refrigeration.

Changes affecting the wall were confined to the layered cell wall (LCW) of all groups, and were more severe among bacteria of heat-inactivated groups suggesting a more severe compromise of the cell wall integrity (consistent with the fluorescent-based results). The internal cell contents were only morphologically affected in bacteria of the heat-inactivated group. Noticeable changes included enlarged nuclear areas that were admixed with a filamentous material and inconspicuous glycogen-like deposits (Fig. 3D). The antigenicity of the e-beam irradiated *R. equi* was demonstrated by western blot (data not shown) using an anti-vapA antibody (kindly provided by Dr. Shinji Takai).

Cell-mediated immune response

Stimulation with ConA significantly ($P < 0.05$) stimulated IFN-γ production in cultured PBMCs on both days 2 and 32 relative to unstimulated cells, and stimulation with *R. equi* resulted in significantly greater IFN-γ production on day 32 ($P < 0.05$) relative to unstimulated control (media only; Fig. 4). The IFN-γ response was significantly ($P < 0.05$) greater for foals in the LVRE, EBRE 1, and EBRE 2 groups than for foals in the saline control group (Fig. 5).

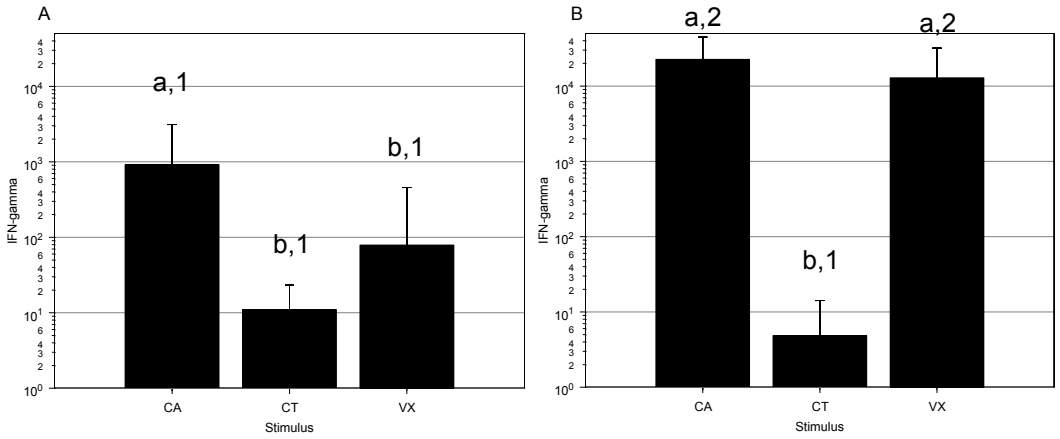


Fig. 4. Effects of stimulus on IFN-γ production in cultured PBMCs of foals. Effects of stimulus (Concavalin A 5 μg/ml, e-beam irradiated *R. equi* MOI 1:10, or saline [unstimulated control]) on concentration of IFN-γ in cell culture supernatant of foals at ages 2 days (panel a.) or 31 days (panel b.), by treatment group. At both ages, Concavalin A stimulated a significant increase in IFN-g concentration. Within a panel, differing letters indicate significant differences among groups. Between panels, different numbers indicate differences between ages.

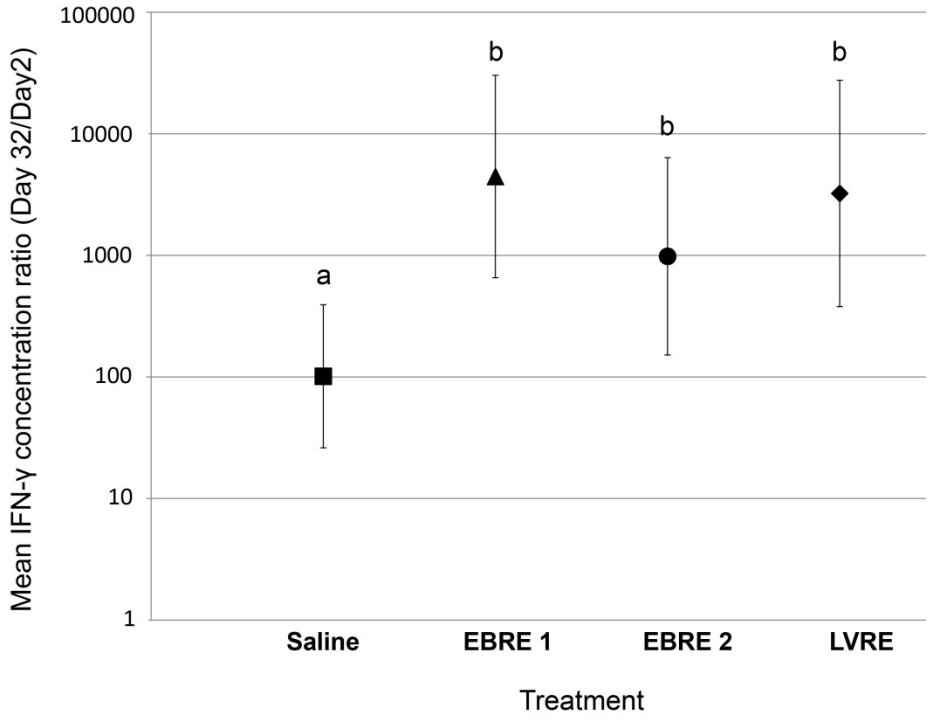


Fig. 5. Mean ratio of IFN-γ concentration in supernatant of cultured PBMCs. Relative quantities on day 32 relative to day 2 (log_{10}-transformed) from 34 foals in 4 treatment groups: 1) Saline: enteral adjuvant only controls (N = 9); 2) EBRE 1: foals receiving 1 x 10^{11} *R. equi* e-beam irradiated with 4 kGy enterally (N = 10); 3) EBRE 2: foals receiving 2 x 10^{10} *R. equi* e-beam irradiated with 5 kGy enterally (N = 9); and, 4) LVRE: foals receiving 1 x 10^{10} live, virulent *R. equi* enterally (N = 6). Symbols with differing letters indicate significant ($P < 0.05$) differences among groups.

Serum antibody immunity

Serum concentrations of total IgA, $IgG_{4/7,}$ and $IgG_{3/5}$ decreased significantly with age for foals in all groups (Fig. 6); however, there were no significant differences in the decline of total immunoglobulins among groups.

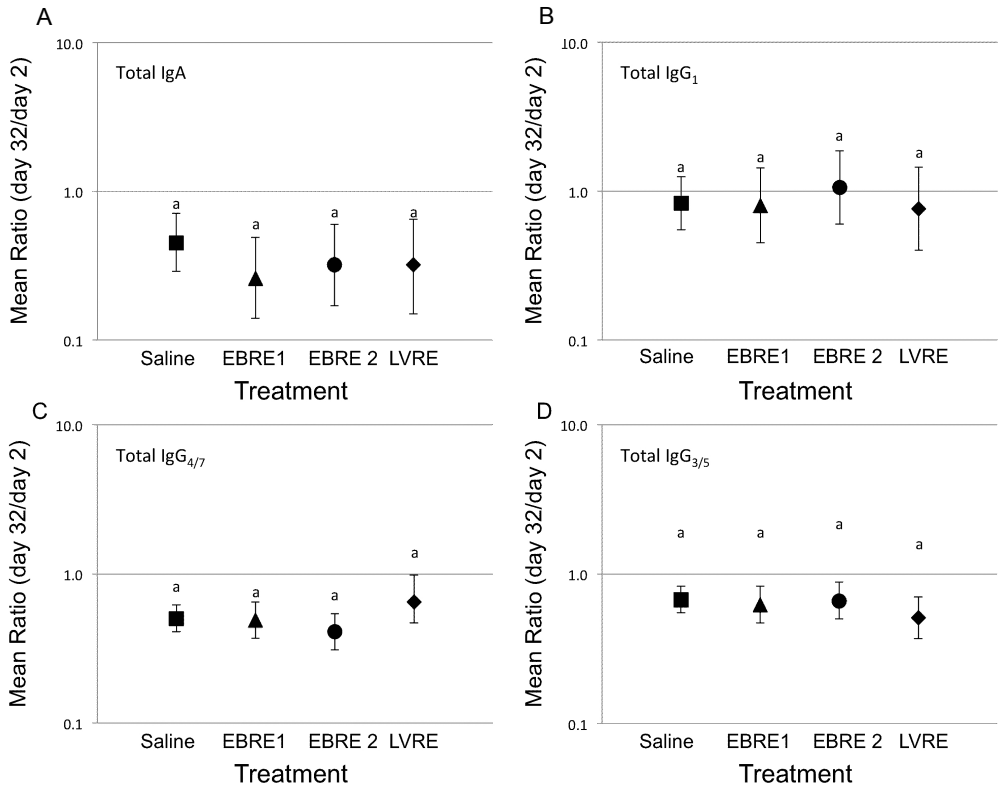


Fig. 6. Mean ratio of total IgA and IgG isotypes concentration from foal serum. Concentration on day 32 relative to day 2 (log_{10}-transformed) from 34 foals in 4 treatment groups: 1) Saline: enteral adjuvant only controls (N = 9); 2) EBRE 1: foals receiving 1 x 10^{11} *R. equi* e-beam irradiated with 4 kGy enterally (N = 10); 3) EBRE 2: foals receiving 2 x 10^{10} *R. equi* e-beam irradiated with 5 kGy enterally (N = 9); and, 4) LVRE: foals receiving 1 x 10^{10} live, virulent *R. equi* enterally (N = 6); Bars with differing letters indicate significant ($P < 0.05$) differences among groups. A) Total IgA; B) Total IgG_1; C) Total $IgG_{4/7}$; D) Total $IgG_{3/5}$.

There were no significant differences among groups in values of the day 32 to day 2 ratio of *R. equi*-specific serum IgA (Fig. 7A); however, the ratios were significantly ($P < 0.05$) less than 1 for all groups. The day 32 to day 2 ratios of serum IgG_1 and $IgG_{4/7}$ were significantly ($P < 0.05$) greater for the LVRE group than other groups (Fig. 7 B and C); there were no other significant differences among groups. Similarly, the ratios were significantly ($P < 0.05$) greater for the LVRE group than the 2

vaccine groups (but not controls; Fig. 7D). Note that the magnitudes of increase were generally small.

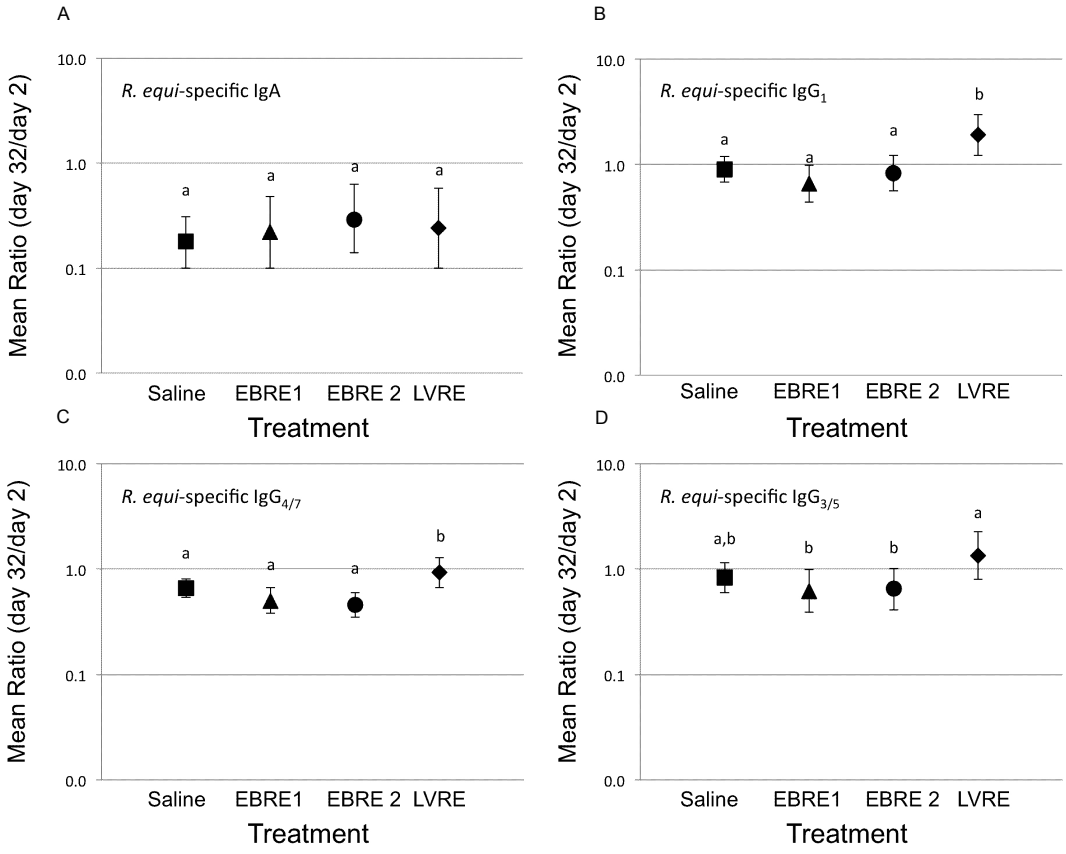


Fig. 7. Mean ratio of *R. equi*-specific IgA and IgG isotypes in serum samples from foals. OD on day 32 relative to day 2 (log_{10}-transformed) from 34 foals in 4 treatment groups as described in Fig. 2. Symbols with differing letters indicate significant ($P < 0.05$) differences among groups. A) *R. equi*-specific IgA; B) *R. equi*-specific IgG_1; C) *R. equi*-specific $IgG_{4/7}$; D) *R. equi*-specific $IgG_{3/5}$.

Mucosal humoral immune response

Naso-pharyngeal samples

Total IgA concentration in naso-pharyngeal samples were increased for all but 1 foal; however, there were no significant differences among groups in either the relative magnitude of increase from day 2 to day 32 (Fig. 8A) or the proportion of foals that had increased IgA following vaccination (Fig. 8B), although the EBRE2 and LVRE groups tended to be increased. Although the relative increase of *R. equi*-specific IgA in NP samples tended to be greater for the EBRE2 and LVRE groups (Fig. 8B), there were no significant differences among groups. The proportions of foals that had increased *R. equi*-specific IgA, however, differed significantly among groups ($P = 0.0223$; Fisher's exact test [FET]; Fig. 8D). Post-hoc specific pair-wise comparisons indicated that the proportion of foals in the EBRE2 group that had increased *R. equi*-specific IgA was significantly ($P < 0.05$) greater than that of the saline controls (Fig. 8D).

BAL fluid

The ratio of total and *R. equi*-specific IgA and IgG isotypes for Day 32 relative to Day 2 were significantly ($P < 0.05$ for all) greater than 1 for all groups, indicating a significant increase with age (Fig. 9); however, there were no significant differences among groups in the values of these ratios. No significant association was observed in either total or *R. equi*-specific IgA between samples of BAL fluid and NP swabs from individual foals (Fig. 10).

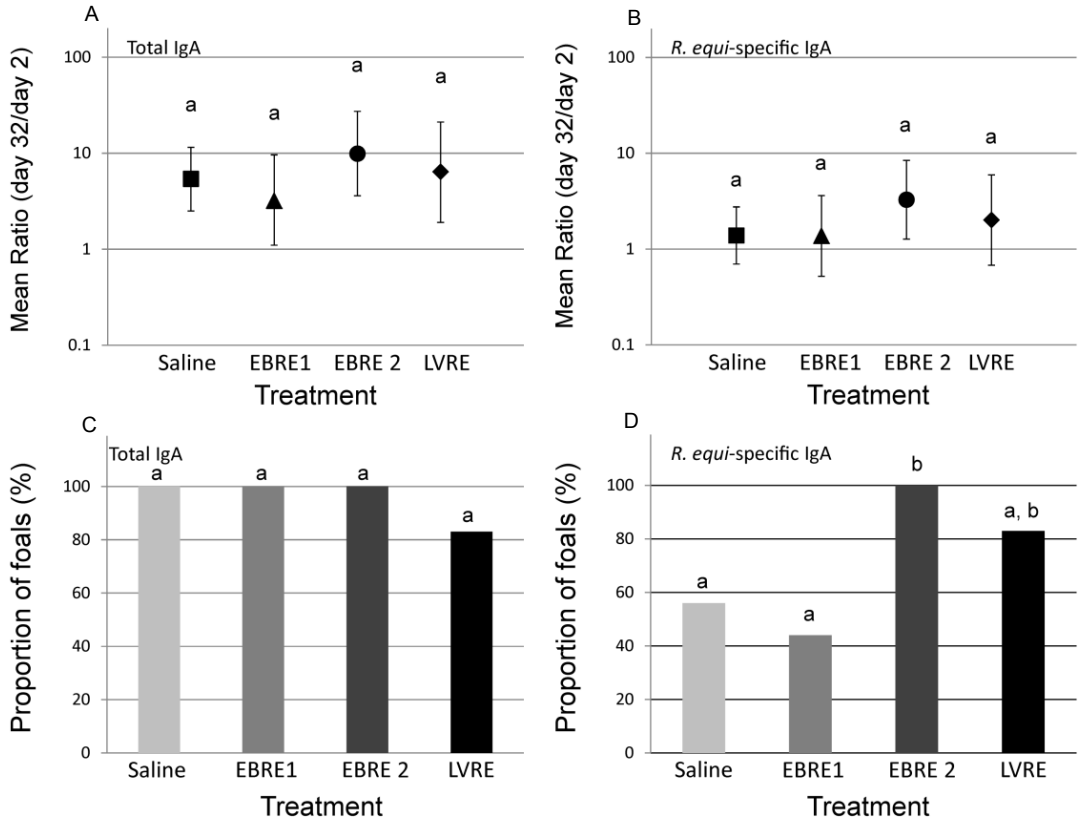


Fig. 8. Mean ratios of total and *R. equi*-specific IgA in naso-pharyngeal samples. Relative quantities on day 32 relative to day 2 (log_{10}-transformed) of IgA from 34 foals in 4 treatment groups as described in Fig. 2. Symbols with differing letters indicate significant ($P < 0.05$) differences among groups. A) Mean ratio (95% confidence interval) concentration total IgA; B) Mean ratio OD *R. equi*-specific IgA; C) Proportion of foals with increase in total IgA from day 32 relative to day 2; D) Proportion of foals with increase in *R. equi*-specific IgA from day 32 relative to day 2; overall distribution differed significantly among groups ($P = 0.0223$; FET); letters indicate results of post-hoc pair-wise testing between groups using FET.

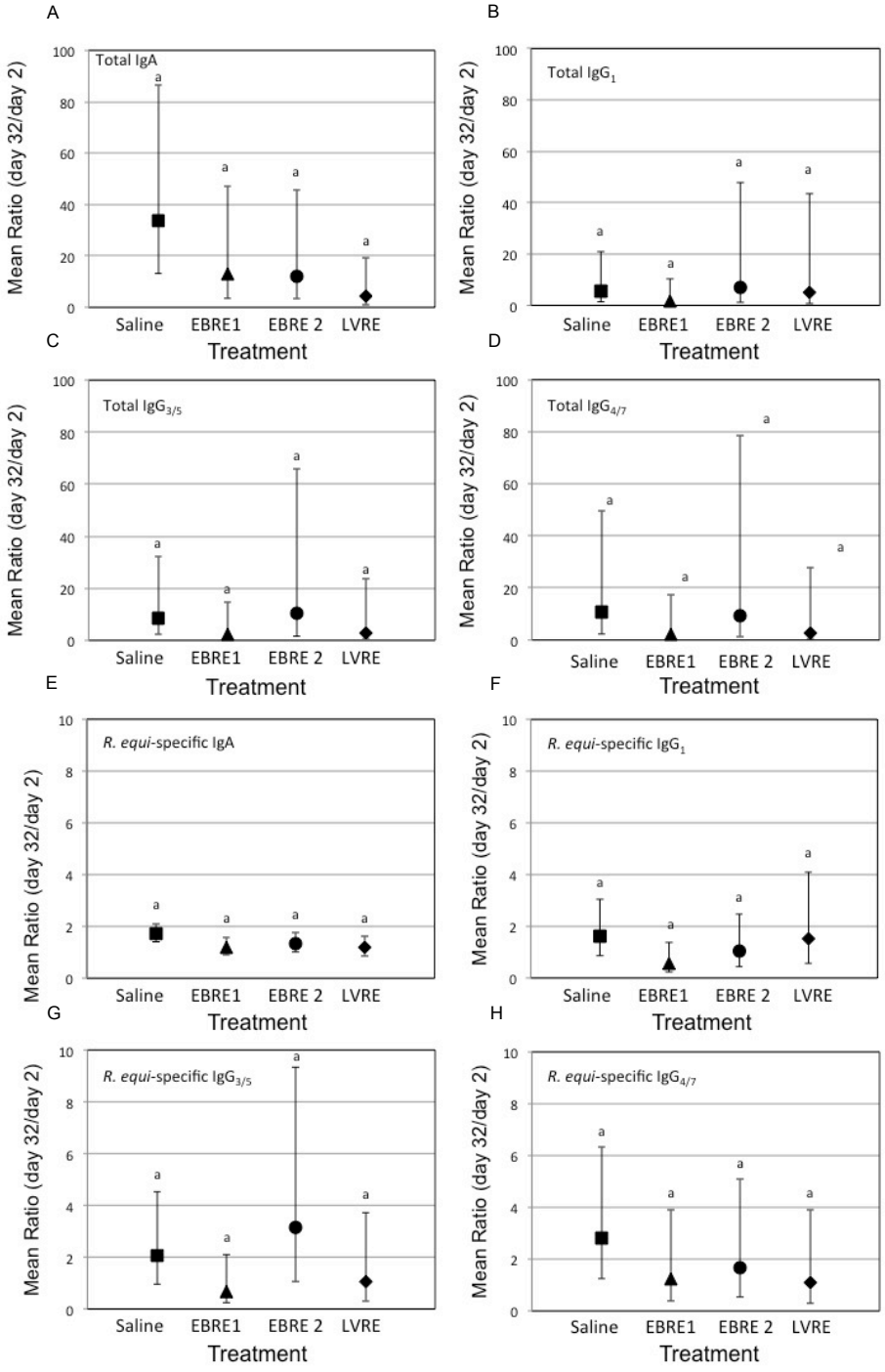


Fig. 9. Mean ratio of total and *R. equi*-specific IgA and IgG isotypes on BAL fluid from foals. Relative quantities concentrations (total) and OD (*R. equi*-specific) on day 32 relative to day 2 (log_{10}-transformed) from 34 foals in 4 treatment groups as described in Suppl. Fig. 4. Bars with differing letters indicate significant ($P < 0.05$) differences among groups. A) Total IgA; B) Total IgG_1; C) Total $IgG_{3/5}$; D) Total $IgG_{4/7}$. E) *R. equi*-specific IgA; F) *R. equi*-specific IgG_1; G) *R. equi*-specific $IgG_{3/5}$; H) *R. equi*-specific $IgG_{4/7}$.

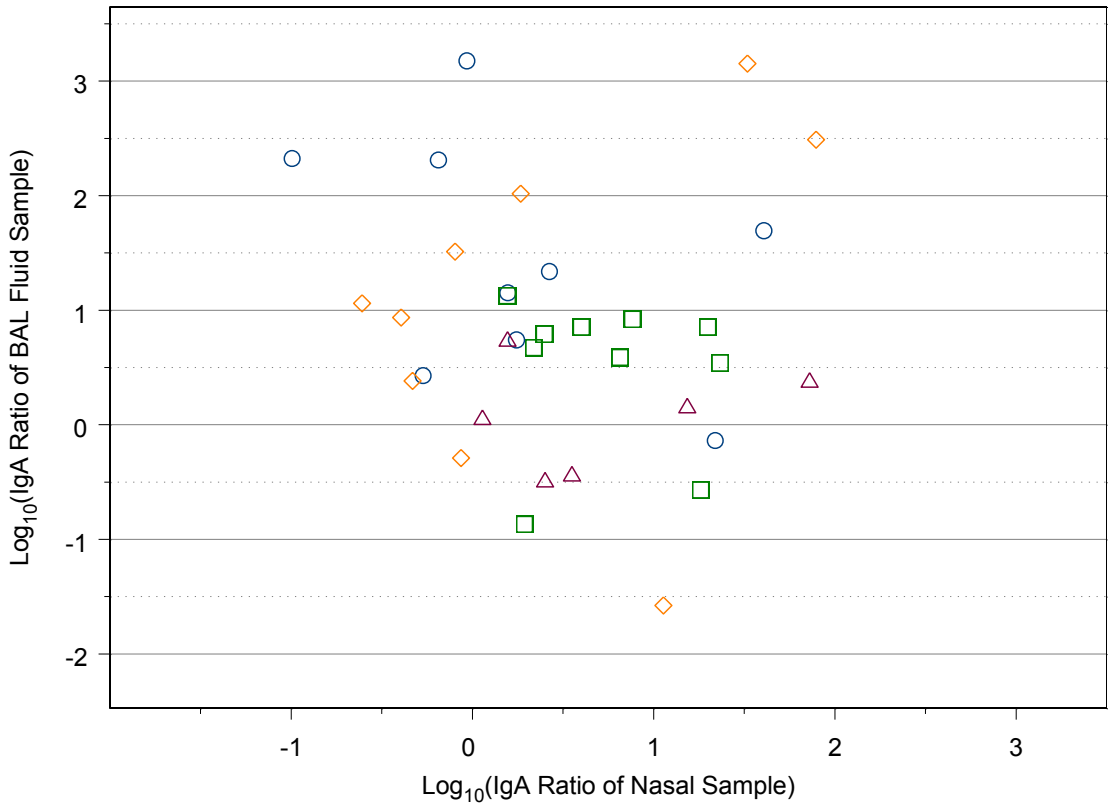


Fig. 10. Association between mean ratio BAL fluid *R. equi*-specific IgA concentration from foal NP swab eluates and BAL fluid. Relative quantities on day 32 relative to day 2 (log_{10}-transformed) from 34 foals in 4 treatment groups as described in Suppl. Fig. 4. There was no significant association (P = 0.5907; Pearson's correlation coefficient = 0.0956) between the BALF *R. equi*-specific IgA and the nasal *R. equi*-specific IgA values for foals.

Maternal influence

All mares had detectable total and *R. equi*-specific immunoglobulins of all classes in both milk and serum from natural exposure to *R. equi* in the environment. There was a significant correlation between milk and serum for total and *R. equi*-specific IgA, IgG_1, $IgG_{4/7}$, $IgG_{3/5}$, except for total concentrations of IgG_1 and $IgG_{4/7}$; however, no differences among treatment groups of foals were observed (Appendix N).

Because milk and serum concentrations were correlated for most isotypes and because colostrum/milk is the vehicle for antibody transfer to foals, we examined the correlation of immunoglobulins in milk with that of foal serum (Appendix O). There was a significant ($P < 0.05$) positive association for IgA and a tendency ($0.05 < P < 0.11$) for a positive association for $IgG_{4/7}$ and $IgG_{3/5}$ between milk and foal serum on day 2; there was no apparent association for IgG_1 (Fig. 11). No significant differences among groups were observed. There was a significant ($P < 0.05$) correlation between mare's milk concentration and foals NP swab concentrations for both total and *R. equi*-specific IgA on day 2 of life (Appendix P); however, there was no significant difference among groups.

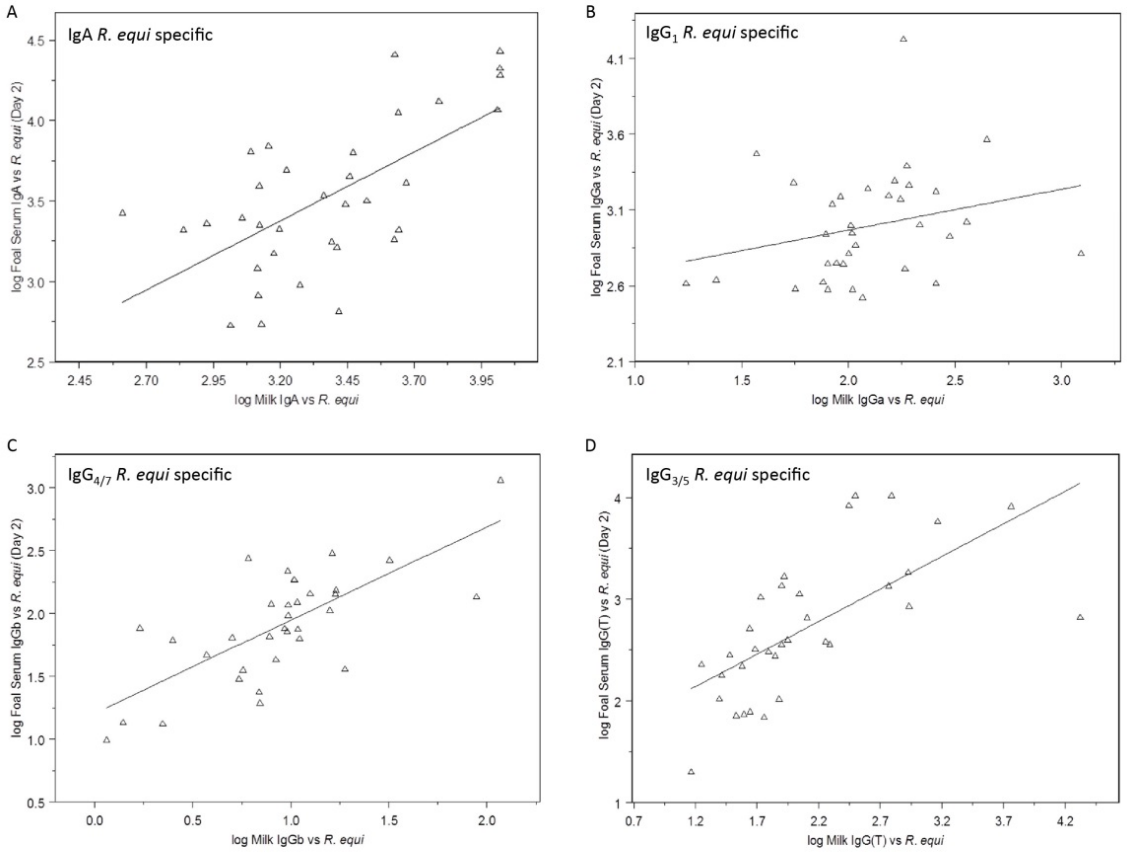


Fig. 11. Association between milk and foal serum samples on day 2 for *R. equi*-specific immunoglobulins. A) IgA; the association was weak but statistically significant; B) IgG_1; the association was weak and not statistically significant (P = 0.1345); C) $IgG_{4/7}$; the association was weak and not statistically significant (P < 0.0001); D) $IgG_{3/5}$; the association was weak but statistically significant (P < 0.0001).

Discussion

The objectives of this study were to render *R. equi* non-replicating with e-beam irradiation and to examine the immunogenicity of the e-beamed *R. equi* administered enterally to foals. Viability of *R. equi* was inversely related to e-beam irradiation dose (Fig. 1), as previously reported for other microorganisms including *Escherichia coli* K-12 [124], *Bacillus atrophaeus* [125], and avian influenza virus [123]. As expected, a higher dose of irradiation was required to completely inhibit replication of a higher concentration of *R. equi*. Thus, the dose of irradiation for a vaccine preparation would need to be established for the concentration of bacteria produced. Depending on the bacterial growth phase, a higher dose might be needed for complete killing because bacterial cells in logarithmic growth phase can have multiple copies of their genomes per cell [172].

At the doses selected, outer cell wall integrity appeared to be conserved. We observed similar ratios for both concentrations of irradiated bacteria tested and live samples for all time-points (day 1, weeks 1, 2, and 4), whereas the heat-inactivated samples were considered damaged at all time-points. This finding was anticipated because e-beam irradiation was expected to damage the bacterial DNA [172], but damage to the cell wall might be dose-dependent and consequently titratable. For example, e-beam irradiation of spores of *Bacillus* spp. caused membrane disruption with cytoplasm leakage when high doses (i.e., ≥10.4 kGy) were used but not at lower doses [131]. We did not evaluate bacterial structure in samples irradiated with > 5 kGy in our

study, however. Using TEM, all control and irradiated samples preserved the overall structural integrity of the cell wall [173], meaning that changes in the cell wall indicative of destruction or perforation of the bacterial cell wall were not observed. However, morphologic changes affecting ultrastructural components of the cell wall (e.g., the layered cell wall) and internal structures (e.g., nuclear area and glycogen-like deposits) were observed. Although variation in severity of changes involving the ultrastructural components of the cell wall was found between the irradiated and heat-inactivated groups, morphologic changes in internal structures only were detected in the heat-inactivated bacteria. Preservation of the cell wall integrity after e-beam irradiation has also been shown for *Paracoccidioides brasiliensis* [130], and membrane damage of *Bacillus* spp. spores was observed only at high doses of e-beam irradiation [131]. Although the evaluation of the ultrastructure in our study did not reveal changes in the cell wall indicative of perforation or destruction of the cell in any of the treatment groups, data from the fluorescence assay, coupled with the presence of more severe ultrastructural changes affecting the layered cell wall of heat-inactivated bacteria, indicate that the cell wall of heat-inactivated bacteria were more severely compromised relative to the other treatment groups. For this study, we used a heat-inactivation protocol of 85^{o}C for 30 min. Other heat-killing protocols using more prolonged exposure, higher temperatures, or both likely would have caused more pronounced changes in the *R. equi* ultrastructure. Nevertheless, we observed that e-beam inactivation resulted in better maintenance of normal membrane integrity and structure.

We observed that these structurally intact but non-replicating bacteria were immunogenic in neonatal foals. We chose to evaluate immune responses during the first month of life on the basis of evidence that natural infection with *R. equi* generally occurs early in life [31, 32]. We elected to use ratios (day 32 values relative to day 2) because of considerable variation in absolute values among individual foals and between ages (e.g., declining total antibody concentrations or increasing IFN-γ production with age).

Cell-mediated immune responses are of irrefutable importance to immunity to intracellular pathogens, including *R. equi* [47]. Neonatal foals, however, are known to have age-related impaired ability to produce IFN-γ, [81] [82], which is essential for activating macrophages to kill intracellular *R. equi* [103]. Consistent with previous findings, we observed that IFN-γ expression by PBMCs increased with age (both basal and stimulated expression). More importantly, neonatal foals vaccinated with either dose of e-beam irradiated *R. equi* produced significantly greater IFN-γ in response to stimulation with *R. equi* than did controls, and this indicator of CMI was similar to that generated by the LVRE group (the positive control). These results indicate that enteral mucosal vaccination with irradiated bacteria can stimulate systemic CMI responses in neonatal foals despite consumption of colostrum with presumably specific antibodies against *R. equi* (based on the presence of *R. equi* specific in milk on day 2 postpartum) and natural exposure to environmental *R. equi* similar to those induced by enterally-administered live *R. equi*. Unpublished data from our laboratory showed that *R. equi* has been isolated from air from the stalls and pastures where the foals were kept in this study, as well as from mare feces. Therefore, we believe all foals are naturally exposed

to *R. equi*, justifying the detection of specific immune responses from control foals. Also, evidence exists that foals are infected in the first 2 weeks of life [31, 32]. Thus, even immunologically naïve newborn foals can be primed to fight intracellular pathogens such as *R. equi* at the very susceptible early age. The results from the LVRE group are of further importance because they extend our knowledge of CMI responses to enteral administration of live, virulent *R. equi*, the only approach repeatedly demonstrated to protect foals against subsequent experimental intrabronchial challenge with virulent *R. equi* [35, 36].

The concentration of total immunoglobulins of all isotypes in foal serum decreased with age but there was no significant difference among treatment groups. Age-related decline in maternal antibody has been demonstrated in foals [79, 174], and was expected as a result of consumption and catabolization of maternally-derived immunoglobulin [174]. Foals from the LVRE group had significantly higher ratios of serum *R. equi*-specific IgG_1 and $IgG_{4/7}$ compared to other groups, and in $IgG_{3/5}$ compared to both groups of vaccinates (but not saline control foals). The importance of these findings remains to be determined. Reported associations of specific IgG isotypes with protection against *R. equi* are inconclusive and conflicting. Initially, it was suggested that IgG_1 was expected to be protective because it represented a Th1 isotype, whereas and $IgG_{3/5}$ and $IgG_{4/7}$ were Th2 isotypes [89], while both IgG_1 and $IgG_{4/7}$ interact with Fc receptors on effector cells and activate complement [88]. Subsequent studies, however, have indicated that IgG isotype dominance is not indicative of protection against *R. equi* in foals [35]. The magnitude of observed increase was modest

for $IgG_{3/5}$ and $IgG_{4/7}$, with neither ratio (day 32 to day 2) being significantly greater than 1. Nonetheless, enteral administration of e-beam inactivated bacteria did not result in similar serum antibody responses as enteral administration of live *R. equi*. The relevance of this result to protection against infection is an important consideration that remains to be determined.

Nasal mucosal *R. equi*-specific IgA appeared to be increased among foals in the higher-dose vaccine group (EBRE 2) than saline controls; the increase for the EBRE 2 foals was similar to the LVRE group (positive controls) in magnitude of the ratio and proportion of foals whose ratio increased between days 2 and 32. IgA is an important immunoglobulin at mucosal surfaces that functions primarily as a neutralizing antibody, but can also opsonize and activate complement [95]. Infection with *R. equi* in foals is thought to occur by inhalation of the bacterium [47], so IgA in nasal secretions may be an important barrier to *R. equi* infections in nasal passages by either neutralizing inhaled bacteria or by opsonizing them for subsequent phagocytosis and killing by macrophages in the lungs. Although CTB is an adjuvant known to induce IgA responses at mucosal surfaces [137], we nonetheless observed a significantly higher proportion of foals from the EBRE2 group with increased *R. equi*-specific nasal IgA compared to the saline control group. We observed that both total and *R. equi*-specific nasal IgA amounts increased significantly with age, consistent with what has been reported previously for total IgA [48]; to our knowledge, this is the first such report for *R. equi*-specific nasal IgA. These findings indicate nasal mucosal immunity may be relatively diminished in

newborn foals, possibly rendering them more susceptible to respiratory mucosal infection.

Because *R. equi* primarily causes pneumonia in foals, we evaluated immunoglobulin concentrations in BAL fluid of study foals. Similar to IgA from NP samples, we observed age-related increases (i.e., day 32 to day 2 ratios significantly greater than 1) in BAL fluid total and *R. equi*-specific IgA, IgG_1, $IgG_{3/5}$, and $IgG_{4/7;}$ however, no significant differences were observed among groups for any isotype. As was observed in the foals of this study, IgG and not IgA is the most abundant antibody in human BAL fluid [175]. The concentrations of *R. equi*-specific immunoglobulins of all isotypes in BAL fluid were very low, and in some instances undetectable, especially on day 2. We thus repeated analyses after concentrating the BAL fluid but results were essentially identical to those presented for the original analysis. It is unclear to what extent our BAL technique affected our results. We chose to use a low-volume BAL (30 ml) on the basis of previous research in sheep, in an attempt to yield more concentrated BAL [176-178]. Typically larger volumes such as 180 ml [179] and 500 ml [26] are used to obtain BAL samples from foals. Conceivably, a larger lavage volume might have provided greater contact with lung tissue, yielding a larger volume of fluid with more immunoglobulins.

Both total and *R. equi*-specific antibodies of all isotypes were identified in both milk and serum from mares, and these antibodies were likely passively transferred to the foal via colostrum and milk. Transfer of antibodies to the foals from colostrum usually occurs in the first 24 h after birth [180]. We acknowledge that milk on day 2 postpartum

will unlikely have the same levels of immunoglobulins as those found in colostrum. There were, nonetheless, positive associations between all immunoglobulin isotypes in the dams' milk and foals serum and NP swab samples from day 2. One frequent concern regarding vaccination of newborns is the presence and interference of maternal antibodies that could neutralize the vaccine, impairing the newborn's response to the vaccine. We did not observe this in the present study; in fact, we observed the generation of a CMI response in spite of the presumed presence of maternal antibodies. This situation has also been demonstrated with vaccination against measles in 6-month-old infants [181], where there was significant generation of IFN-γ producing $CD4^+$ T cells in spite of the presence of maternal antibodies, demonstrating that mucosal vaccination in neonates can be efficacious in priming the immune system.

Our study has a number of limitations. First, enteral administration of 4 doses of vaccine is impractical for large-scale use at farms. It may nevertheless represent a strategy that is less cumbersome, labor-intensive and risky for the foals than the accepted and widespread use of prophylactic IV plasma. However, that does not preclude us from pursuing a new formulation for use as an oral paste less frequently in the future. Second, we determined the IFN-γ concentration using supernatant of cultured PBMCs stimulated *in vitro* with either ConA or e-beam inactivated *R. equi.* We are aware that different populations of cells could be producing IFN-γ, nevertheless, we were able to demonstrate that vaccinated foals responded better than control to e-beam inactivated *R. equi*, irrespective of the cell source, and in a similar way to foals receiving LVRE, known for inducing protective immune responses against *R. equi* challenge. Third, we

recognize that no vaccine is proven efficacious until protecting foals from challenge with the bacterium, and challenge studies are currently being performed.

In summary, we demonstrated that e-beam can safely inactivate *R. equi* for potential use in vaccines, without compromising cell wall integrity. We also showed that *R. equi* inactivated with e-beam doses of either 4 or 5 kGy can be immunogenic in foals when administered enterally with CTB as adjuvant.

EFFECTS OF ADMINISTRATION OF LIVE OR INACTIVATED VIRULENT *RHODOCOCCUS EQUI* AND AGE ON THE FECAL MICROBIOME OF NEONATAL FOALS*

Introduction

Rhodococcus equi is a facultative intracellular pathogen that primarily infects macrophages [6]. Although human beings may be infected (primarily those who are immunocompromised by HIV infection or immunosuppressive treatments), *R. equi* is most commonly recognized clinically as a leading cause of severe pneumonia in foals [5-8]. The disease occurs among foals worldwide [5-8]. Isolates that are virulent in foals bear a plasmid that encodes for a pathogenicity island, which includes the gene for the virulence-associated protein A (vapA); vapA is necessary but not sufficient to cause disease [33, 51].

Despite the global importance of the disease, an effective vaccine is lacking for control and prevention of *R. equi* pneumonia in foals. The lack of an effective vaccine is likely attributable to the complexity of immunity to *R. equi* [28-30], and the finding that foals appear to be infected very early in life [31, 32], when immune responses are naïve

---

*Reprinted with permission from "Effects of Administration of Live or Inactivated Virulent Rhodococccus equi and Age on the Fecal Microbiome of Neonatal Foals" by Bordin AI, Suchodolski JS, Markel ME, et al. PLoS ONE 2013; 8(6):e66640, Copyright [2013] by Public Library of Science (PLOS).

or deficient. It is generally accepted that a vaccine must be able to provide foals with protection against infection with *R. equi* during early life [33].

To date, the only vaccination strategy that has been demonstrated repeatedly to be effective for protecting against experimental intrabronchial challenge with virulent *R. equi* has been oral administration of live, virulent *R. equi* [35, 36, 157]. Protection against respiratory pathogens induced by oral vaccination also has been documented in mice [114-116], and evidence exists that bacillus Calmette-Guerin (BCG) administered orally is protective against tuberculosis in people and animals [182-184]. Moreover, inactivated bacteria and viruses also can elicit protective immune responses against systemic infections, including those of the respiratory tract [158-161]. Despite the success of oral administration of live organisms to protect foals against experimental challenge, very limited information is available regarding immune and other biological responses to the enteral route of vaccination.

One issue of importance with regard to enteral vaccination with live organisms is the impact of enteral administration of bacteria on the intestinal microbiome. This question might be particularly important for neonates. Although the microbiome of foals has not been systematically evaluated, evidence exists in other species, including humans, that the intestinal microbiome of neonates develops with age [148-150], and is linked to the functional development of the gut and gut immunity [148-150, 152, 153]. Thus, the purpose of the study reported here was to determine whether age-related changes in the microbiome occur in foals and whether age-associated changes are impacted by administration of either live virulent *R. equi* at a dose documented to protect

foals against experimental challenge or 2 doses of inactivated virulent *R. equi* higher than the dose of live *R. equi*.

Materials and methods

Ethics statement

All procedures for this study, including collection of rectal swab samples and enteral treatments/vaccinations, were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee (protocol number AUP # 2011-124) and the Texas A&M University Institutional Biosafety Committee (permit number 20110183-Cohen). The foals used in this study are owned by Texas A&M University, and permission for their use was provided in compliance with the Institutional Animal Care and Use Committee procedures.

Animals and housing

Forty-two healthy Quarter Horse foals were used for this study. All foals were born healthy and had age-appropriate results of complete blood count (CBC) on day 2 of life, and adequate transfer of passive immunity as assessed by a commercially-available qualitative immunoassay for serum concentration of total IgG (SNAP Foal IgG test; IDEXX, Inc., Westbrook, ME). The foals were assigned into 1 of 5 experimental groups prior to birth (please see section on Vaccine Preparation and Treatment Groups below). All foals were monitored daily by Texas A&M University Horse Center staff for clinical

signs of disease, and inspected at least twice weekly by a veterinarian for clinical signs of disease. All foals remained free of clinical signs of disease and in good health throughout the study.

Mare diet

The respective dams were fed 6.4 kg per horse per day of a 13% horse pellet (crude protein: 13.5%; crude fat: 4.5%; crude fiber: 10%). Also, the foals and their mares were allowed free access to coastal Bermuda grass hay, plus grazing of pastures at the Texas A&M University Horse Center where the mares were maintained.

Vaccine preparation and treatment groups

Rhodococcus equi strain EIDL 5-331, obtained from a Texas foal confirmed to have *R. equi* pneumonia, was used to prepare live and inactivated vaccines used for this project. Physiological saline (NaCl 0.9%) was used as a diluent to achieve the specified concentration of all vaccine preparations, as well as for the negative control. The vaccine was produced by inoculating blood agar plates with 1 colony forming unit (CFU) of *R. equi* strain 5-331 and incubating at 37^{o}C for 48 hours. One colony from this pure culture was selected and used to inoculate 1,000 ml of brain heart infusion (BHI, Bacto^{TM} Brain Heart Infusion, BD Diagnostic Systems, Sparks, MD) broth. The flask with inoculated broth was placed on an orbital shaker (VWR OS-500, VWR, Radnor, PA) at 200 rpm for 24h at 37^{o}C to allow bacterial growth. Isolates were repeatedly tested by PCR for the vapA gene to confirm that the isolates were virulent [185]. The

bacterial culture was inactivated by electron-beam irradiation (irradiation dose between 4 and 5 kGy). After inactivation, the irradiated bacterial cells were plated out on BHI agar plates and incubated for 2 weeks at 37^{o}C to confirm inactivation.

The number of foals in each group was determined *a priori*, and foals were assigned randomly to each of the groups. The study groups were as follows: 1) low-dose inactivated virulent *R. equi* group (n = 9), receiving 2×10^{10} CFUs of inactivated *R. equi* combined with 100 µg of cholera toxin subunit B (CTB, List Biological Laboratories, Campbell, CA) as a mucosal adjuvant, diluted in 100 ml of saline administered via nasogastric intubation; 2) high-dose inactivated virulent *R. equi* group (n=10), receiving 1×10^{11} CFUs of inactivated *R. equi* with 100 µg of CTB diluted in 100 ml of saline via nasogastric intubation; 3) live virulent *R. equi* group (n=6), receiving 1×10^{10} CFUs of live *R. equi* diluted in 100 ml of saline administered via nasogastric intubation; 4) control with CTB group (n=9), receiving 100 µg of CTB diluted in 100 ml of saline via nasogastric intubation; and, 5) control without CTB group (n = 8), receiving 100 ml of saline via nasogastric intubation. Treatments (i.e., live bacteria, inactivated bacteria, and negative controls) were administered by nasogastric intubation to foals at 2, 9, 16, and 23 days of age.

Fecal swabbing

Rectal swabs were collected by inserting a 16-inch, cotton-tipped swab that was pre-moistened with 3 ml of sterile saline approximately 2 to 3 inches into the rectum, and swabbing the rectal mucosa circumferentially by rotating the swab. Once the cotton

swab was removed, the cotton tip was separated from the handle using scissors and the tip was placed inside the barrel of a 35-ml catheter-tip syringe; the syringe plunger was used to squeeze the liquid from the swab tip, and the liquid was collected into a sterile tube. Fecal swab samples were collected on days 2 and 32 of life from foals in all groups. For 2 foals in the control group without CTB, fecal swab samples were collected on days 2, 9, 16, 23, 32, and 56 following birth. All fecal solutions were frozen at -80^{o}C until processed.

Fecal DNA extraction

DNA was extracted by a bead-beating method using the ZR Fecal DNA MiniPrep Kit (Zymo Research Corporation) per the manufacturer's instructions. The bead-beating step was performed using a homogenizer (FastPrep-24, MP Biomedicals) for 60 s at speed of 4 m/s.

Microbiome analysis

Bacterial tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) was performed as described previously [186] based upon the V4-V6 region (*E. coli* position 530 – 1100) of the 16S rRNA gene, with primers forward 530F: GTGCCAGCMGCNGCGG and reverse 1100R: GGGTTNCGNTCGTTR.

Raw sequence data were screened, trimmed, filtered, de-noised, and chimera-depleted with default settings using the QIIME pipeline version 1.6.0 (http://qiime.sourceforge.net) and with USEARCH using the OTU pipeline

(www.drive5.com). Operational taxonomic units (OTUs) were defined as sequences with at least 97% similarity using QIIME. For classification of sequences on a genus level the naïve Bayesian classifier within the Ribosomal Database Project (RDP, v10.28) was used [186].

The obtained data were compiled to determine the relative proportions of bacteria for each individual sample. The subsequent analysis was performed on a randomly selected subset of 1,300 sequences per sample to account for unequal sequencing depth across samples. Alpha diversity and beta diversity measures were calculated and plotted using QIIME. To determine differences in microbiota composition between the animal groups, the analysis of similarities (ANOSIM) function in the statistical software package PRIMER 6 (PRIMER-E Ltd., Lutton, UK) was used on the unweighted Unifrac distances matrices. This analysis measures the phylogenetic distance among bacterial communities in a phylogenetic tree, and thereby provides a measure of similarity among microbial communities present in different biological samples. The linear discriminant analysis (LDA) effect size (LEfSe) method was used to represent taxonomic relevant age-related differences in foal fecal swabs [187].

qPCR

To validate the pyrosequencing results, quantitative PCR (qPCR) assays were performed as described previously [188]. Briefly, EvaGreen-based reaction mixtures (total 10 µL) contained 5 µL of SsoFast^{TM} EvaGreen® supermix (Biorad Laboratories), 2.2 µL of water, 0.4 µL of each primer (final concentration: 400 nM), and 2 µL of DNA

(normalized to 5ng/µl). PCR conditions were 98°C for 2 min, and 40 cycles at 98°C 5 s, and 5 s at the optimized annealing temperature (Table 1). A melt curve analysis was performed for under the following conditions: beginning at 65 °C, gradually increasing 0.5 °C/ 5 s to 95 °C with acquisition data every 5 s. The qPCR data was expressed as log amount of DNA (fg) for each particular bacterial group per 10 ng of isolated total DNA [189].

Table 1
Oligonucleotide primers/probes used for this study

qPCR primers/probe	**Sequence (5'- 3')**	**Target**	**Annealing (°C)**	**Reference**
CFB555f	CCGGAWTYATTGGGTTTAAAGGG	Bacteroidetes	60	[190]
CFB968r	GGTAAGGTTCCTCGCGTA			
Fuso-F	KGGGCTCAACMCMGTATTGCGT	Fusobacteria	51	[189]
Fuso-R	TCGCGTTAGCTTGGGCGCTG			
341-F	CCTACGGGAGGCAGCAGT	Universal Bacteria	59	[191]
518-R	ATTACCGCGGCTGCTGG			
EntF	CCCTTATTGTTAGTTGCCATCATT	*Enterococcus*	61	[192]
EntR	ACTCGTTGTACTTCCCATTGT			
EcolRT_F	GTTAATACCTTTGCTCATTGA	*E. coli*	55	[193]
EcolRT R	ACCAGGGTATCTAATCCTGTT			

Data analysis

Pairwise comparisons between ages 2 days and 32 days were made at the levels of phylum, class, order, and family of bacteria for 2 outcomes: the observed percentage of sequences of bacteria at a given level, and the proportion of foals in which any amount of a given sequence for a given level was observed (i.e., the dichotomous outcome of whether or not a specific phylum [or class or order or family] was represented). The paired differences in percentages were compared using a Wilcoxon sign-rank test, and the paired proportions were compared using McNemar's test. Because of the multiplicity of comparisons, P values at a given level (e.g., order) were adjusted using the method of Hochberg [194]. An adjusted P value < 0.05 was considered significant for these analyses. Analyses were conducted using S-PLUS (Version 8.0; Insightful, Inc.) and R (Version 2.12.1; R Statistical Project). To assess the diversity of the GI microbiota, the Shannon-Weaver [195] and Chao 1 [196] diversity indices were calculated in QIIME.

Results

Sequence analysis

The 454-pyrosequencing pipeline yielded 499,419 quality sequences for the 42 samples analyzed. For technical reasons attributed to random error, 5 foals (2 foals from the control group without CTB, and 3 foals from the live *R. equi* group) did not generate sufficient sequences (cut-off value of 1,300 sequences) in at least 1 sample from 1

sampling time-point (either 2 or 32 days) by 454-pyrosequencing. Those foals were included in the descriptive analysis (Fig. PCoA and rarefaction). For comparing age-related changes of the microbiome, however, the analysis was restricted to 37 foals with samples available from both collection time-points (2 and 32 days).

Across all vaccination groups and ages, sequences were classified into 18 phyla (Table Q-1 and R-1, please see appendices Q and R). For the rarefaction curves of all vaccination groups (Fig. 12A and 12B) and age groups (Fig. 13), 1,300 sequences per sample yielded stable estimates of sample diversity.

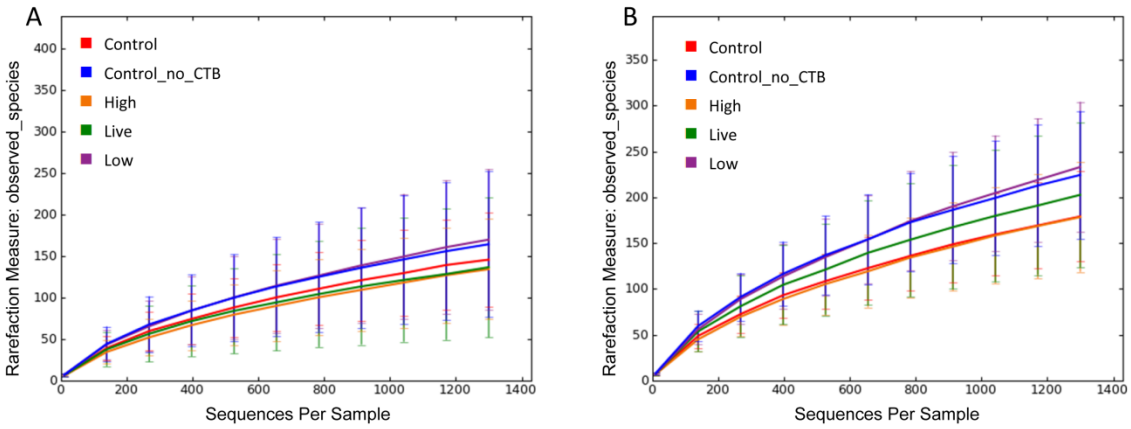


Fig. 12. Rarefaction analysis of 16 S rRNA gene sequences obtained from fecal swabs from foals of different treatment vaccination groups. Lines represent the average of each vaccination group at all ages (panel A) or at 32 days only (panel B), while the error bars represent the standard deviations. The analysis was performed on a randomly selected subset of 1,300 sequences per sample and included samples from 42 foals. Note that both the greatest and least number of species observed occurred among foals that received no enteral bacteria (live or inactivated), indicating an absence of evidence of treatment effect. Control = control plus CTB group; Control_no_CTO = control without CTB group; High = high-dose inactivated *R. equi* group; Live = live *R. equi* group; Low = low-dose inactivated *R. equi* group.

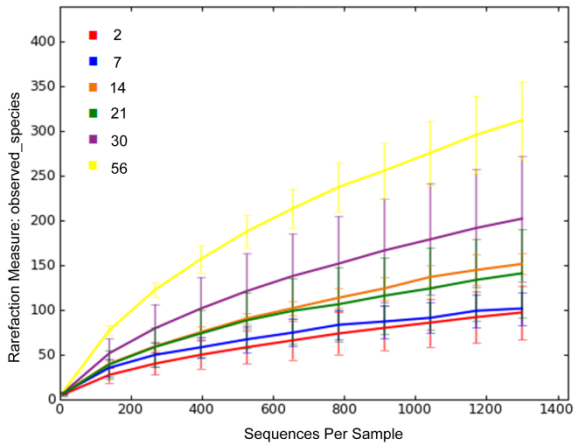


Fig. 13. Rarefaction analysis of 16 S rRNA gene sequences obtained from fecal swabs from foals at different ages. Lines represent the average numbers obtained at each age (legend numbers refer to the age in days), while the error bars represent the standard deviations. The analysis was performed on a randomly selected subset of 1,300 sequences per sample and included samples from 42 foals. Note the progressive increase in observed species (representing microbial diversity) with sequential age. The numbers for the legend represent age (in days).

Microbial communities in control and vaccinated foals

No differences in microbial composition were observed among animals from control, live and inactivated treatment/vaccination groups (Fig. 12A, 12B, and 14). The rarefaction curves for the treatment groups revealed no clear pattern of greater number of observed species (i.e., diversity) among foals receiving either live or inactivated *R. equi*, or those foals in the 3 control groups that did not receive *R. equi* (Fig. 12A). Because the samples at age 2 days were not affected by treatment (because treatment was administered after sample collection on day 2), we also performed analysis restricting data to samples collected at age 32 days (Fig. 12B). Once again, there was no pattern of differences in the rarefaction curves among treatment groups receiving either live or inactivated *R. equi* or the control groups. Using PCoA (Fig. 14 and 15), there was no

qualitative evidence of differences among groups; the clustering observed in Fig. 7 panel A was attributable to effects of age (please see next section). When considering only the data from foals at 32 days of age (because samples on day 2 were collected prior to treatment administration), the PCoA plots revealed no clustering by group and the ANOSIM test statistic for differences among groups was not significant (P = 0.494).

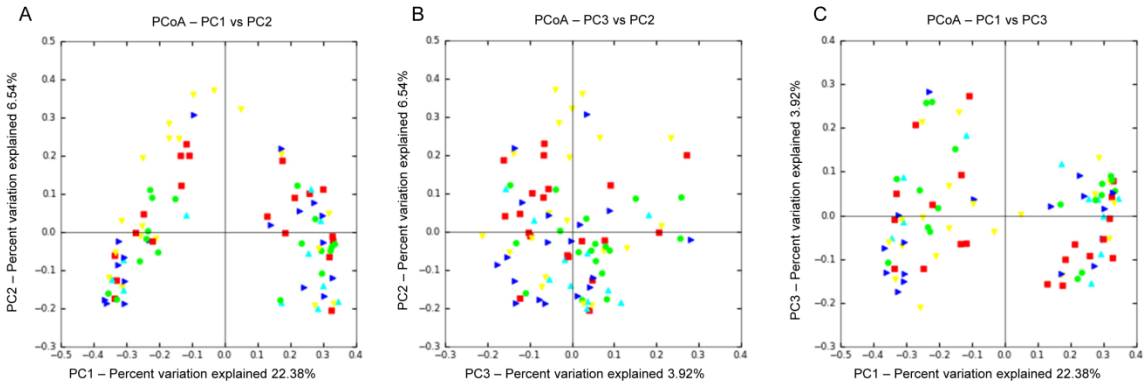


Fig. 14. Principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16 S rRNA genes for foals of different treatment vaccination groups at all ages. Analysis for 42 foals in groups control with CTB (red square), control without CTB (yellow triangle), low-dose inactivated *R. equi* (dark blue triangle), high-dose inactivated *R. equi* 2 (green dot), and live *R. equi* (light blue triangle) at 2 and 32 days of age (ANOSIM, P = 0.236). The 3 panels represent the comparison of the first 2 principal components (A), the second and third principal components (B), and the first and third principal components (C). The pattern in the panel A is attributable to effects of age (please see Fig. 15 and 16).

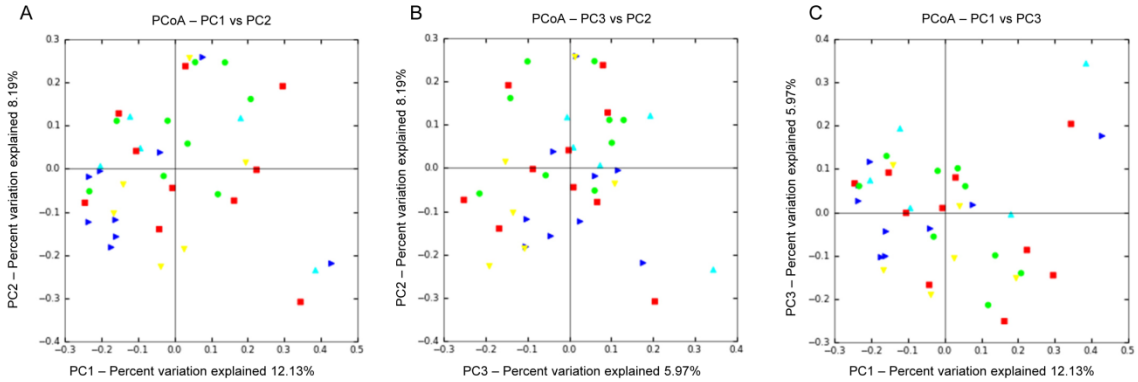


Fig. 15. Principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16 S rRNA genes for foals of different treatment vaccination groups at 32 days of age. Analysis for 42 foals in groups control with CTB (red square), control without CTB (yellow triangle), low-dose inactivated *R. equi* (dark blue triangle), high-dose inactivated *R. equi* 2 (green dot), and live *R. equi* (light blue triangle) at 32 days of age only. Differences among groups were not significant (ANOSIM, P = 0.449). The 3 panels represent the comparison of the first 2 principal components (A), the second and third principal components (B), and the first and third principal components (C).

Age-related changes in microbial communities in foals

There were strong and significant differences in the fecal microbiome of foals associated with age. The rarefaction curves demonstrated a pattern of increasing number of species (diversity) with increasing age (Fig. 13). These results should be interpreted with caution because there were only 2 foals for which data for ages other than 2 days and 32 days were available. The PCoA plots by age revealed an obvious separation of samples by age, attributable to differences between the time-points of days 2 and 32 (Fig. 16); the ANOSIM test statistic for differences between day 2 and day 32 was significant (P = 0.0010).

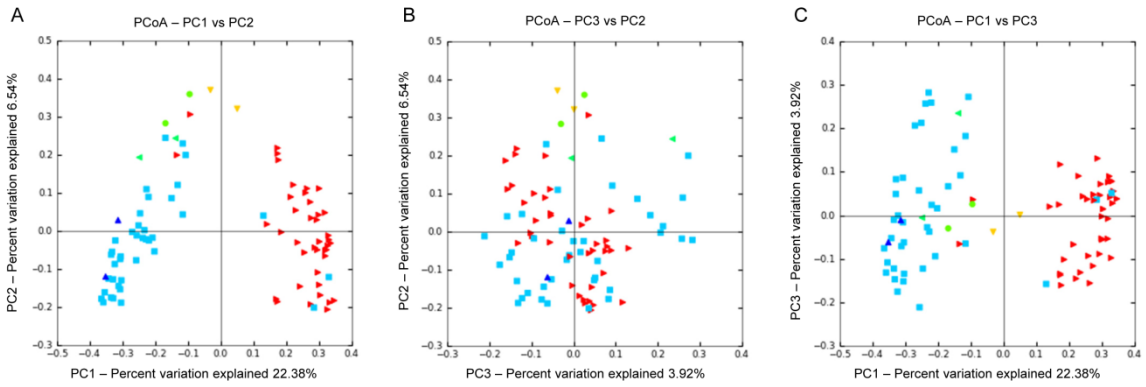


Fig. 16. Principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16 S rRNA genes for foals at different ages. Analysis for 42 foals at 2 (red triangle), 7 days old (yellow triangle), 14 (green dot), 21 (green triangle), 32 (light blue square), and 56 days of age (dark blue triangle). The 3 panels represent the comparison of the first 2 principal components (A), the second and third principal components (B), and the first and third principal components (C). Strong effects of age can be seen in panels A and C, and differences among age groups were significant (ANOSIM, P =0.0010).

Significant differences in the number of OTUs, the Shannon index, and the Chao1 metric were observed between the age groups (Table 2). The median number of OTUs for 2day-old foals (92 OTUs; range, 50 to 195 OTUs) was significantly ($P < 0.0001$) lower than that for 32-day-old foals (201 OTUs; range, 94 to 318 OTUs). The Shannon Index for the foals studied also increased significantly ($P < 0.0001$) from 2 days of life (median, 2.37; range, 1.24 to 3.97) to 32 days of life (median, 3.7; range, 1.90 to 4.80). Similarly, there was a significant ($P < 0.0001$) age-related increase in Chao 1 values between 2-day-old foals (median, 206.54; range, 128.16 to 415.70) and 32-day-old foals (median, 362.38; range, 197.42 to 581.43).

Table 2
Summary of alpha diversity measures

Index	2 day-old	32 day-old	P
Chao 1 (median, range)	206.54 (128.16 to 415.70)	362.38 (197.42 to 581.43)	**<0.0001**
OTUs (median, range)	92 (50 to 195)	201 (94 to 318)	**<0.0001**
Shannon H (median, range)	2.37 (1.24 to 3.97)	3.7 (1.90 to 4.80)	**<0.0001**

Because of the apparent differences of the microbiota between age groups, we also compared the distribution of bacteria by phylum, class, order, and family between foals aged 2 days and 32 days. In total, 18 phyla were detected in fecal samples from foals (Table Q-1, Appendix Q). Of those, Bacteroidetes (40.6%, day 32), Firmicutes (40.4%, day 2), and Proteobacteria (36.6%, day 2) had the highest percentages of sequences reported. Proteobacteria and Firmicutes were detected in all samples from 2-day-old foals, followed by Bacteroidetes (92%) and Actinobacteria (73%) (Table R-1, Appendix R). Among 32-day-old foals, Bacteroidetes and Firmicutes were detected in all fecal samples, followed by Actinobacteria (97%) and Proteobacteria (97%), Verrucomicrobia (89%), and Fusobacteria (84%) (Table R-1, please see Appendix R). The following phyla increased significantly with age (i.e., from 2 days to 32 days of age): Euryarchaeota, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Planctomycetes, Spirochaetes, TM7, and Verrucomicrobia. Proteobacteria was the only phylum that decreased significantly with age. Other classes, orders, and families also showed statistically significant age-related changes (Table Q-1 and Fig. 17).

Within the phylum Proteobacteria, the class Gammaproteobacteria ($P < 0.0001$) and the family Enterobacteriaceae ($P < 0.0001$) decreased significantly with age. Other classes of Proteobacteria, such as Deltaproteobacteria ($P = 0.0084$) and Epsilonproteobacteria ($P < 0.0001$) significantly increased with age (Table Q-1).

To confirm results of pyrosequencing, we also performed real-time quantitative PCR. Significant differences were observed in specific microbial communities between the 2 age groups based on qPCR analysis, with age-related decreases for *Escherichia coli* ($P < 0.0001$) and for *Enterococcus* ($P < 0.0001$). These data were consistent with genus-level results observed by pyrosequencing for *Enterococcus* ($P = 0.0009$) and for *Escherichia* ($P < 0.0001$). We also found agreement for a lack of evidence of a significant difference between the pyrosequencing and the qPCR results for Bacteroidetes ($P = 0.9519$ by qPCR and $P = 0.5376$ by pyrosequencing) and Fusobacteria ($P = 0.1051$ on qPCR and $P = 0.1000$ on pyrosequencing).

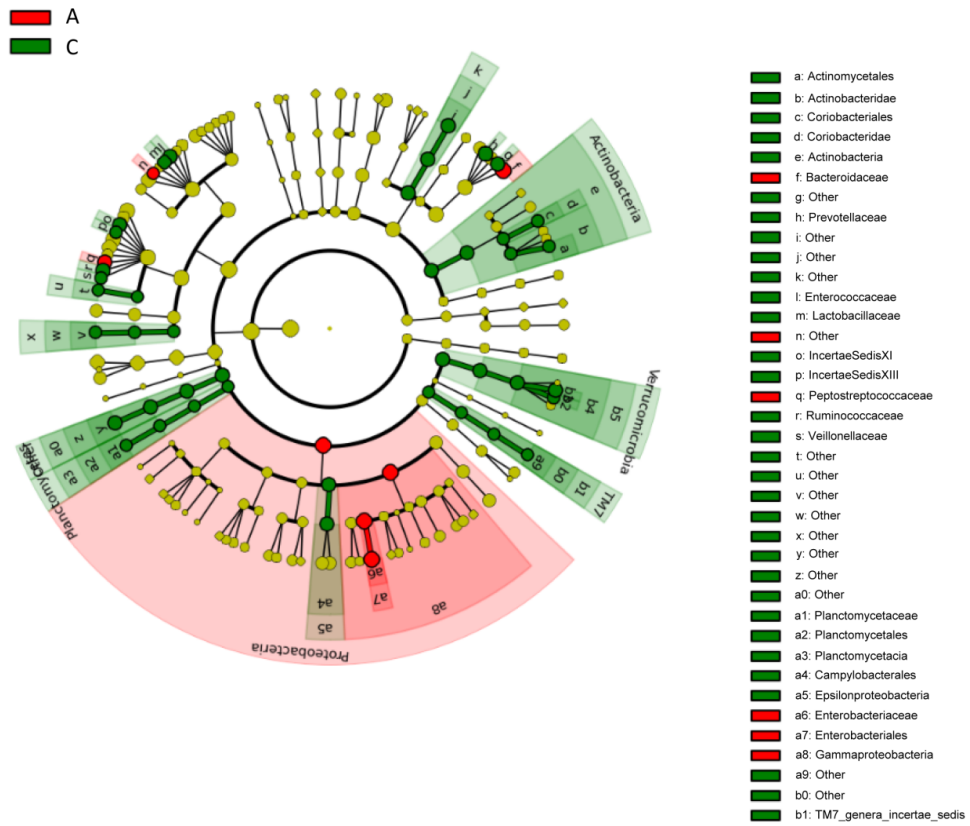


Fig. 17. LEfSe results on foal microbiome. Rotary phylogenetic representation of the predominate microbial composition of fecal samples from foals at 2 days of age (A, red) and 32 days of age (C, green) [187].

Discussion

In this study, our first objective was to evaluate changes in the microbiome of foals following vaccination with both live and inactivated *R. equi*. Although the number of CFUs administered were as high (for the live *R. equi* group) or higher than the number of CFU documented to protect foals against intrabronchial challenge with virulent *R. equi* (viz., 1 x 10^{10} CFU), no apparent differences in microbial communities

were observed among vaccinated groups (Fig. 12A and 14). Because all but 2 foals had samples collected only on days 2 and 32, and because fecal samples on day 2 were not influenced by treatment (because they were collected immediately prior to treatment), the effect of group was also examined among only samples collected at 32 days of age. Results restricted to 32 days of age also revealed no pattern distinguishing vaccinated and non-vaccinated foals (Fig. 12B and 15). Thus, we failed to detect evidence of a significant effect of enteral administration of either live or inactivated *R. equi* on microbial populations in neonatal foals. These results are consistent with reports in which probiotics (administered at similar or higher numbers of CFUs) have failed to alter the intestinal/fecal microbiome [197-199]. Our results should be interpreted with caution because of the relatively small number of foals, particularly in the live *R. equi* group. For technical reasons attributed to random error, pyrosequencing failed for samples from 3 foals from the live *R. equi* group and 2 foals from the control group without CTB group; therefore, only 3 foals from the live *R. equi* group and 6 from the controls without CTB group were included in the analysis.

A significant difference between the fecal microbial populations between day 2 and day 32 of age was observed (Table Q-1 and R-1; Fig. 13, 16, and 17). For descriptive purposes, we included the results from the 2 foals from which we had data at other ages (these data were not included in the statistical analysis comparing ages). The resident intestinal or fecal microbiota has been described for neonates of other species, such as cats [145, 146], dogs [147], and humans [148-151]. To the authors' knowledge, this is the first report of age-related changes of the fecal microbiome in foals. Significant

changes in the number of OTUs, the Shannon index, and the Chao1 metric were observed between the age groups (Table 2), showing clear evidence of strong diversification of bacterial populations between 2 and 32 days of age.

Firmicutes were detected in 100% of foals at both 2 and 32 days of age, with reported median sequences of 40% in 2-day-old foals decreasing (albeit not significantly) to 23% in 32-day-old foals. In 2 previous studies using fecal samples from adult horses, Firmicutes represented 44% [200] and 72% [201] of the bacteria. Within the Firmicutes, the family Enterococcaceae significantly decreased with age ($P = 0.0080$), which was likely attributable at least in part to decreases in the genus *Enterococcus* that were observed to decrease significantly by qPCR ($P < 0.0001$) and by pyrosequencing. Proteobacteria were detected in the feces of all 2-day-old foals and 97% of 32-day-old foals, a difference that was not significant; however, the median percentage of sequences decreased significantly ($P < 0.0001$) between day 2 (median, 36.3%; range, 0.5 to 85.8%) and day 32 (median, 2.7%; range, 0 to 40.9%). In adult horses, Proteobacteria have been reported to represent 6% [200] and 12% [202] of fecal sequences. These results from adult horses are interesting in light of our findings, particularly our observation that the family Enterobacteriaceae decreased with age, a finding substantiated by our qPCR results with a significant decrease in the amount of *E. coli* ($P < 0.0001$) between ages 2 and 32 days.

The sterile GI tract of newborn puppies and kittens is presumably colonized by bacteria present in the birth canal and from the environment [155], and human neonates appear to become colonized by these sources as well as through the intestinal microbiota

of the mother [148, 154]. In humans, the initial microbes colonizing infants are facultative anaerobic bacteria, such as *E. coli* and *Streptococcus* spp. [154], which was also observed in 2-day old foals by the presence of Enterobacteriaceae (*E. coli*) and Streptococcaceae families (*Streptococcus* spp.). We observed a significant decrease in both these families by 32 days of age, suggesting that a similar phenomenon might happen in foals. In human beings, after the initial colonization by facultative anaerobic bacteria, colonization occurs by *Staphylococcus*-, *Enterococcus*-, and *Lactobacillus*-like species, and this change might contribute to generating an anaerobic environment [151]. The development during the first month of life in foals of an anaerobic environment is supported by the age-related increase in the detection of the phylum of Bacteroidetes ($P = 0.0066$), which is also a common constituent of the gut microbiota of dogs and cats [155]. However, we also observed a significant decrease in the Enterococcaceae family ($P = 0.0080$) and *Enterococcus* spp. by qPCR ($P < 0.0001$), as well as the Lactobacillaceae family ($P = 0.0281$).

Our study has a number of important limitations. One limitation is the use of fecal swab samples for analysis, because feces might not be representative of other compartments of the gut. In humans, the composition of the mucosal-surface microbiota is distinct from that recovered in the feces [175]. The situation is probably similar in the horse, because of the complexity of the equine gastrointestinal tract. For example, the microbial population of adult horse fecal samples is likely to represent that of the right dorsal colon, but not that of the cecum [156].

A second limitation of our study is the small number of foals enrolled. Our sample size was limited both by financial considerations and the number of foals available to us during the study period. Because of the small sample size, we were only able to observe large changes in fecal microbial populations. Nevertheless, our results provide useful data for those exploring enteral vaccination of foals [157, 203]. It is worth noting that there were significant differences in immune responses that were detectable among these groups of foals despite the small sample size (please refer to chapter II). Also, we were able to detect significant age-related differences in the microbiome of foals, irrespective of the treatment groups.

Another limitation of our study is that we only characterized age-related changes at 2 ages during the first month of life. Although our data from 2 foals with more frequent sampling appears to demonstrate a progressive diversification of microbial flora with age (Fig. 16), further studies using more foals with more frequent sampling times are needed to better characterize microbial diversification. Our focus on the first month of life was based on current understanding that vaccination of foals against *R. equi* will have to occur during early life [204].

In conclusion, no differences were observed in the fecal microbiome of foals following enteral vaccination with either live or inactivated *R. equi*. These results demonstrate that administration of the doses of bacteria used in this study does not likely cause an alteration of the fecal microbiome of foals. More notably, the results indicate significant age-related changes in the microbiome composition of foals during the first month of life.

SUMMARY AND CONCLUSIONS

Summary

Rhodococcus equi pneumonia is an important cause of pneumonia in foals. Despite considerable effort, to date there is no vaccine that will protect foals against intrabronchial challenge with *R. equi.* The only approach that has been demonstrated repeatedly to protect foals from challenge is the administration of live, virulent *R. equi*. This strategy, however, is not acceptable for use in farms because of its potential to cause disease in the host, as well as concerns with environmental contamination and manufacturing of live vaccines. This dissertation describes development and initial assessment of the immunogenicity of e-beam irradiated bacteria as a candidate for an effective vaccine against *R. equi* pneumonia in foals.

Two concentrations of *R. equi* were used to evaluate e-beam irradiation as a method of inactivation: 1×10^8 colony-forming units/ml (CFU/ml) and 1×10^9 CFU/ml. Both concentrations were submitted to a range of e-beam radiation doses, ranging from 0 to 7 kGy. All microorganisms of both the lower and higher concentrations were completely inactivated by 4 and 5 kGy, respectively. Moreover, the cell walls of the irradiated bacteria remained structurally intact, whereas heat-inactivated samples developed compromised cell walls. It was demonstrated that e-beam irradiation completely inactivated *R. equi* samples, while retaining their structural integrity.

The immunogenicity of 2 doses of vaccine were examined: the doses selected were 2- and 5-fold greater than the number of bacteria (1×10^{10} CFU) previously reported to protect foals against intra-bronchial challenge with live, virulent *R. equi*. Mucosal (NP swab eluates and BAL fluid) and serum antibody responses were studied, as well as cell-mediated immune responses (IFN-γ production by PBMCs in culture). The e-beam vaccine appeared to stimulate a robust cell-mediated immune response and, at the higher dose, upper respiratory tract mucosal IgA, in the face of maternally-transferred antibody to foals. These results encourage us to evaluate the effectiveness of this candidate vaccine for efficacy in protecting foals against *R. equi* pneumonia.

The e-beam inactivated *R. equi* vaccine appeared safe, as no adverse effects were noted in study foals; however, vaccine safety will require hundreds of animals to identify common side-effects and thousands of animals to identify less common adverse effects. One aspect of safety consideration for enteral vaccines is the impact on the intestinal microbiome. Although there were profound changes in the composition and diversity of the neonatal microbiome during the first month of life, enteral administration of either live *R. equi* or e-beam irradiated *R. equi* did not appear to alter the fecal microbiome. These results help us feel confident that neonatal enteral vaccination is not likely to impair the natural age-related expansion of composition and diversity of the intestinal microbiome.

Conclusions

In summary, our conclusions were:

1. *Rhodococcus equi* can be inactivated by e-beam irradiation without losing membrane integrity;
2. enteral administration of live virulent *R. equi* induces detectable total and anti-*R. equi* IgA in nasal swab eluates and IgA and IgG_1, $IgG_{4/7}$, $IgG_{3/5}$ isotypes in serum, as well as IFN-γ in supernatant of cultured PBMCs; it does not induce anti-*R. equi*-specific antibodies in BAL fluid;
3. intragastric administration of either dose of enteral e-beam inactivated virulent *R. equi* to foals induced significant increases in the production of soluble IFN-γ in supernatant of cultured PBMCs, but does not induce significant changes in the amount of total and anti-*R. equi* IgA and IgG_1, $IgG_{4/7}$, $IgG_{3/5}$ isotypes antibodies in BAL fluid and serum samples;
4. the proportion of foals with increased *R. equi*-specific IgA is higher in foals receiving the higher dose of enteral e-beam inactivated virulent *R. equi* compared to those receiving saline (plus adjuvant);
5. the 2 doses of enteral e-beam inactivated virulent *R. equi* induced similar serum and mucosal humoral responses, except the higher dose appears to induce stronger nasal *R. equi*-specific IgA;
6. intragastric administration of either dose of enteral e-beam inactivated virulent *R. equi* or live *R. equi* to foals does not substantively alter the microbiome of foals;

however, significant age-related changes in the fecal microbiome composition and diversity were detected during the first month of life in foals; and,

7. enteral administration of e-beam irradiated *R. equi* merits evaluation for efficacy to protect against *R. equi* challenge infection in foals.

REFERENCES

[1] Anonymous. Part I: Baseline Reference of 1998 Equine Health Management. USDA-APHIS: Veterinary Services Report; 1998. p. 34-46.

[2] Anonymous. Report of Foal Pneumonia Panel. J Equine Med Surg. 1978;412:400-4.

[3] Ainsworth DM, Eicker SW, Yeagar AE, Sweeney CR, Viel L, Tesarowski D, et al. Associations between physical examination, laboratory, and radiographic findings and outcome and subsequent racing performance of foals with *Rhodococcus equi* infection: 115 cases (1984-1992). J Am Vet Med Assoc. 1998;213:510-5.

[4] Giguère S, Prescott JF. Clinical manifestations, diagnosis, treatment, and prevention of *Rhodococcus equi* infections in foals. Vet Microbiol. 1997;56:313-34.

[5] Prescott JF. *Rhodococcus equi*: an animal and human pathogen. Clin Microbiol Rev. 1991;4:20-34.

[6] Meijer WG, Prescott JF. *Rhodococcus equi*. Vet Res. 2004;35:383-96.

[7] Muscatello G, Leadon DP, Klayt M, Ocampo-Sosa A, Lewis DA, Fogarty U, et al. *Rhodococcus equi* infection in foals: the science of 'rattles'. Equine Vet J. 2007;39:470-8.

[8] Takai S. Epidemiology of *Rhodococcus equi* infections: a review. Vet Microbiol. 1997;56:167-76.

[9] Cohen ND, O'Conor MS, Chaffin MK, Martens RJ. Farm characteristics and management practices associated with development of *Rhodococcus equi* pneumonia in foals. J Am Vet Med Assoc. 2005;226:404-13.

[10] Chaffin MK, Cohen ND, Martens RJ, Edwards RF, Nevill M. Foal-related risk factors associated with development of *Rhodococcus equi* pneumonia on farms with endemic infection. J Am Vet Med Assoc. 2003;223:1791-9.

[11] Giguère S, Hernandez J, Gaskin J, Miller C, Bowman JL. Evaluation of white blood cell concentration, plasma fibrinogen concentration, and an agar gel immunodiffusion test for early identification of foals with *Rhodococcus equi* pneumonia. J Am Vet Med Assoc. 2003;222:775-81.

[12] Giguère S, Gaskin JM, Miller C, Bowman JL. Evaluation of a commercially available hyperimmune plasma product for prevention of naturally acquired pneumonia caused by *Rhodococcus equi* in foals. J Am Vet Med Assoc. 2002;220:59-63.

[13] Hurley JR, Begg AP. Failure of hyperimmune plasma to prevent pneumonia caused by *Rhodococcus equi* in foals. Aust Vet J. 1995;72:418-20.

[14] Slovis NM MJ, Mundy GD. How to use thoracic ultrasound to screen foals for *Rhodococcus equi* at affected farms. In: Brokken TD, editor. AAEP 2005: Proceedings of the 51st Annual Convention of the American Association of Equine Practitioners; 2005 Dec 3-7; Seattle, USA. Lexington, KY: American Association of Equine Practitioners. p. 274-8.

[15] Martens RJ, Martens JG, Fiske RA, Hietala SK. *Rhodococcus equi* foal pneumonia: protective effects of immune plasma in experimentally infected foals. Equine Vet J. 1989;21:249-55.

[16] Caston SS, McClure SR, Martens RJ, Chaffin MK, Miles KG, Griffith RW, et al. Effect of hyperimmune plasma on the severity of pneumonia caused by *Rhodococcus equi* in experimentally infected foals. Vet Ther. 2006;7:361-75.

[17] Madigan JE, Hietala S, Muller N. Protection against naturally acquired *Rhodococcus equi* pneumonia in foals by administration of hyperimmune plasma. J Reprod Fertil Suppl. 1991;44:571-8.

[18] Martens RJ, Martens JG, Fiske RA. Failure of passive immunisation by colostrum from immunised mares to protect foals against *Rhodococcus equi* pneumonia. Equine Vet J (Suppl) 1991;12:19-22.

[19] Varga J, Fodor L, Rusvai M, Soos I, Makrai L. Prevention of *Rhodococcus equi* pneumonia of foals using two different inactivated vaccines. Vet Microbiol. 1997;56:205-12.

[20] Becu T, Polledo G, Gaskin JM. Immunoprophylaxis of *Rhodococcus equi* pneumonia in foals. Vet Microbiol. 1997;56:193-204.

[21] Cauchard J, Sevin C, Ballet JJ, Taouji S. Foal IgG and opsonizing anti-*Rhodococcus equi* antibodies after immunization of pregnant mares with a protective VapA candidate vaccine. Vet Microbiol. 2004;104:73-81.

[22] Takai S, Kobayashi C, Murakami K, Sasaki Y, Tsubaki S. Live virulent *Rhodococcus equi*, rather than killed or avirulent, elicits protective immunity to *R. equi* infection in mice. FEMS Immunol Med Microbiol. 1999;24:1-9.

[23] Prescott JF, Nicholson VM, Patterson MC, Zandona Meleiro MC, Caterino de Araujo A, Yager JA, et al. Use of *Rhodococcus equi* virulence-associated protein

for immunization of foals against *R. equi* pneumonia. Am J Vet Res. 1997;58:356-9.

[24] Lopez AM, Hines MT, Palmer GH, Knowles DP, Alperin DC, Hines SA. Analysis of anamnestic immune responses in adult horses and priming in neonates induced by a DNA vaccine expressing the vapA gene of *Rhodococcus equi*. Vaccine. 2003;21:3815-25.

[25] Mealey RH, Stone DM, Hines MT, Alperin DC, Littke MH, Leib SR, et al. Experimental *Rhodococcus equi* and equine infectious anemia virus DNA vaccination in adult and neonatal horses: effect of IL-12, dose, and route. Vaccine. 2007;25:7582-97.

[26] Lopez AM, Townsend HG, Allen AL, Hondalus MK. Safety and immunogenicity of a live-attenuated auxotrophic candidate vaccine against the intracellular pathogen *Rhodococcus equi*. Vaccine. 2008;26:998-1009.

[27] Pei Y, Nicholson V, Woods K, Prescott JF. Immunization by intrabronchial administration to 1-week-old foals of an unmarked double gene disruption strain of *Rhodococcus equi* strain 103+. Vet Microbiol. 2007;125:100-10.

[28] Dawson TR, Horohov DW, Meijer WG, Muscatello G. Current understanding of the equine immune response to *Rhodococcus equi*. An immunological review of *R. equi* pneumonia. Vet Immunol Immunopathol. 2010;135:1-11.

[29] Harris SP, Fujiwara N, Mealey RH, Alperin DC, Naka T, Goda R, et al. Identification of *Rhodococcus equi* lipids recognized by host cytotoxic T lymphocytes. Microbiology. 2010;156:1836-47.

[30] Patton KM, McGuire TC, Hines MT, Mealey RH, Hines SA. *Rhodococcus equi*-specific cytotoxic T lymphocytes in immune horses and development in asymptomatic foals. Infect Immun. 2005;73:2083-93.

[31] Horowitz ML, Cohen ND, Takai S, Becu T, Chaffin MK, Chu KK, et al. Application of Sartwell's model (lognormal distribution of incubation periods) to age at onset and age at death of foals with *Rhodococcus equi* pneumonia as evidence of perinatal infection. J Vet Intern Med. 2001;15:171-5.

[32] Chaffin MK, Cohen ND, Martens RJ. Chemoprophylactic effects of azithromycin against *Rhodococcus equi*-induced pneumonia among foals at equine breeding farms with endemic infections. J Am Vet Med Assoc. 2008;232:1035-47.

[33] Giguère S, Cohen ND, Chaffin MK, Hines SA, Hondalus MK, Prescott JF, et al. *Rhodococcus equi*: clinical manifestations, virulence, and immunity. J Vet Int Med. 2011;25:1221-30.

[34] Magnusson H. Spezifische infektiose Pneumonie beim Fohlen. Ein neuer Eitererreger beim Pferde. Archiv f wissensch u prakt Tierh. 1923;50:22-38. German.

[35] Hooper-McGrevy KE, Wilkie BN, Prescott JF. Virulence-associated protein-specific serum immunoglobulin G-isotype expression in young foals protected against *Rhodococcus equi* pneumonia by oral immunization with virulent *R. equi*. Vaccine. 2005;23:5760-7.

[36] Chirino-Trejo JM, Prescott JF, Yager JA. Protection of foals against experimental *Rhodococcus equi* pneumonia by oral immunization. Can J Vet Res. 1987;51:444-7.

[37] Hines SA, Kanaly ST, Byrne BA, Palmer GH. Immunity to *Rhodococcus equi*. Vet Microbiol. 1997;56:177-85.

[38] Cohen ND, Giguère S. *Rhodococcus equi* Foal Pneumonia. In: Mair TS, Hutchinson RE, editors. Infectious Diseases of the Horse. Cambridgeshire, UK: Equine Veterinary Journal Ltd; 2009, p. 235-46.

[39] Ellenberger MA, Kaeberle ML, Roth JA. Equine humoral immune response to *Rhodococcus (Corynebacterium) equi*. Am J Vet Res. 1984;45:2428-30.

[40] Wilks CR, Barton MD, Allison JF. Immunity to and immunotherapy for *Rhodococcus equi*. J Reprod Fertil Suppl. 1982;32:497-505.

[41] Takai S, Kawazu S, Tsubaki S. Immunoglobulin and specific antibody responses to *Rhodococcus (Corynebacterium) equi* infection in foals as measured by enzyme-linked immunosorbent assay. J Clin Microbiol. 1986;23:943-7.

[42] Hines MT. *Rhodococcus equi*. In: Sellon DC, Long MT, editors. Equine Infectious Diseases. St. Louis: Elsevier; 2007, p. 281-295.

[43] Takai S, Chaffin MK, Cohen ND, Hara M, Nakamura M, Kakuda T, et al. Prevalence of virulent *Rhodococcus equi* in soil from five R. equi-endemic horse-breeding farms and restriction fragment length polymorphisms of virulence plasmids in isolates from soil and infected foals in Texas. J Vet Diagn Invest. 2001;13:489-94.

[44] Morton AC, Begg AP, Anderson GA, Takai S, Lammler C, Browning GF. Epidemiology of *Rhodococcus equi* strains on Thoroughbred horse farms. Appl Environ Microbiol. 2001;67:2167-75.

[45] Morton AC, Baseggio N, Peters MA, Browning GF. Diversity of isolates of *Rhodococcus equi* from Australian thoroughbred horse farms. Antonie Van Leeuwenhoek. 1998;74:21-5.

[46] Bolton T, Kuskie K, Halbert N, Chaffin K, Healy M, Lawhon S, et al. Detection of strain variation in isolates of *Rhodococcus equi* from an affected foal using repetitive sequence-based polymerase chain reaction. J Vet Diagn Invest. 2010;22:611-5.

[47] Giguère S, Cohen ND, Chaffin MK, Hines SA, Hondalus MK, Prescott JF, et al. *Rhodococcus equi*: clinical manifestations, virulence, and immunity. J Vet Intern Med. 2011;25:1221-30.

[48] Takai S, Michizoe T, Matsumura K, Nagai M, Sato H, Tsubaki S. Correlation of in vitro properties of *Rhodococcus (Corynebacterium) equi* with virulence for mice. Microbiol Immunol. 1985;29:1175-84.

[49] Zink MC, Yager JA, Prescott JF, Fernando MA. Electron microscopic investigation of intracellular events after ingestion of *Rhodococcus equi* by foal alveolar macrophages. Vet Microbiol. 1987;14:295-305.

[50] Hondalus MK, Mosser DM. Survival and replication of *Rhodococcus equi* in macrophages. Infect Immun. 1994;62:4167-75.

[51] Jain S, Bloom BR, Hondalus MK. Deletion of vapA encoding virulence associated protein A attenuates the intracellular actinomycete *Rhodococcus equi*. Mol Microbiol. 2003;50:115-28.

[52] Takai S, Sekizaki T, Ozawa T, Sugawara T, Watanabe Y, Tsubaki S. Association between a large plasmid and 15- to 17-kilodalton antigens in virulent *Rhodococcus equi*. Infect Immun. 1991;59:4056-60.

[53] Giguère S, Hondalus MK, Yager JA, Darrah P, Mosser DM, Prescott JF. Role of the 85-kilobase plasmid and plasmid-encoded virulence-associated protein A in intracellular survival and virulence of *Rhodococcus equi*. Infect Immun. 1999;67:3548-57.

[54] Coulson GB, Agarwal S, Hondalus MK. Characterization of the role of the pathogenicity island and vapG in the virulence of the intracellular actinomycete pathogen *Rhodococcus equi*. Infect Immun. 2010;78:3323-34.

[55] Hondalus MK, Diamond MS, Rosenthal LA, Springer TA, Mosser DM. The intracellular bacterium *Rhodococcus equi* requires Mac-1 to bind to mammalian cells. Infect Immun. 1993;61:2919-29.

[56] Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. Annu Rev Immunol. 1999;17:593-623.

[57] Hietala SK, Ardans AA. Interaction of *Rhodococcus equi* with phagocytic cells from *R. equi*-exposed and non-exposed foals. Vet Microbiol. 1987;14:307-20.

[58] Barton MD, Hughes KL. Is *Rhodococcus equi* a soil organism? J Reprod Fertil Suppl. 1982;32:481-9.

[59] Barton MD, Hughes KL. Comparison of three techniques for isolation of *Rhodococcus (Corynebacterium) equi* from contaminated sources. J Clin Microbiol. 1981;13:219-21.

[60] Nakazawa M, Sugimoto C, Isayama Y. Quantitative culture of *Rhodococcus equi* from the feces of horse. Natl Inst Anim Health Q (Tokyo). 1983;23:67-8.

[61] Takai S, Iimori S, Tsubaki S. Quantitative fecal culture for early diagnosis of *Corynebacterium (Rhodococcus) equi* enteritis in foals. Can J Vet Res. 1986;50:479-84.

[62] Takai S, Ohkura H, Watanabe Y, Tsubaki S. Quantitative aspects of fecal *Rhodococcus (Corynebacterium) equi* in foals. J Clin Microbiol. 1986;23:794-6.

[63] Grimm MB, Cohen ND, Slovis NM, Mundy GD, Harrington JR, Libal MC, et al. Evaluation of fecal samples from mares as a source of *Rhodococcus equi* for their foals by use of quantitative bacteriologic culture and colony immunoblot analyses. Am J Vet Res. 2007;68:63-71.

[64] Buntain S, Carter C, Kuskie K, Smith J, Stepusin R, Chaffin MK, et al. Frequency of *Rhodococcus equi* in feces of mares in central Kentucky. J Equine Vet Sci. 2010;30:191-5.

[65] Cohen ND, Carter CN, Scott HM, Chaffin MK, Smith JL, Grimm MB, et al. Association of soil concentrations of *Rhodococcus equi* and incidence of pneumonia attributable to *Rhodococcus equi* in foals on farms in central Kentucky. Am J Vet Res. 2008;69:385-95.

[66] Cohen ND, Smith KE, Ficht TA, Takai S, Libal MC, West BR, et al. Epidemiologic study of results of pulsed-field gel electrophoresis of isolates of *Rhodococcus equi* obtained from horses and horse farms. Am J Vet Res. 2003;64:153-61.

[67] Takai S, Narita K, Ando K, Tsubaki S. Ecology of *Rhodococcus (Corynebacterium) equi* in soil on a horse-breeding farm. Vet Microbiol. 1986;12:169-77.

[68] Muscatello G, Gerbaud S, Kennedy C, Gilkerson JR, Buckley T, Klay M, et al. Comparison of concentrations of *Rhodococcus equi* and virulent *R. equi* in air of stables and paddocks on horse breeding farms in a temperate climate. Equine Vet J. 2006;38:263-5.

[69] Cohen ND, Kuskie KR, Smith JL, Slovis NM, Brown SE, 2nd, Stepusin RS, et al. Association of airborne concentration of virulent *Rhodococcus equi* with location (stall versus paddock) and month (January through June) on 30 horse breeding farms in central Kentucky. Am J Vet Res. 2012;73:1603-9.

[70] Chaffin MK, Cohen ND, Martens RJ. Evaluation of equine breeding farm management and preventative health practices as risk factors for development of *Rhodococcus equi* pneumonia in foals. J Am Vet Med Assoc. 2003;222:476-85.

[71] Grondahl G, Johannisson A, Demmers S, Jensen Waern M. Influence of age and plasma treatment on neutrophil phagocytosis and CD18 expression in foals. Vet Microbiol. 1999;65:241-54.

[72] Martens JG, Martens RJ, Renshaw HW. *Rhodococcus (Corynebacterium) equi*: bactericidal capacity of neutrophils from neonatal and adult horses. Am J Vet Res. 1988;49:295-9.

[73] McTaggart C, Yovich JV, Penhale J, Raidal SL. A comparison of foal and adult horse neutrophil function using flow cytometric techniques. Res Vet Sci. 2001;71:73-9.

[74] Nerren JR, Payne S, Halbert ND, Martens RJ, Cohen ND. Cytokine expression by neutrophils of adult horses stimulated with virulent and avirulent *Rhodococcus equi* in vitro. Vet Immunol Immunopathol. 2009;127:135-43.

[75] Nerren JR, Martens RJ, Payne S, Murrell J, Butler JL, Cohen ND. Age-related changes in cytokine expression by neutrophils of foals stimulated with virulent *Rhodococcus equi* in vitro. Vet Immunol Immunopathol. 2009;127:212-9.

[76] Flaminio MJ, Rush BR, Davis EG, Hennessy K, Shuman W, Wilkerson MJ. Characterization of peripheral blood and pulmonary leukocyte function in healthy foals. Vet Immunol Immunopathol. 2000;73:267-85.

[77] Flaminio MJ, Borges AS, Nydam DV, Horohov DW, Hecker R, Matychak MB. The effect of CpG-ODN on antigen presenting cells of the foal. J Immune Based Ther Vaccines. 2007;5:1.

[78] Lunn DP, Holmes MA, Duffus WP. Equine T-lymphocyte MHC II expression: variation with age and subset. Vet Immunol Immunopathol. 1993;35:225-38.

[79] Sheoran AS, Timoney JF, Holmes MA, Karzenski SS, Crisman MV. Immunoglobulin isotypes in sera and nasal mucosal secretions and their neonatal transfer and distribution in horses. Am J Vet Res. 2000;61:1099-105.

[80] Ryan C, Giguère S, Hagen J, Hartnett C, Kalyuzhny AE. Effect of age and mitogen on the frequency of interleukin-4 and interferon gamma secreting cells in foals

and adult horses as assessed by an equine-specific ELISPOT assay. Vet Immunol Immunopathol. 2010;133:66-71.

[81] Boyd NK, Cohen ND, Lim WS, Martens RJ, Chaffin MK, Ball JM. Temporal changes in cytokine expression of foals during the first month of life. Vet Immunol Immunopathol. 2003;92:75-85.

[82] Breathnach CC, Sturgill-Wright T, Stiltner JL, Adams AA, Lunn DP, Horohov DW. Foals are interferon gamma-deficient at birth. Vet Immunol Immunopathol. 2006;112:199-209.

[83] Flaminio MJ, Rush BR, Shuman W. Immunologic function in horses after non-specific immunostimulant administration. Vet Immunol Immunopathol. 1998;63:303-15.

[84] Chaffin MK, Cohen ND, Martens RJ, Edwards RF, Nevill M, Smith R, 3rd. Hematologic and immunophenotypic factors associated with development of *Rhodococcus equi* pneumonia of foals at equine breeding farms with endemic infection. Vet Immunol Immunopathol. 2004;100:33-48.

[85] Ryan C, Giguère S. Equine neonates have attenuated humoral and cell-mediated immune responses to a killed adjuvanted vaccine compared to adult horses. Clin Vaccine Immunol. 2010;17:1896-902.

[86] Nelson KM, Schram BR, McGregor MW, Sheoran AS, Olsen CW, Lunn DP. Local and systemic isotype-specific antibody responses to equine influenza virus infection versus conventional vaccination. Vaccine. 1998;16:1306-13.

[87] Lopez AM, Hines MT, Palmer GH, Alperin DC, Hines SA. Identification of pulmonary T-lymphocyte and serum antibody isotype responses associated with protection against *Rhodococcus equi*. Clin Diagn Lab Immunol. 2002;9:1270-6.

[88] Lewis MJ, Wagner B, Woof JM. The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. Mol Immunol. 2008;45:818-27.

[89] Hooper-McGrevy KE, Wilkie BN, Prescott JF. Immunoglobulin G subisotype responses of pneumonic and healthy, exposed foals and adult horses to *Rhodococcus equi* virulence-associated proteins. Clin Diagn Lab Immunol. 2003;10:345-51.

[90] Tan C, Prescott JF, Patterson MC, Nicholson VM. Molecular characterization of a lipid-modified virulence-associated protein of *Rhodococcus equi* and its potential in protective immunity. Can J Vet Res. 1995;59:51-9.

[91] Vaerman JP, Querinjean P, Heremans JF. Studies on the IgA system of the horse. Immunology. 1971;21:443-54.

[92] McGuire TC, Crawford TB. Identification and quantitation of equine serum and secretory immunoglobulin A. Infect Immun. 1972;6:610-5.

[93] Pahud JJ, Mach JP. Equine secretory IgA and secretory component. Int Arch Allergy Appl Immunol. 1972;42:175-86.

[94] Macpherson AJ, McCoy KD, Johansen FE, Brandtzaeg P. The immune geography of IgA induction and function. Mucosal Immunol. 2008;1:11-22.

[95] Murphy K. The Humoral Immune Response. In: Murphy K, editor. Janeway's Immunobiology. New York: Garland Science; 2012. p. 387-428.

[96] Sheoran AS, Sponseller BT, Holmes MA, Timoney JF. Serum and mucosal antibody isotype responses to M-like protein (SeM) of *Streptococcus equi* in convalescent and vaccinated horses. Vet Immunol Immunopathol. 1997;59:239-51.

[97] Higuchi T, Arakawa T, Hashikura S, Inui T, Senba H, Takai S. Effect of prophylactic administration of hyperimmune plasma to prevent *Rhodococcus equi* infection on foals from endemically affected farms. Zentralbl Veterinarmed B. 1999;46:641-8.

[98] Meijer WG, Prescott JF. *Rhodococcus equi*. Vet Res. 2004;35:383-96.

[99] Hines SA, Stone DM, Hines MT, Alperin DC, Knowles DP, Norton LK, et al. Clearance of virulent but not avirulent *Rhodococcus equi* from the lungs of adult horses is associated with intracytoplasmic gamma interferon production by CD4+ and CD8+ T lymphocytes. Clin Diagn Lab Immunol. 2003;10:208-15.

[100] Kanaly ST, Hines SA, Palmer GH. Cytokine modulation alters pulmonary clearance of *Rhodococcus equi* and development of granulomatous pneumonia. Infect Immun. 1995;63:3037-41.

[101] Kanaly ST, Hines SA, Palmer GH. Transfer of a CD4+ Th1 cell line to nude mice effects clearance of *Rhodococcus equi* from the lung. Infect Immun. 1996;64:1126-32.

[102] Kanaly ST, Hines SA, Palmer GH. Failure of pulmonary clearance of *Rhodococcus equi* infection in CD4+ T-lymphocyte-deficient transgenic mice. Infect Immun. 1993;61:4929-32.

[103] Darrah PA, Hondalus MK, Chen Q, Ischiropoulos H, Mosser DM. Cooperation between reactive oxygen and nitrogen intermediates in killing of *Rhodococcus equi* by activated macrophages. Infect Immun. 2000;68:3587-93.

[104] Jacks S, Giguère S, Crawford PC, Castleman WL. Experimental infection of neonatal foals with *Rhodococcus equi* triggers adult-like gamma interferon induction. Clin Vaccine Immunol. 2007;14:669-77.

[105] Jacks S, Giguère S, Prescott JF. In vivo expression of and cell-mediated immune responses to the plasmid-encoded virulence-associated proteins of *Rhodococcus equi* in foals. Clin Vaccine Immunol. 2007;14:369-74.

[106] Sturgill TL, Giguère S, Berghaus LJ, Hurley DJ, Hondalus MK. Comparison of antibody and cell-mediated immune responses of foals and adult horses after vaccination with live *Mycobacterium bovis* BCG. Vaccine. 2014;32:1362-7.

[107] Giguère S, Lee E, Williams E, Cohen ND, Chaffin MK, Halbert N, et al. Determination of the prevalence of antimicrobial resistance to macrolide antimicrobials or rifampin in *Rhodococcus equi* isolates and treatment outcome in foals infected with antimicrobial-resistant isolates of *R. equi*. J Am Vet Med Assoc. 2010;237:74-81.

[108] Burton AJ, Giguère S, Sturgill TL, Berghaus LJ, Slovis NM, Whitman JL, et al. Macrolide- and rifampin-resistant *Rhodococcus equi* on a horse breeding farm, Kentucky, USA. Emerg Infect Dis. 2013;19:282-5.

[109] Venner M, Reinhold B, Beyerbach M, Feige K. Efficacy of azithromycin in preventing pulmonary abscesses in foals. Vet J. 2009;179:301-3.

[110] Haghighi HR, Prescott JF. Assessment in mice of vapA-DNA vaccination against *Rhodococcus equi* infection. Vet Immunol Immunopathol. 2005;104:215-25.

[111] van der Geize R, Grommen AW, Hessels GI, Jacobs AA, Dijkhuizen L. The steroid catabolic pathway of the intracellular pathogen *Rhodococcus equi* is important for pathogenesis and a target for vaccine development. PLoS Pathog. 2011;7:e1002181.

[112] Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med. 2005;11:S45-53.

[113] Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol. 2006;6:148-58.

[114] Arnold H, Bumann D, Felies M, Gewecke B, Sorensen M, Gessner JE, et al. Enhanced immunogenicity in the murine airway mucosa with an attenuated *Salmonella* live vaccine expressing OprF-OprI from *Pseudomonas aeruginosa*. Infect Immun. 2004;72:6546-53.

[115] Villena J, Medina M, Raya R, Alvarez S. Oral immunization with recombinant *Lactococcus lactis* confers protection against respiratory pneumococcal infection. Can J Microbiol. 2008;54:845-53.

[116] KuoLee R, Harris G, Conlan JW, Chen W. Oral immunization of mice with the live vaccine strain (LVS) of *Francisella tularensis* protects mice against respiratory challenge with virulent type A *F. tularensis*. Vaccine. 2007;25:3781-91.

[117] Dziedzic-Goclawska A, Kaminski A, Uhrynowska-Tyszkiewicz I, Stachowicz W. Irradiation as a safety procedure in tissue banking. Cell Tissue Bank. 2005;6:201-19.

[118] Poon PW, Dubeski P, Kitts DD. Effect of electron beam irradiation on microbial growth, lipid oxidation and color of ground beef patties upon refrigerated storage. Adv Exp Med Biol. 2004;542:101-11.

[119] Rivera CS, Blanco D, Marco P, Oria R, Venturini ME. Effects of electron-beam irradiation on the shelf life, microbial populations and sensory characteristics of summer truffles (*Tuber aestivum)* packaged under modified atmospheres. Food Microbiol. 2011;28:141-8.

[120] Cabeza MC, Cambero MI, Nunez M, Medina M, de la Hoz L, Ordonez JA. Lack of growth of *Listeria monocytogenes* and *Staphylococcus aureus* in temperature abuse of E-beam treated ready-to-eat (RTE) cooked ham. Food Microbiol. 2010;27:777-82.

[121] Pillai SD, Braby LA. Eletronic Pasteurization. In: Tewari G, Juneja VK, editors. Advances in Thermal and Non-Thermal Food Preservation. Ames, IA: Blackwell Publishing; 2007. doi: 10.1002/9780470277898.ch11.

[122] Sommers C, Fan X. Food Irradiation Research and Technology. Ames, IA: Blackwell; 2006.

[123] Brahmakshatriya V, Lupiani B, Brinlee JL, Cepeda M, Pillai SD, Reddy SM. Preliminary study for evaluation of avian influenza virus inactivation in contaminated poultry products using electron beam irradiation. Avian Pathol. 2009;38:245-50.

[124] Grasso EM, Uribe-Rendon RM, Lee K. Inactivation of *Escherichia coli* inoculated onto fresh-cut chopped cabbage using electron-beam processing. J Food Protect. 2011;74:115-8.

[125] Helfinstine SL, Vargas-Aburto C, Uribe RM, Woolverton CJ. Inactivation of *Bacillus* endospores in envelopes by electron beam irradiation. Appl Environ Microbiol. 2005;71:7029-32.

[126] Alpen EL. Radiation Chemistry. In: Alpen EL, editor. Radiation Biophysics. San Diego, CA: Academic Press, 1998. p. 104-31.

[127] Khan FH. The Elements of Immunology. Delhi, India: Pearson Education; 2009.

[128] Werner M, Chott A, Fabiano A, Battifora H. Effect of formalin tissue fixation and processing on immunohistochemistry. Am J Surg Pathol. 2000;24:1016-9.

[129] Brown F. Review of accidents caused by incomplete inactivation of viruses. Dev Biol Stand. 1993;81:103-7.

[130] Demicheli MC, Goes AM, de Andrade AS. Ultrastructural changes in *Paracoccidioides brasiliensis* yeast cells attenuated by gamma irradiation. Mycoses. 2007;50:397-402.

[131] Fiester SE, Helfinstine SL, Redfearn JC, Uribe RM, Woolverton CJ. Electron beam irradiation dose dependently damages the *Bacillus* spore coat and spore membrane. Int J Microbiol. 2012;2012:579593.

[132] Siegrist C-A. Vaccine Immunology. In: Plotkin SA, Orenstein W, Offit PA, editors. Vaccines. Philadelphia, PA: Saunders; 2008. p. 1335-55.

[133] Holmgren J, Czerkinsky C, Lycke N, Svennerholm AM. Mucosal immunity: implications for vaccine development. Immunobiology. 1992;184:157-79.

[134] Langridge W, Denes B, Fodor I. Cholera toxin B subunit modulation of mucosal vaccines for infectious and autoimmune diseases. Curr Opin Investig Drugs. 2010;11:919-28.

[135] Lei H, Sheng Z, Ding Q, Chen J, Wei X, Lam DM, et al. Evaluation of oral immunization with recombinant avian influenza virus HA1 displayed on the *Lactococcus lactis* surface and combined with the mucosal adjuvant cholera toxin subunit B. Clin Vaccine Immunol. 2011;18:1046-51.

[136] Asahi-Ozaki Y, Itamura S, Ichinohe T, Strong P, Tamura S, Takahashi H, et al. Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. Microbes Infect. 2006;8:2706-14.

[137] Anosova NG, Chabot S, Shreedhar V, Borawski JA, Dickinson BL, Neutra MR. Cholera toxin, *E. coli* heat-labile toxin, and non-toxic derivatives induce dendritic cell migration into the follicle-associated epithelium of Peyer's patches. Mucosal Immunol. 2008;1:59-67.

[138] MM OD, Harris HM, Jeffery IB, Claesson MJ, Younge B, PW OT, et al. The core faecal bacterial microbiome of Irish Thoroughbred racehorses. Lett Appl Microbiol. 2013;57:492-501.

[139] Cordeiro RA, Teixeira CE, Brilhante RS, Castelo-Branco DS, Paiva MA, Giffoni Leite JJ, et al. Minimum inhibitory concentrations of amphotericin B, azoles and caspofungin against *Candida* species are reduced by farnesol. Med Mycol. 2013;51:53-9.

[140] Dougal K, de la Fuente G, Harris PA, Girdwood SE, Pinloche E, Geor RJ, et al. Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. PLoS One. 2014;9:e87424.

[141] Moreau MM, Eades SC, Reinemeyer CR, Fugaro MN, Onishi JC. Illumina sequencing of the V4 hypervariable region 16S rRNA gene reveals extensive changes in bacterial communities in the cecum following carbohydrate oral infusion and development of early-stage acute laminitis in the horse. Vet Microbiol. 2014;168:436-41.

[142] Steelman SM, Chowdhary BP, Dowd S, Suchodolski J, Janecka JE. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. BMC Vet Res. 2012;8:231.

[143] Schoster A, Arroyo LG, Staempfli HR, Weese JS. Comparison of microbial populations in the small intestine, large intestine and feces of healthy horses using terminal restriction fragment length polymorphism. BMC Res Notes. 2013;6:91.

[144] Costa MC, Weese JS. The equine intestinal microbiome. Anim Health Res Rev. 2012;13:121-8.

[145] Jia J, Frantz N, Khoo C, Gibson GR, Rastall RA, McCartney AL. Investigation of the faecal microbiota of kittens: monitoring bacterial succession and effect of diet. FEMS Microbiol Ecol. 2011;78:395-404.

[146] Minamoto Y, Hooda S, Swanson KS, Suchodolski JS. Feline gastrointestinal microbiota. Anim Health Res Rev. 2012;13:64-77.

[147] Buddington RK. Postnatal changes in bacterial populations in the gastrointestinal tract of dogs. Am J Vet Res. 2003;64:646-51.

[148] Mshvildadze M, Neu J. The infant intestinal microbiome: friend or foe? Early Hum Dev. 2010;86 Suppl 1:67-71.

[149] Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biology. 2007;5:e177.

[150] Murgas Torrazza R, Neu J. The developing intestinal microbiome and its relationship to health and disease in the neonate. J Perinatol. 2011;31 Suppl 1:S29-34.

[151] Scholtens PA, Oozeer R, Martin R, Amor KB, Knol J. The early settlers: intestinal microbiology in early life. Annu Rev Food Sci Technol. 2012;3:425-47.

[152] Inman CF, Laycock GM, Mitchard L, Harley R, Warwick J, Burt R, et al. Neonatal colonisation expands a specific intestinal antigen-presenting cell subset prior to CD4 T-cell expansion, without altering T-cell repertoire. PLoS One. 2012;7:e33707.

[153] Willing BP, Van Kessel AG. Enterocyte proliferation and apoptosis in the caudal small intestine is influenced by the composition of colonizing commensal bacteria in the neonatal gnotobiotic pig. J Anim Sci. 2007;85:3256-66.

[154] Fanaro S, Chierici R, Guerrini P, Vigi V. Intestinal microflora in early infancy: composition and development. Acta Paediatr Suppl. 2003;91:48-55.

[155] Suchodolski JS. Companion animals symposium: microbes and gastrointestinal health of dogs and cats. J Anim Sci. 2011;89:1520-30.

[156] Dougal K, Harris PA, Edwards A, Pachebat JA, Blackmore TM, Worgan HJ, et al. A comparison of the microbiome and the metabolome of different regions of the equine hindgut. FEMS Microbiol Ecol. 2012;82:642-52.

[157] van der Geize R, Grommen AW, Hessels GI, Jacobs AA, Dijkhuizen L. The steroid catabolic pathway of the intracellular pathogen *Rhodococcus equi* is important for pathogenesis and a target for vaccine development. PLoS Pathog. 2011;7:e1002181.

[158] Garrido JM, Sevilla IA, Beltran-Beck B, Minguijon E, Ballesteros C, Galindo RC, et al. Protection against tuberculosis in Eurasian wild boar vaccinated with heat-inactivated *Mycobacterium bovis*. PLoS One. 2011;6:e24905.

[159] Quan FS, Compans RW, Kang SM. Oral vaccination with inactivated influenza vaccine induces cross-protective immunity. Vaccine. 2012;30:180-8.

[160] Sealy R, Webby RJ, Crumpton JC, Hurwitz JL. Differential localization and function of antibody-forming cells responsive to inactivated or live-attenuated influenza virus vaccines. Int Immunol. 2012; 25:183-95.

[161] Paillot R, Prowse L, Montesso F, Huang CM, Barnes H, Escala J. Whole inactivated equine influenza vaccine: efficacy against a representative clade 2 equine influenza virus, IFNgamma synthesis and duration of humoral immunity. Vet Microbiol. 2013;162:396-407.

[162] Baffa O, Kinoshita A. Clinical applications of alanine/electron spin resonance dosimetry. Radiat Environ Biophys. 2014. [Epub ahead of print] 10.1007/s00411-013-0509-2.

[163] Takai S, Iie M, Watanabe Y, Tsubaki S, Sekizaki T. Virulence-associated 15- to 17-kilodalton antigens in *Rhodococcus equi*: temperature-dependent expression and location of the antigens. Infect Immun. 1992;60:2995-7.

[164] Probes M (2004). LIVE/DEAD BacLight bacterial viability kit. Life Technologies. Available from: http://www.lifetechnologies.com/order/catalog/product/L7012?CID=search-product .

[165] Soboll G, Nelson KM, Leuthner ES, Clark RJ, Drape R, Macklin MD, et al. Mucosal co-administration of cholera toxin and influenza virus hemagglutinin-DNA in ponies generates a local IgA response. Vaccine. 2003;21:3081-92.

[166] van der Meulen KM, Nauwynck HJ, Pensaert MB. Mitogen stimulation favours replication of equine herpesvirus-1 in equine blood mononuclear cells by inducing cell proliferation and formation of close intercellular contacts. J Gen Virol. 2001;82:1951-7.

[167] Hackett CH, Flaminio MJ, Fortier LA. Analysis of CD14 expression levels in putative mesenchymal progenitor cells isolated from equine bone marrow. Stem Cells Dev. 2011;20:721-35.

[168] Rennard SI, Basset G, Lecossier D, O'Donnell KM, Pinkston P, Martin PG, et al. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. J Appl Physiol. 1986;60:532-8.

[169] Pinheiro J, Bates D. Mixed-Effects Models in S and S-PLUS. New York: Springer; 2000.

[170] Rodriguez O, Castell-Perez ME, Ekpanyaskun N, Moreira RG, Castillo A. Surrogates for validation of electron beam irradiation of foods. Int J Food Microbiol. 2006;110:117-22.

[171] Sidak Z. Rectangular confidence regions for the means of multivariate normal distributions. J Am Statist Assoc. 1967;62:626-33.

[172] Pillai SD. Food Irradiation. In: Beier RC, Pillai SD, Phillips TD, editors. Preharvest and Postharvest Food Safety: Contemporary Issues and Future Directions. Ames, IA: Blackwell Publishing; 2008. p. 375-88. doi: 10.1002/9780470752579.ch28

[173] Garrison RG, Mirikitani FK, Lane JW. Fine structural studies of *Rhodococcus* species. Microbios. 1983;36:183-90.

[174] Flaminio MJ, Rush BR, Shuman W. Peripheral blood lymphocyte subpopulations and immunoglobulin concentrations in healthy foals and foals with *Rhodococcus equi* pneumonia. J Vet Intern Med. 1999;13:206-12.

[175] Smith P, MacDonald TT, Blumerg RS. Principles of Mucosal Immunology. New York: Garland Science; 2013.

[176] Collie DD, Baker A, Mauchline S, Porteous D, Mc LG. Ovine bronchoalveolar lavage cellularity: reproducibility and the effect of multiple repeated lavage. Res Vet Sci. 1999;67:137-40.

[177] Fernandez-Bustamante A, Easley RB, Fuld M, Mulreany D, Chon D, Lewis JF, et al. Regional pulmonary inflammation in an endotoxemic ovine acute lung injury model. Respir Physiol Neurobiol. 2012;183:149-58.

[178] Walters EH, Gardiner PV. Bronchoalveolar lavage as a research tool. Thorax. 1991;46:613-8.

[179] Hines MT, Paasch KM, Alperin DC, Palmer GH, Westhoff NC, Hines SA. Immunity to *Rhodococcus equi*: antigen-specific recall responses in the lungs of adult horses. Vet Immunol Immunopathol. 2001;79:101-14.

[180] Jeffcott LB. Studies on passive immunity in the foal. II. The absorption of 125I-labelled PVP (polyvinyl pyrrolidone) by the neonatal intestine. J Comp Pathol. 1974;84:279-89.

[181] Gans HA, Maldonado Y, Yasukawa LL, Beeler J, Audet S, Rinki MM, et al. IL-12, IFN-gamma, and T cell proliferation to measles in immunized infants. J Immunol. 1999;162:5569-75.

[182] Clark SO, Kelly DL, Badell E, Castello-Branco LR, Aldwell F, Winter N, et al. Oral delivery of BCG Moreau Rio de Janeiro gives equivalent protection against tuberculosis but with reduced pathology compared to parenteral BCG Danish vaccination. Vaccine. 2010;28:7109-16.

[183] Tompkins DM, Buddle BM, Whitford J, Cross ML, Yates GF, Lambeth MR, et al. Sustained protection against tuberculosis conferred to a wildlife host by single dose oral vaccination. Vaccine. 2013;31:893-9.

[184] Ancelet LR, Aldwell FE, Rich FJ, Kirman JR. Oral vaccination with lipid-formulated BCG induces a long-lived, multifunctional CD4(+) T cell memory immune response. PLoS One. 2012;7:e45888.

[185] Halbert ND, Reitzel RA, Martens RJ, Cohen ND. Evaluation of a multiplex polymerase chain reaction assay for simultaneous detection of *Rhodococcus equi* and the vapA gene. Am J Vet Res. 2005;66:1380-5.

[186] Mashoof S, Goodroe A, Du CC, Eubanks JO, Jacobs N, Steiner JM, et al. Ancient T-independence of mucosal IgX/A: gut microbiota unaffected by larval thymectomy in *Xenopus laevis*. Mucosal Immunol. 2012; 6:358-68

[187] Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12:R60.

[188] Garcia-Mazcorro JF, Dowd SE, Poulsen J, Steiner JM, Suchodolski JS. Abundance and short-term temporal variability of fecal microbiota in healthy dogs. Microbiologyopen. 2012;1:340-7.

[189] Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, et al. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. PLoS One. 2012;7:e51907.

[190] Muhling M, Woolven-Allen J, Murrell JC, Joint I. Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. ISME J. 2008;2:379-92.

[191] Lubbs DC, Vester BM, Fastinger ND, Swanson KS. Dietary protein concentration affects intestinal microbiota of adult cats: a study using DGGE and qPCR to evaluate differences in microbial populations in the feline gastrointestinal tract. J Anim Physiol Anim Nutr (Berl). 2009;93:113-21.

[192] Malinen E, Rinttila T, Kajander K, Matto J, Kassinen A, Krogius L, et al. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. Am J Gastroenterol. 2005;100:373-82.

[193] Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol. 2004;97:1166-77.

[194] Hochberg Y. A sharper Bonferroni procedure for multiple tests of significance. Biometrika. 1988;75:800-2.

[195] Shannon CE, Weaver W. The Mathematical Theory of Communication. Urbana, IL: University of Illinois Press; 1963.

[196] Chao A. Estimating the population size for capture-recapture data with unequal catchability. Biometrics. 1987;43:783-91.

[197] Garcia-Mazcorro JF, Lanerie DJ, Dowd SE, Paddock CG, Grutzner N, Steiner JM, et al. Effect of a multi-species synbiotic formulation on fecal bacterial microbiota of healthy cats and dogs as evaluated by pyrosequencing. FEMS Microbiol Ecol. 2011;78:542-54.

[198] Venturi A, Gionchetti P, Rizzello F, Johansson R, Zucconi E, Brigidi P, et al. Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. Aliment Pharmacol Ther. 1999;13:1103-8.

[199] Vitali B, Ndagijimana M, Cruciani F, Carnevali P, Candela M, Guerzoni ME, et al. Impact of a synbiotic food on the gut microbial ecology and metabolic profiles. BMC Microbiol. 2010;10:4.

[200] Shepherd ML, Swecker WS, Jr., Jensen RV, Ponder MA. Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. FEMS Microbiol Lett. 2012;326:62-8.

[201] Daly K, Stewart CS, Flint HJ, Shirazi-Beechey SP. Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes. FEMS Microbiol Ecol. 2001;38:141-51.

[202] Weese JS. The second genome of the horse: metagenomic investigation of the equine intestinal microbiome. In: McFarlene D, Toll J, editors. Proceedings of the Annual Forum of the American College of Veterinary Internal Medicine Denver; 2011 Jun 15-18; Denver, CO. Lakewood, CO: Curran Associates, Inc; 2011.

[203] Whitehead AE, Parreira VR, Hewson J, Watson JL, Prescott JF. Development of a live, attenuated, potential vaccine strain of *R. equi* expressing vapA and the virR operon, and virulence assessment in the mouse. Vet Immunol Immunopathol. 2012;145:479-84.

[204] Giguère S, Cohen ND, Chaffin MK, Slovis NM, Hondalus MK, Hines SA, et al. Diagnosis, treatment, control, and prevention of infections caused by *Rhodococcus equi* in foals. J Vet Int Med. 2011;25:1209-20.

APPENDIX A

Protocol for growing *Rhodococcus equi* strain 5-331 and vaccine preparation

1. Inoculate blood agar plates with 1 colony-forming unit (CFU) of *R. equi* strain 5-331 from an existing plate or from frozen beads kept at -80^{o}C.
2. Incubate at 37^{o}C for 48 hours.
3. Pick one colony from this pure culture and inoculate 1,000 ml of brain heart infusion (BHI, Bacto^{TM} Brain Heart Infusion, BD Diagnostic Systems, Sparks, MD) broth.
4. Put the flask with inoculated broth on an orbital shaker (VWR OS-500, VWR, Radnor, PA) at 200 rpm for 24h at 37^{o}C to allow bacterial growth.
5. Transfer bacteria to 200 ml tubes and centrifuge at 3840 × g for 20 min.
6. Discard supernatant, resuspend pellet in 100 ml of PBS.
7. Centrifuge at 3840 × g for 20 min.
8. Resuspend pellets in 20 ml of PBS.
9. Combine all pellets in one tube and measure OD using a spectrophotometer.
10. Dilute until either OD 1.0 or 2.0 is reached (depending on desired concentration).
11. Once desired OD is reached, make serial dilutions and plate out in BHI agar plates to confirm bacteria concentration.

APPENDIX B

Standard-operating procedure (SOP) for inactivation of biohazards using high energy electron-beam

1. Purpose:

    1.1 The purpose of this SOP is to outline the steps involved in the inactivation of biohazardous materials using high-energy electron beam.

2. Scope:

    2.1. This SOP applies to inactivation of biohazardous materials using high energy electron beam in the National Center for Electron Beam Research at Texas A&M University.

3. Responsibilities

    3.1. The E-Beam Center guarantees only the delivery of defined doses of E-Beam and X-ray. The E-Beam Center does not guarantee the inactivation of target organisms.

    3.2. It is the responsibility of the investigator to inform the E-Beam Center the biological agent in question and the irradiation doses that are needed.

    3.3. The E-Beam Center will ensure that appropriate doses of E-Beam and X-ray are delivered.

3.4. The Office of Biosafety will approve this SOP.

3.5. The Director of the National Center for Electron Beam Research will ensure that all personnel performing the procedures will be trained on the contents of this SOP.

3.6. The Director of the National Center for Electron Beam Research will ensure that all work with biohazardous materials has been reviewed and approved by the Office of Biosafety /IBC. This will be confirmed by the E-beam Center Director or his designate by calling the Office of Biosafety and confirming that each PI requesting E-beam services has IBC approval for this aspect of the research project and for use of the E-beam Center.

3.6.1. All Investigators will provide the E-beam Center Director a copy of their IBC approval letter to ensure that all necessary Office of Biosafety / IBC approvals have been obtained prior to working with biohazardous materials.

3.7. All investigators will ensure that materials to be transported to the facility for inactivation are packed in accordance with applicable Office of Biosafety / IBC, local, state, and federal regulations.

3.8. All personnel involved in the procedures are required to read and follow this SOP.

4. Reference Documents and Forms:

4.1. SOP# 001 Emergency Plan for Biohazard Leaking or Spill.

5. Training

    5.1. Personnel whose job involves inactivation of biohazards in the center must receive training on the contents of all Standard Operating Procedures.

6. Equipment and Materials

    6.1. Cardboard “carrier” trays

    6.2. 10 MeV E-Beam and 5 MeV X-ray linear accelerators (LINACS) and control systems.

    6.3. Material handling conveyor belt system.

7. General Instructions

    7.1. Biohazardous samples will be transported in Office of Biosafety / IBC approved durable, leak proof containers during handling, processing, storage, and transport to and within the facility. Depending on the experimental procedures, the infectious samples will be contained in heavy duty specimen transport bags. (An approved bag for this use: Infecon Heavy Duty Specimen Transport Bags, ML0419-20, 31, 32, 33, 34, and 35, Market Labs) These transport bags will be placed within DOT approved transport boxes and transported to the E-beam center.

    7.2. Potentially infectious samples must be contained within the sealed, leakproof specimen bag throughout the irradiation process.

7.3. All external surfaces of the sample containers and sample bags must be disinfected prior to the transportation to the E-Beam Center.

7.4. All containers containing biohazardous samples for processing must be delivered to the sample lab (room #102) located on the west end of the facility.

7.5. All containers containing biohazardous samples must be properly labeled with:

7.5.1. Name of PI, Phone number or contact information.

7.5.2. Biohazard Symbol.

7.5.3. Description of the biohazard/Contents of container (i.e. Agent name).

7.5.4. Process degree desired if known.

7.6. Upon arrival at the E-beam center, biohazardous samples contained in heavy duty, leakproof specimen bags will be removed from the DOT transport carrier and will be placed in a cardboard container, provided by the E-beam center, for processing and delivery of E-beam/X-ray dose and transport throughout the facility, preventing direct contact of the sample with the equipment.

8. Procedure:

8.1. Sample receiving process for containers of biohazardous samples to be irradiated:

8.1.1. E-beam personnel will visually inspect the specimen bag containers containing biohazardous samples for integrity and evidence of any damage or leakage.

8.1.2. If there is any evidence that the biohazardous material may be leaking from the specimen bag container, follow the SOP 001 Emergency Plan for Biohazard Leaking or Spill.

8.1.3. If no leakage is observed, log in sample in log book.

8.2. Run speed check samples to ensure delivery of target dose.

8.3. Perform dosimetry to verify delivered dose.

8.4. Adjust belt speed as needed to control dose.

8.5. Place samples, in cardboard carrier trays, on material handling conveyer belt system.

8.6. Deliver target E-Beam/X-ray doses to the research samples.

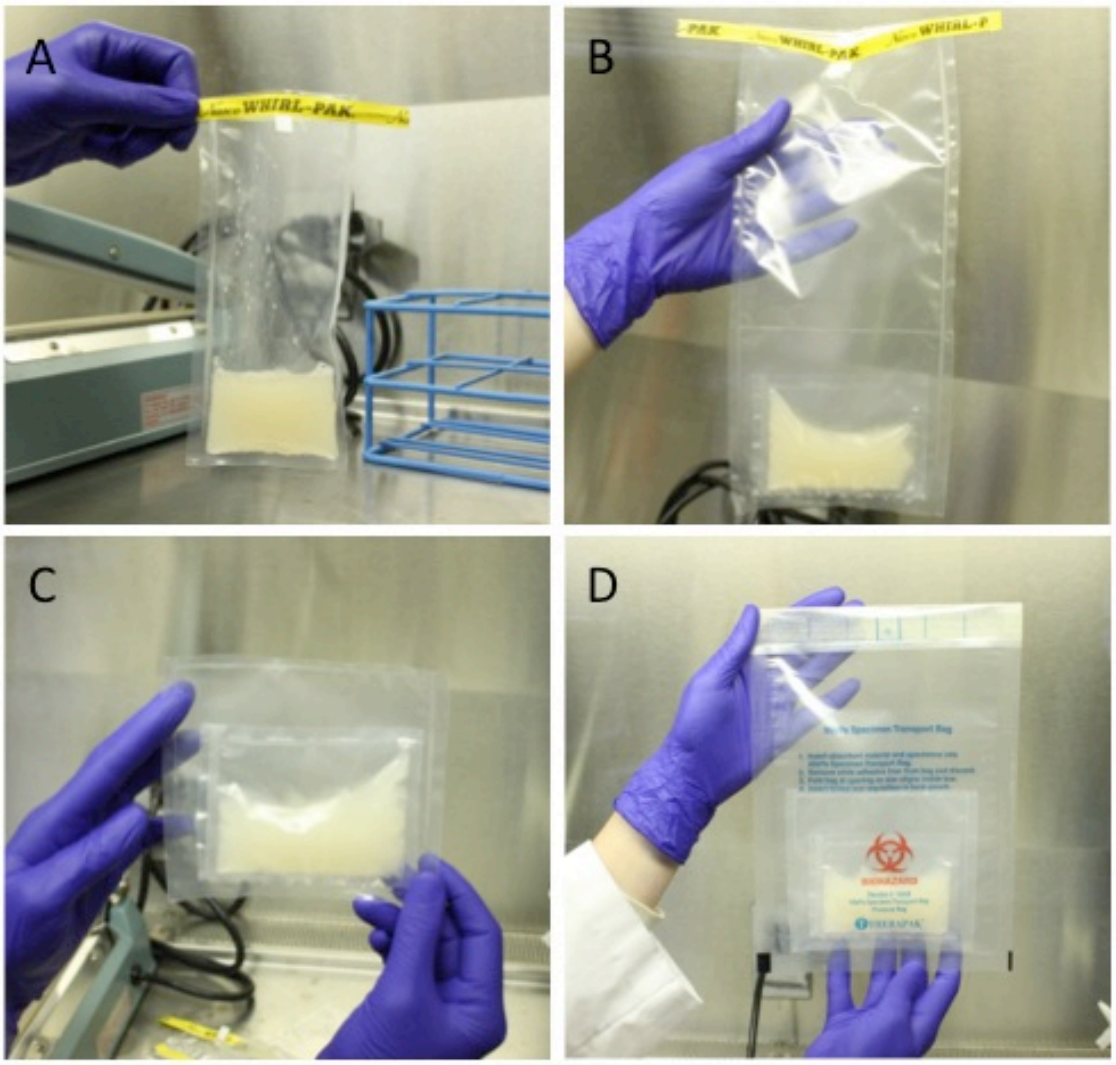
8.7. Remove samples from carrier trays and transfer the primary container back into the secondary (DOT) container for transport out of the facility.

8.8. Call responsible PI to pick up irradiated samples.

8.9. Irradiated samples are not to be stored within the facility. Irradiated samples are returned to the PI's lab and autoclaved for 1 hour at 121^{o}C prior to disposal.

APPENDIX C

Vaccine preparation for electron-beam irradiation



A) Vaccine in desired concentration was added to the first plastic container;

B) Second plastic container with the first container after being heat-sealed and trimmed;

C) Second container was heat-sealed and trimmed to fit the transport bag;

D) Biohazard transport bag containing double-bagged vaccine.

APPENDIX D

Fluorescence-based bacterial viability kit (LIVE/DEAD® BacLight. Bacterial Viability, kit L7012, protocol adapted from manufacturer's instructions, Life Technology, Molecular Probes, Eugene, OR, USA)

Contents

- SYTO 9 dye, 3.34 mM (Component A), 300 µL solution in DMSO
- Propidium iodide, 20 mM (Component B), 300 µL solution in DMSO

Storage and Handling:

- DMSO stock solutions should be stored frozen at ≤–20°C and protected from light.
- Allow reagents to warm to room temperature and centrifuge briefly before opening the vials. Before refreezing, seal all vials tightly. When stored properly, these stock solutions are stable for at least one year.

1. Grow 30 mL cultures of *Rhodococcus equi* to late log phase in nutrient broth.
2. Concentrate 25 mL of the bacterial culture by centrifugation at 10,000 × *g* for 10–15 minutes.
3. Remove the supernatant and resuspend the pellet in 2 mL of PBS.
4. Add 1 mL of this suspension to each of two 50 mL centrifuge tubes containing either 20 mL of PBS (intact bacteria tube) or 20 mL of 70% isopropyl alcohol (damaged bacteria tube).

5. Incubate both samples at RT for 1 hour, mixing every 15 minutes.
6. Pellet both samples by centrifugation at 10,000 × g for 10–15 minutes.
7. Resuspend the pellets in 20 mL of PBS and centrifuge again as in step 6.
8. Resuspend both pellets in separate tubes with 10 mL of PBS.
9. Determine the optical density at 670 nm (OD_{670}) of a 3 mL aliquot of the bacterial suspensions in acrylic absorption cuvettes (1 cm pathlength).
10. Dilute suspension in PBS to reach OD of 0.4.
11. Mix five different proportions of *R. equi* according to (table below) in 16 × 125 mm borosilicate glass culture tubes. The total volume of each of the five samples will be 2 ml.

Table D-1
Volume of cell suspension for each ratio undamaged:damaged cells

Ratio of undamaged:damaged cells	Volume (ml) intact-cell suspension	Volume (ml) damaged-cell suspension
0:100	0	2.0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

12. Mix 6 µL of Component A with 6 µL of Component B in a microfuge tube.
13. Prepare a 2X stain solution by adding the entire 12 µL of the above mixture to 2.0 mL of filter-sterilized dH_2O in a 16 × 125 mm borosilicate glass culture tube and mix well.

14. Pipet 100 µL of each of the bacterial cell suspension mixtures into separate wells of a 96-well flat-bottom microplate.
15. Using a new tip for each well, pipet 100 µL of the 2X staining solution (from step 7.4) to each well and mix thoroughly by pipetting up and down several times.
16. Incubate at room temperature in the dark for 15 minutes.
17. With the excitation wavelength centered at about 485 nm, measure the fluorescence intensity at a wavelength centered at about 530 nm (emission 1; green) for each well of the entire plate.
18. With the excitation wavelength still centered at about 485 nm, measure the fluorescence intensity at a wavelength centered about 630 nm (emission 2; red) for each well of the entire plate.
19. Analyze the data by dividing the fluorescence intensity of the stained bacterial suspensions (F cell) at emission 1 by the fluorescence intensity at emission 2
20. Record the data and save it for subsequent analysis.

APPENDIX E

Protocol for isolation and culture of peripheral blood mononuclear cells (PBMCs) using Carbonyl-iron and Ficoll-Paque (modified from the original protocol kindly provided by Dr. Julia Felippe, Cornell University, Ithaca, NY)

1. Collect blood in heparinized tubes.
2. Allow samples to stand at RT until plasma separates from red cells (no more than 30 min).
3. Under the hood, pipet plasma to sterile 50 ml conical tubes containing 600 mg sterile CN-Fe.
4. Incubate tubes in a rotator at 37^{o} C.
5. Remove plasma off the CN-Fe with a magnet (run magnet around the tube to attract iron a few times; then pour plasma into a fresh tube slowly, holding the magnet outside the lower wall of the tube, close to the opening).
6. Centrifuge plasma at 300 × g, 4^{o} C, 10 min.
7. Discard supernatant.
8. Resuspend pellet in 8 ml cold PBS.
9. Aliquot 4 ml of Ficoll 1077 in 12 ml tubes [use the proportion 2:1 (cell suspension: Ficoll)].
10. Layer 8ml of the cell suspension over Ficoll, carefully.
11. Centrifuge tubes at 700 × g, RT, 15 min.

12. Transfer the cloudy interface layer between Ficoll and suspension into a clean 15 conical tube.
13. Add cold PBS to the capacity of the tube.
14. Centrifuge tubes at 1200 rpm, 4^{o} C, 10 min.
15. Aspirate (discard) supernatant.
16. Wash the cell pellet 3 times with 12 ml cold PBS.
17. Resuspend the final cell pellet in 2 ml PBS – count cells.
18. Stimulate 1x10^{6} cells with antigen (vaccine, MOI 10) or mitogen (ConA final concentration 5mcg/ml, stock 0.5 mg/ml, use 10 µl/ml of culture) in culture medium (RPMI + 15% fetal bovine serum and 1.5% penicillin-streptomycin).
19. Incubate plate at 37^{o} C, 5% CO_2 for 48 h.
20. Harvest supernatant in 2 ml tubes.
21. Store in -80^{o}C until assayed.

APPENDIX F

Protocol for IFN-γ ELISA in supernatant of cultured PBMCs, modified from manufacturer's instructions (product code 3117-1H-6, Mabtech Inc., Cincinnati, OH, USA)

1. Coat a high protein binding ELISA plate with mAb bIFN-γ, diluted to 2 μg/ml in PBS, pH 7.4, by adding 100 μl/well. Incubate overnight at 4-8^{o}C.
2. Wash twice with PBS (200 μl/well).
3. Block plate by adding 200 μl/well of incubation buffer.
4. Wash five times with PBS containing 0.05% Tween.
5. Prepare equine IFN-g standard by reconstituting contents of vial 4 in 1 ml PBS with 1% BSA and leave at RT for 15 minutes, then vortex the tube and spin down. This gives a concentration of 0.2 μg/ml. Use immediately or store in aliquots at -20^{o}C for future use. We recommend the aliquots not to be refrozen after initial use. For the test, prepare dilutions of the stock using the standard range as a guideline.
6. Add 100 μl/well of samples or standards diluted in incubation buffer and incubate for 2 hours at RT.
7. Wash as in step 4.
8. Add 100 μl/well of mAb PAN-biotin at 0.1 μg/ml in incubation buffer. Incubate for 1 hour at RT.
9. Wash as in step 4.

10. Add 100 µl/well of Streptavidin-HRP diluted in 1:1000 in incubation buffer. Incubate for 1 hour at RT.
11. Wash as in step 4.
12. Add 100 µl/well of TMB substrate solution.
13. Add 100 µl/well of stop solution.
14. Measure the optical density in a microplate reader at 450 nm.

Reagents

Incubation buffer: PBS with 0.05% Tween 20 with 0.1% BSA

TMB substrate solution: ready to use substrate (Bethyl Laboratories, Montgomery, TX, USA)

Stop solution: 0.18M H_2SO_4

APPENDIX G

Protocol for total immunoglobulin ELISA determination (protocol from Bethyl Laboratories, Inc., Montgomery, TX)

1. Add 100 mcl of diluted coating antibody to each well.
1. Note: Run each standard or sample in duplicate.
2. Incubate at room temperature (20-25° C) for 1 hour.
3. Wash plate FIVE times.
4. Add 200 mcl of Blocking Solution to each well.
5. Incubate at room temperature for 30 minutes.
6. Wash plate FIVE times.
7. Add 100 mcl of standard or sample to well.
8. Incubate at room temperature for 1 hour.
9. Wash plate FIVE times.
10. Add 100 mcl of diluted HRP detection antibody to each well (See table below).
11. Incubate at room temperature for 1 hour.
12. Wash plate FIVE times.
13. Add 100 mcl of TMB Substrate Solution to each well.
14. Develop the plate in the dark at room temperature for 15 minutes.
15. Stop reaction by adding 100 mcl of Stop Solution to each well.
16. Measure absorbance on a plate reader at 450 nm.

Table G-1
Sources of polyclonal antibodies against horse immunoglobulins

Immunoglobulin isotype	HRP detection polyclonal antibody source
IgA	Goat
IgG_1	Goat
$IgG_{4/7}$	Sheep
$IgG_{3/5}$	Goat

Reagents

Blocking buffer: 1% BSA in 1x room temperature PBS

Washing buffer: 1x PBS with 0.5% Tween 20

Stop solution: 0.18 M H_2SO_4.

APPENDIX H

Protocol for *Rhodococcus equi*-specific immunoglobulin ELISA (modified from the original protocol kindly provided by Dr. Steeve Giguère from University of Georgia, Athens, GA)

1. To make coating buffer, empty contents of one capsule of carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, MO, USA) into 100 ml of deionized water. Mix until dissolved. Dilute 2.5 µg/ml of *R. equi* lysate in fresh coating buffer to desired concentration and put 100µl per well(s) in your ELISA plate(s). Put sealing cover over the wells and store overnight at 4°C.
2. Prepare blocking solution.
3. Take plate from 4°C and remove protein/coating buffer solution from the wells.
4. Wash plate 4 times with washing buffer.
5. Block the plate using 300µl/well of the blocking solution. Reseal plate and let sit at room temperature for 60 minutes.
6. Wash plate 4 times with washing buffer.
7. Apply 100µl/well of anti-equine Ig (see table below for specific information about the antibody). Allow plate to incubate at RT for 60 minutes.
8. Wash plate 4 times with washing buffer.
9. Apply 100µl/well of the substrate solution to your plate and cover the plate. Incubate the plate in the dark for 15 min.

10. Add 100µl/well of stop solution to the wells.

11. Measure absorbance on a microplate reader at 450 nm.

Reagents

Blocking buffer: 1% BSA in 1x room temperature PBS

Washing buffer: 1x PBS with 0.5% Tween 20

Stop solution: 0.18 M H_2SO_4.

APPENDIX I

Protocol for antigen production for *Rhodococcus equi*-specific ELISA (modified from the original protocol kindly provided by Dr. Steeve Giguère from University of Georgia, Athens, GA)

1. Inoculate BHI plates with *R. equi* 5-331.
2. Incubate at 37°C for 48 hours.
3. Select one colony from plates and inoculate 250mls BHI broth in a 500 ml Erlenmeyer flask.
4. Place in shaker with a speed of 160 RPMs at 37°C for 48 hours.
5. Aliquot broth into 50ml centrifuge tubes
6. Centrifuge at 3840 × g (500 RPMs) for 10 min.
7. Decant off supernatant, retaining the pellet.
8. Resuspend pellet with 100 ml PBS.
9. Centrifuge at 3840 × g for 10 min
10. Decant off supernatant and resuspend in remainder fluid.
11. Take 2mls and add to 10 ml of PBS.
12. Do three freeze/thaw cycles by freezing at -80°C and thawing in 37°C water bath.
13. Centrifuge at 12,000 × *g* (10,000 RPMs) for 15 min at 4°C.
14. SAVE SUPERNATANT
15. Centrifuge supernatant at 25,000 × g (14,500 RPMs) for 20 min at 4°C.

16. Place on ice and test for protein concentration with BCA protein assay kit (Pierce) using microplate technique and read in the microplate reader at 540nm. Working reagent 10mls of part A to 200µl of part B.

APPENDIX J

Primary data from IFN-γ production on cultured PBMCs (median and range) of vaccinated foals on both day2 2 and 32 of life.

Table J-1
IFN-γ (pg/ml) production median (range) on cultured PBMCs from vaccinated foals on days 2 and 32 of life.

Group	IFN-γ (pg/ml)	
	Day 2	Day 32
Saline Control	13.9 (0 to 2,211)	854.9 (200.5 to 56,893.2)
EBRE 1	0 (0 to 66.7)	16,399.8 (3,086.98 to 77,480.1)
EBRE 2	0 (0 to 47.6)	3,145.4 (81.1 to 9,221.4)
LVRE	0 (0 to 14.8)	6,065.4 (1,637.4 to 12,766.9)

APPENDIX K

Primary data from serum antibody levels of vaccinated foals on both day 2 and day 32 of life.

Table K-1
Distribution of total IgA (ng/ml) from serum by group and age: median (range)

Group	Total IgA	
	Day 2	Day 32
Saline Control	234,230 (79,731 – 762,820)	145,758 (24,852 – 1,176,302)
EBRE 1	735,818 (377,076 – 2,098,880)	86,292 (29,078 – 536,244)
EBRE 2	509,942 (101,067 – 2,033,148)	223,511 (24,693 – 498,557)
LVRE	582,256 (170,958 – 1,876,689)	302,229 (43,604 – 715,973)

Table K-2
Distribution of Total IgG_1 (ng/ml) from serum by group and age: median (range)

Group	Total IgG_1	
	Day 2	Day 32
Saline Control	4,692,804 (2,448,892 – 6,026,232)	3,321,586 (1,985,946 – 13,752,336)
EBRE 1	3,569,435 (1,534,894 – 6,460,604)	3,269,665 (1,757,995 – 8,768,651)
EBRE 2	4,917,821 (2,271,937 – 8,404,795)	5,171,167 (2,787,443 – 8,941,273)
LVRE	10,279,961 (3,363,242 – 13,752,336)	8,703,443 (6,155,403 –11,921,392)

Table K-3
Distribution of Total $IgG_{4/7}$ (ng/ml) from serum by group and age: median (range)

Group	Total $IgG_{4/7}$	
	Day 2	Day 32
Saline Control	7,952,728 (852,402– 10,292,461)	3,659,334 (529,879 – 6,461,719)
EBRE 1	4,608,000 (1,256,550 – 14,398,587)	2,609,406 (884,535 – 6,934,430)
EBRE 2	8,408,957 (4,355,850 – 15,101,657)	3,285,647 (2,596,798 – 5,656,674)
LVRE	10,950,509 (7,461,929 – 15,222,571)	5,724,701 (2,734,293– 8,027,475)

Table K-4
Distribution of Total IgG $G_{3/5}$ (ng/ml) from serum by group and age: median (range)

Group	Total $IgG_{3/5}$	
	Day 2	Day 32
Saline Control	3,062,260 (1,007,223– 4,927,078)	1,819,580 (623,153– 9,058,173)
EBRE 1	1,839,858 (1,282,800 – 5,549,351)	1,409,532 (1,048,560 – 2,632,221)
EBRE 2	2,616,035 (775,120 – 6,566,822)	1,435,738 (556,940 –3,189,244)
LVRE	6,453,936 (1,938,040 – 13,001,753)	3,305,300 (1,684,840 – 5,948,785)

Table K-5
Distribution of *R. equi*-specific IgA from serum by group and age: median (range) ratio OD sample (- negative control)/OD positive (- negative control)

Group	*R. equi*-specific IgA	
	Day 2	Day 32
Saline Control	0.9 (0.4 – 2.1)	0.1 (0.1 – 0.6)
EBRE 1	1.1 (0.4 – 2.1)	0.2 (0.1 – 1.0)
EBRE 2	0.8 (0.5 – 1.4)	0.1 (0.1 – 1.0)
LVRE	0.8 (0.5 – 1.4)	0.2 (0.1 – 0.9)

Table K-6
Distribution of *R. equi*-specific IgG_1 from serum by group and age: median (range) ratio OD sample (- negative control)/OD positive (- negative control)

Group	*R. equi*-specific IgG_1	
	Day 2	Day 32
Saline Control	0.8 (0.2 – 0.9)	0.5 (0.3 – 2.3)
EBRE 1	0.7 (0.5 – 1.1)	0.5 (0.3 – 0.9)
EBRE 2	0.6 (0.5 – 1.7)	0.6 (0.4 – 0.9)
LVRE	0.7 (0.2 – 1.3)	1.3 (0.8 – 1.5)

Table K-7
Distribution of *R. equi*-specific $IgG_{4/7}$ from serum by group and age: median (range) ratio OD sample (- negative control)/OD positive (- negative control)

Group	*R. equi*-specific $IgG_{4/7}$	
	Day 2	Day 32
Saline Control	0.5 (0.1 – 0.9)	0.3 (0.1 – 1.1)
EBRE 1	0.5 (0.2 – 0.6)	0.2 (0.1 – 0.5)
EBRE 2	0.5 (0.2 – 0.8)	0.2 (0.1 – 0.5)
LVRE	0.3 (0.2 – 0.8)	0.4 (0.2 – 0.7)

Table K-8
Distribution of *R. equi*-specific $IgG_{3/5}$ from serum by group and age: median (range) ratio OD sample (- negative control)/OD positive (- negative control)

Group	*R. equi*-specific $IgG_{3/5}$	
	Day 2	Day 32
Saline Control	0.3 (0.1 – 1.3)	0.2 (0.1 – 2.0)
EBRE 1	0.7 (0.2 – 0.6)	0.4 (0.1 – 1.2)
EBRE 2	0.6 (0.2 – 1.7)	0.3 (0.2 – 0.8)
LVRE	0.4 (0.2 – 0.9)	0.5 (0.3 – 0.8)

APPENDIX L

Results from naso-pharyngeal antibody levels of vaccinated foals on both day 2 and day 32 of life.

Table L-1
Distribution of Total IgA (ng/ml) from NP eluates by Group and age: median (range)

Group	Total IgA	
	Day 2	Day 32
Saline Control	7,294 (1,887 – 19,052)	30,377 (7,444 – 377,659)
EBRE 1	6,325 (3,550 – 17,107)	17,798 (11,973 – 80,645)
EBRE 2	3,187 (890 – 14,876)	43,153 (10,065 – 103,359)
LVRE	4,091 (835 – 19,112)	21,479 (6,330 – 76,775)

Table L-2
Distribution of *R. equi*-specific IgA from Nasal Swabs by Group and age: median (range) ratio OD sample (- negative control)/OD positive (- negative control)

Group	*R. equi*-specific IgA	
	Day 2	Day 32
Saline Control	39.7 (0.3 – 81.9)	44.3 (3.7 – 741.2)
EBRE 1	10.5 (1.5 – 172.4)	14.6 (1.3 – 524.9)
EBRE 2	16.0 (6.0 – 163.5)	98.6 (16.5 – 908.4)
LVRE	16.6 (3.4 – 90.6)	77.1 (11.1 – 676.8)

APPENDIX M

Results from BAL fluid antibody levels of vaccinated foals on both day 2 and day 32 of life.

Table M-1
Distribution of Total IgA (ng/ml) from BAL fluid by group and age: median (range) adjusted to urea values

Group	Total IgA (ng/ml)	
	Day 2	Day 32
Saline Control	106.5 (22.1 – 262.7)	2,342.5 (928.4 – 17,527.8)
EBRE 1	61.5 (36.7 – 355.5)	1,374.0 (353.2 – 3,787.7)
EBRE 2	113.4 (17.3 – 1,040.6)	3,142.9 (26.8 – 10,883.6)
LVRE	190.6 (48.8 – 798.4)	1,014.4 (79.2 – 1,778.3)

Table M-2
Distribution of Total IgG_1 (ng/ml) from BAL fluid by group and age: median (range) adjusted to urea values

Group	Total IgG_1 (ng/ml)	
	Day 2	Day 32
Saline Control	774.4 (24.1 – 10,086.3)	2,688.0 (990.7 – 19,249.6)
EBRE 1	2,243.6 (635.6 – 12,436.5)	2,099.5 (130.0 – 8055.2)
EBRE 2	3,442.4 (1.0 – 7,324.0)	5,394.9 (1,602.6 – 26,860.0)
LVRE	4,765.0 (1.0 – 26,860.0)	16,325.7 (2,896.4 – 26,860.0)

Table M-3
Distribution of Total $IgG_{4/7}$ (ng/ml) from BAL fluid by group and age: median (range) adjusted to urea values

Group	Total $IgG_{4/7}$ (ng/ml)	
	Day 2	Day 32
Saline Control	792.8 (2.6 – 6,412.6)	4,789.0 (1,738.2 – 16,608.0)
EBRE 1	1,823.9 (820.6 – 4,108.7)	3,223.5 (662.9 – 15,992.5)
EBRE 2	831.3 (4.9 – 4,089.3)	5,766.9 (248.8 – 14,445.7)
LVRE	1,719.5 (7.3 – 6,725.9)	1,197.9 (280.0 – 9,395.4)

Table M-4
Distribution of Total IgG $G_{3/5}$ (ng/ml) from BAL fluid by group and age: median (range) adjusted to urea values

Group	Total $IgG_{3/5}$ (ng/ml)	
	Day 2	Day 32
Saline Control	388.6 (2.6 to 2,082.7)	1,724.8 (598.3 – 11,169.9)
EBRE 1	510.3 (226.1 – 1,884.1)	1,877.8 (185.0 – 3,309.2)
EBRE 2	207.0 (4.9 – 1,015.4)	1,926.4 (50.2 – 5,118.1)
LVRE	892.4 (7.3 – 2,301.3)	1,297.4 (500.9 – 2,671.2)

Table M-5
Distribution of *R. equi*-specific IgA from BAL fluid by group and age: median (range) ratio of OD sample (- negative control)/OD positive (- negative control)

Group	*R. equi*-specific IgA	
	Day 2	Day 32
Saline Control	<0.01 (<0.01 – 0.08)	0.07 (0.02 – 0.23)
EBRE 1	<0.01 (<0.01 – 0.09)	0.04 (<0.01 – 0.15)
EBRE 2	<0.01 (<0.01 – 0.02	0.04 (<0.01 – 0.12)
LVRE	<0.01 (<0.01 – 0.01)	0.02 (<0.01 – 0.08)

Table M-6
Distribution of *R. equi*-specific IgG_1 from BAL fluid by group and age: median (range) ratio of OD sample (- negative control)/OD positive (- negative control)

Group	*R. equi*-specific IgG_1	
	Day 2	Day 32
Saline Control	0.02 (0.00 – 0.21)	0.06 (0.01 – 0.31)
EBRE 1	0.08 (0.00 – 0.24)	0.01 (0.00 – 0.43)
EBRE 2	0.02 (0.00 – 0.51)	0.04 (<0.01 – 0.15)
LVRE	0.04 (0.00 – 0.26)	0.12 (<0.01 – 0.24)

Table M-7
Distribution of *R. equi*-specific $IgG_{4/7}$ from BAL fluid by group and age: median (range) ratio of OD sample (- negative control)/OD positive (- negative control)

Group	*R. equi*-specific $IgG_{4/7}$	
	Day 2	Day 32
Saline Control	0.05 (0.01 – 0.28)	0.21 (0.04 – 0.46)
EBRE 1	0.10 (0.03 – 0.56)	0.19 (0.01 – 0.79)
EBRE 2	0.05 (0.00 – 1.36)	0.09 (0.02 – 1.37)
LVRE	0.06 (0.00 – 0.44)	0.07 (<0.01 – 0.34)

Table M-8
Distribution of *R. equi*-specific $IgG_{3/5}$ from BAL fluid by group and age: median (range) ratio of OD sample (- negative control)/OD positive (- negative control)

Group	*R. equi*-specific $IgG_{3/5}$	
	Day 2	Day 32
Saline Control	0.85 (0.01 – 3.81)	2.63 (0.16 – 5.07)
EBRE 1	3.36 (0.74 – 4.98)	3.38 (0.22 – 8.59)
EBRE 2	0.98 (0.02 – 4.60)	3.04 (0.56 – 5.04)
LVRE	0.16 (0.01 – 2.15)	0.12 (0.10 – 2.64)

APPENDIX N

I. Summary of Data Analysis of Mare Serum and Milk Values

A. Data: Data are Mares12

B. Methods: Association between immunoglobulin concentrations in serum and milk for the 34 mares was made using linear regression analysis. When appropriate, data were logarithmically (base 10) transformed to ensure assumptions of linear modeling were not violated. Comparisons of immunoglobulin concentrations among treatment groups were performed using Kruskal-Wallis testing.

C. Results:

1. General summary: All mares had detectable Ig of all classes in both milk and serum, and all mares had detectable Ig specific against *R. equi* in milk and serum. There was significant correlation between milk and serum for total and *R. equi*-specific IgA, IgG_1, $IgG_{4/7}$, and $IgG_{3/5}$ except for total concentrations of IgG_1 and $IgG_{4/7}$; correlations were generally moderate in magnitude (Table N-1). There were no significant differences among treatment groups in milk concentrations of IgA, IgG_1, $IgG_{4/7}$, or $IgG_{3/5}$ against *R. equi*.

For all isotypes for both total and *R. equi*-specific antibodies, concentrations were higher in serum than in milk (Table N-2). Total isotype distribution was relatively similar in milk, although $IgG_{4/7}$ and $IgG_{3/5}$ tended to be higher in serum. *R. equi*-specific

concentrations of IgA were relatively higher and concentrations of $IgG_{4/7}$ were relatively lower in both milk and serum (Table N-2).

Table N-1
R-squared values and P values for correlations between milk and serum for total and *R. equi*-specific immunoglobulins among 34 mares

Isotype	R-squared	P value
IgA		
Total	0.31	0.0010
R. equi-specific	0.65	< 0.0001
IgG_1		
Total	0.09	0.0798
R. equi-specific	0.37	0.0001
$IgG_{4/7}$		
Total	0.05	0.2019
R. equi-specific	0.44	< 0.0001
$IgG_{3/5}$		
Total	0.26	0.0021
R. equi-specific	0.56	< 0.0001

Table N-2
Median (range) of concentrations of immunoglobulins in milk and serum

a. Total (x 10^3; ng/ml)

Source	IgA	*IgG_1*	*$IgG_{4/7}$*	*$IgG_{3/5}$*
Milk	511 (193-1,049)	646 (312-2,098)	724 (341-2,098)	590 (191-1,290)
Serum	1,390 (587-6,711)	1,970 (784-4,274)	7,640 (2,592-5,169)	7,843 (3,724-16,507)

b. *R. equi*-specific ratio of OD sample (- negative control)/OD positive (- negative control)

Source	IgA	*IgG_1*	*$IgG_{4/7}$*	*$IgG_{3/5}$*
Milk	2,382 (408-10,499)	107 (17-1,234)	10 (1-34)	80 (15-20,990)
Serum	15,424 (4,634-67,197)	536 (175-5,742)	73 (16-963)	2,417 (70 to 182,902)

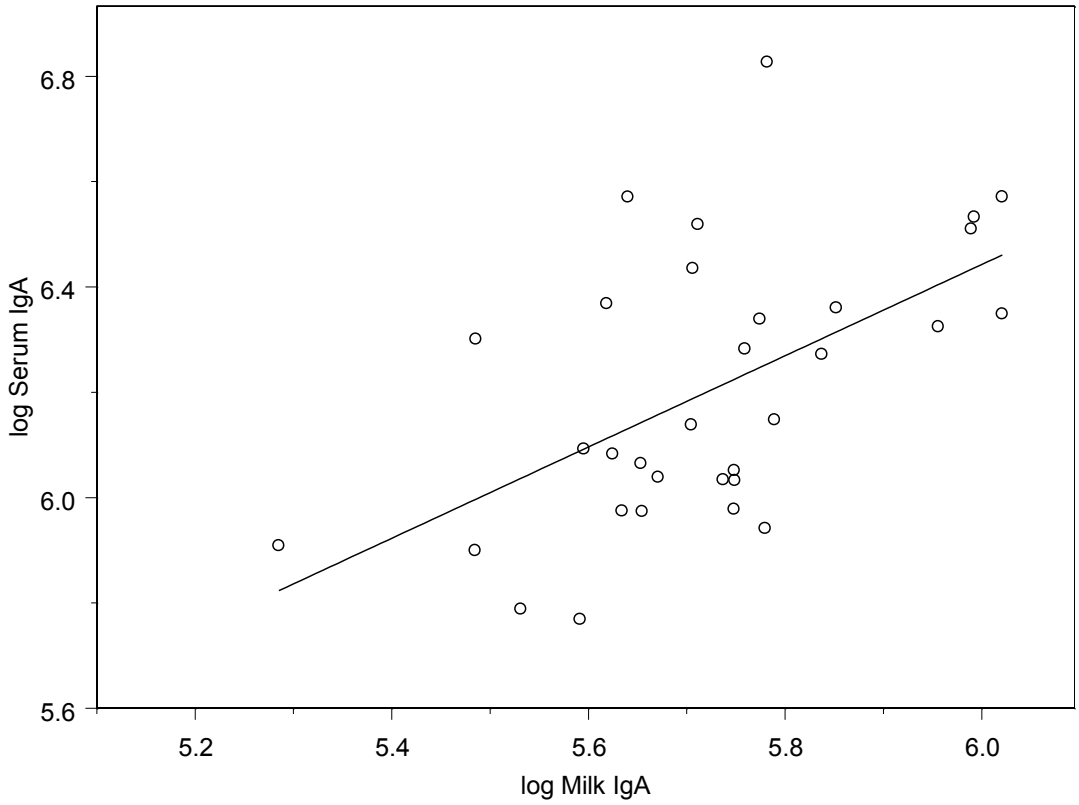


Fig. N-1. Total IgA

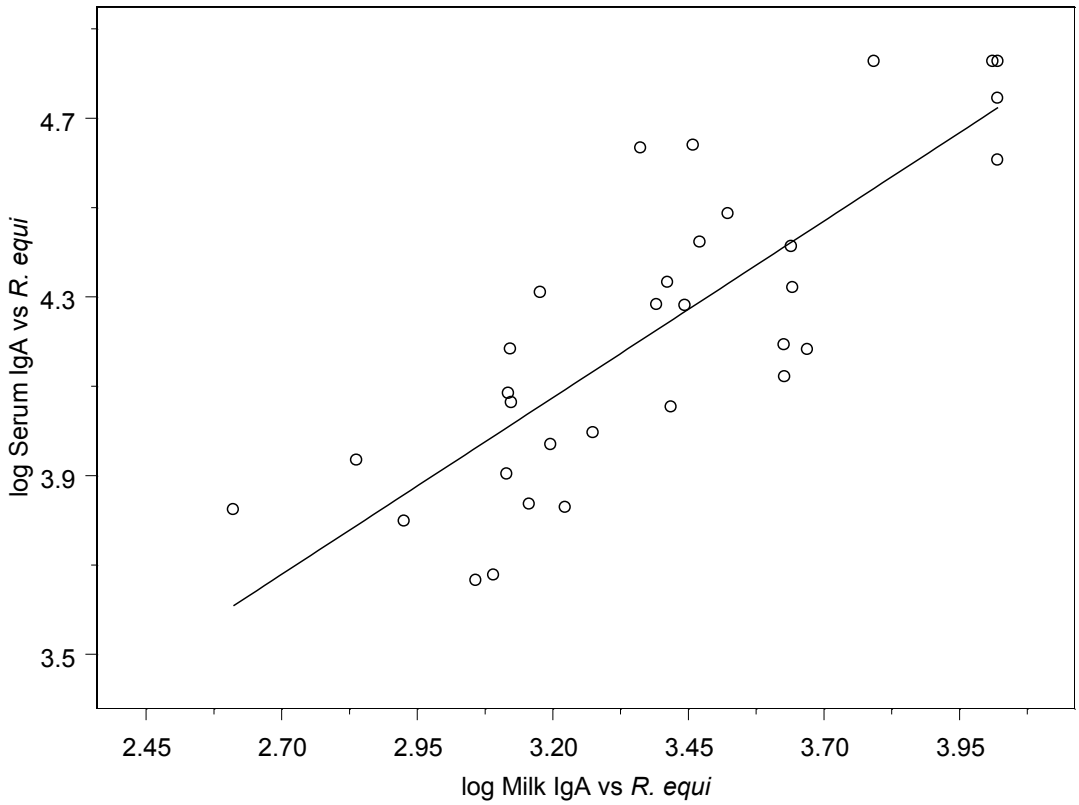


Fig. N-2.Specific IgA

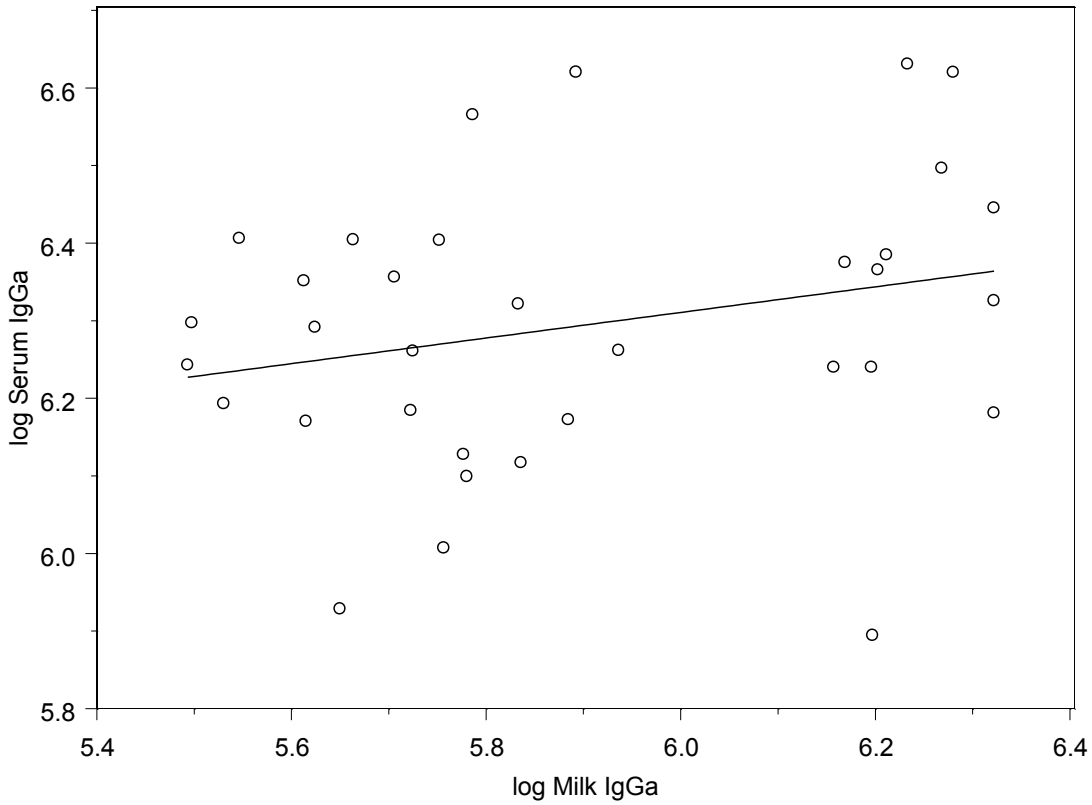


Fig. N-3.Total IgG_1 (not significant)

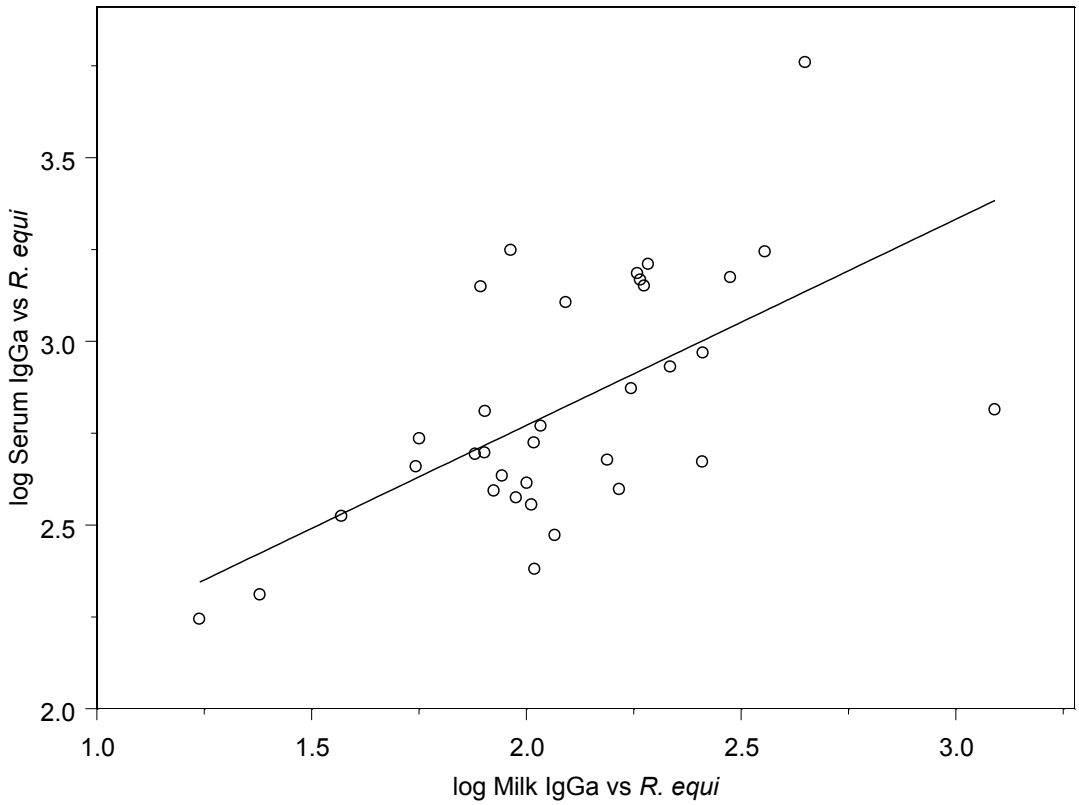


Fig. N-4.Specific IgG_1

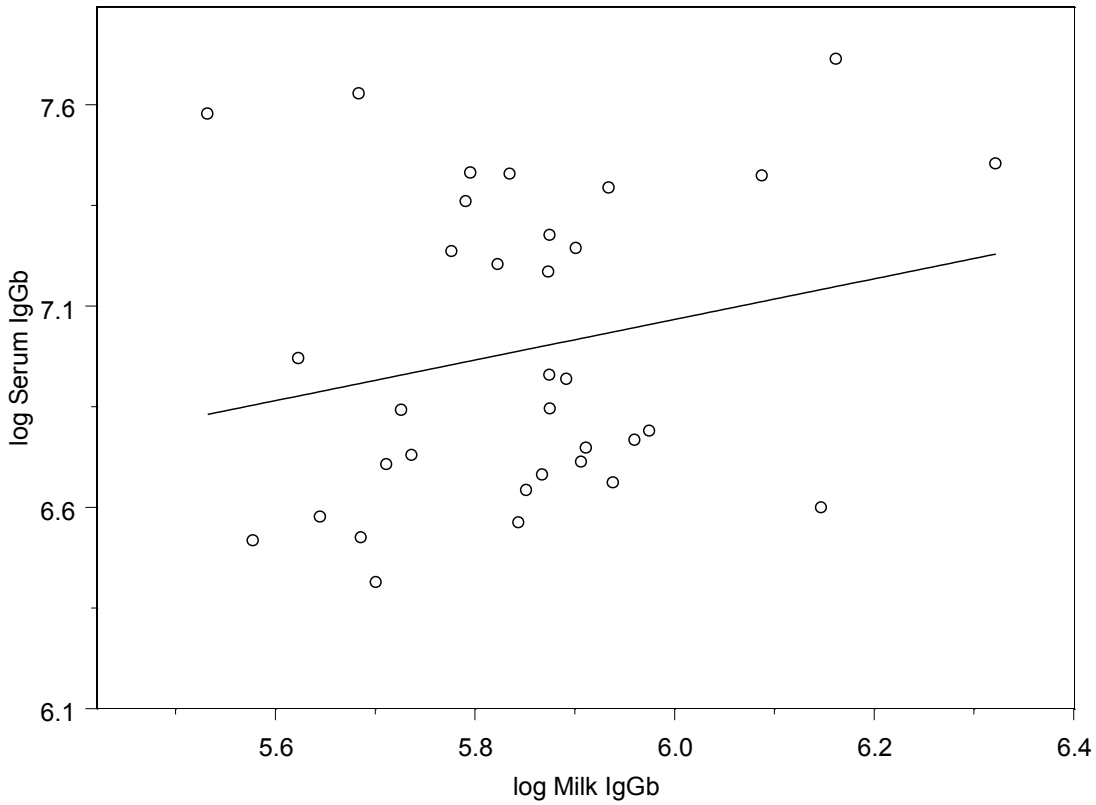


Fig. N-5.Total $IgG_{4/7}$ (not significant)

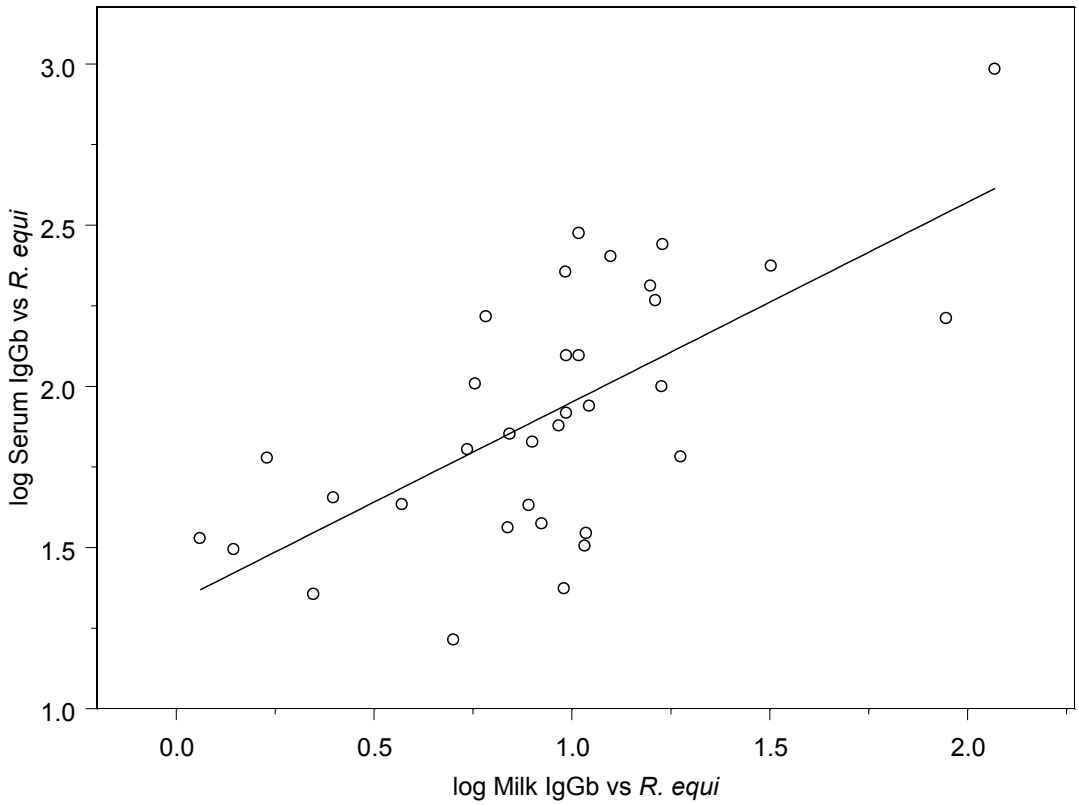


Fig. N-6.Specific $IgG_{4/7}$

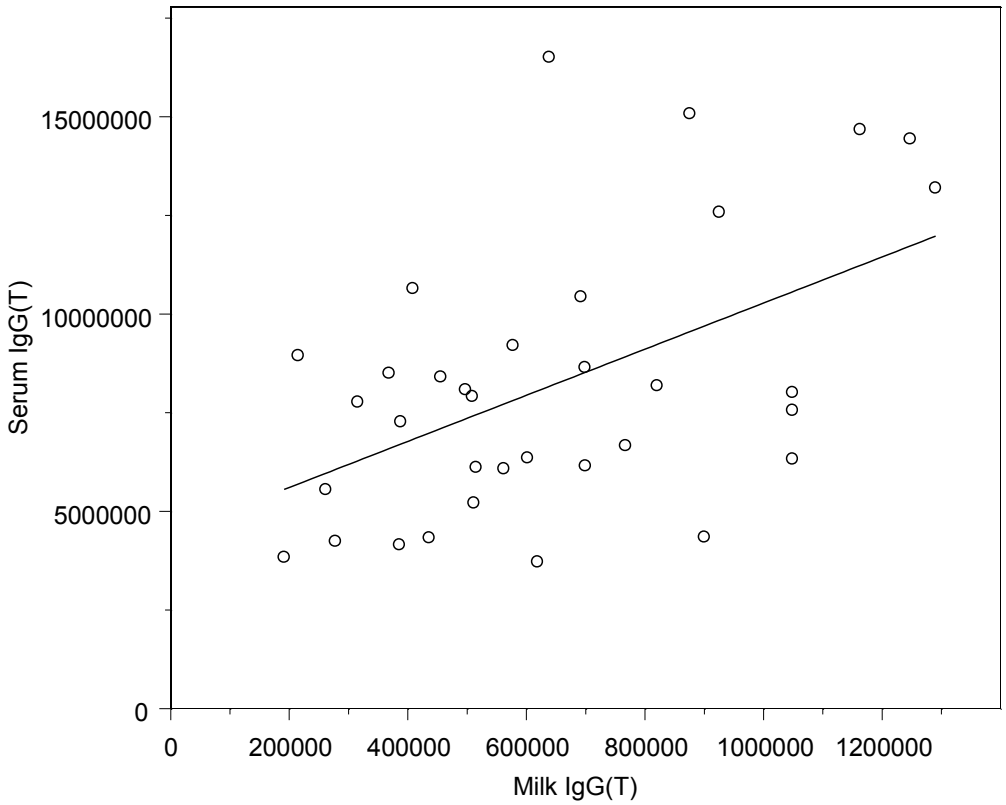


Fig. N-7.Total $IgG_{3/5}$

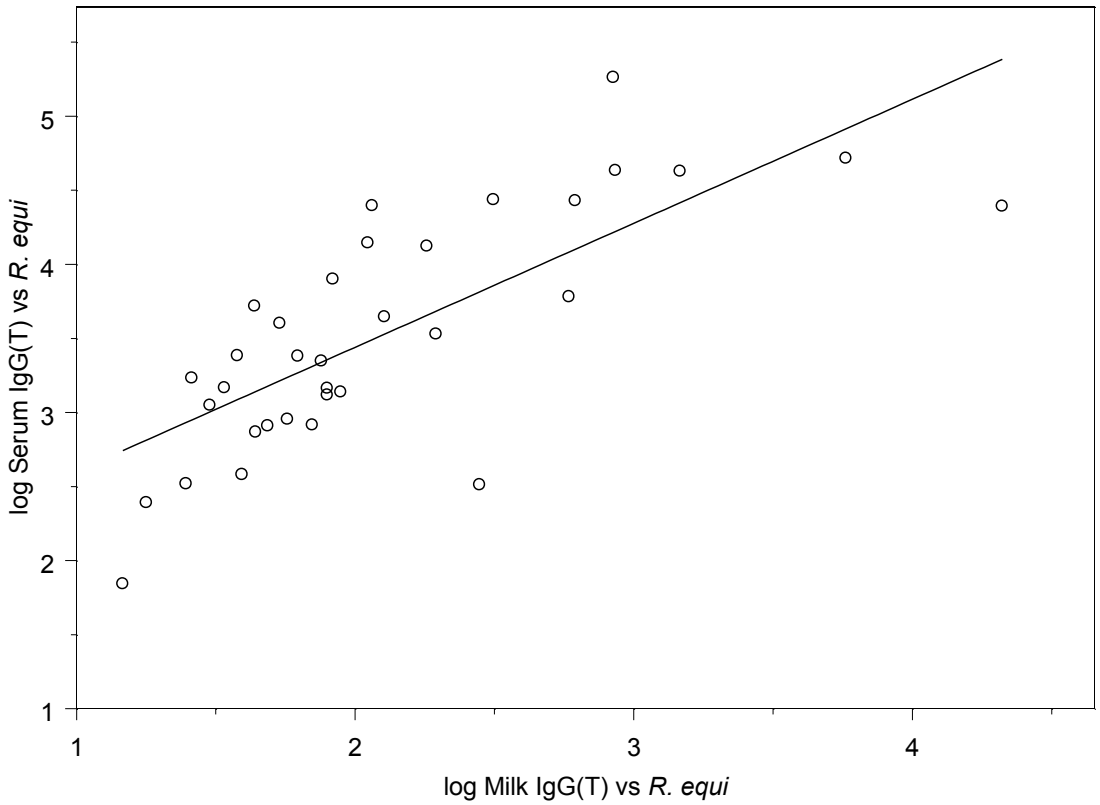


Fig. N-8.Specific $IgG_{3/5}$

APPENDIX O

I. Association of mare milk concentrations with foal serum concentrations

A. Data: Data are MFS13

B. Methods: Association between immunoglobulin concentrations in mares milk and foal serum were performed for the 34 mare-foal pairs. When appropriate, data were logarithmically (base 10) transformed to ensure assumptions of linear modeling were not violated. Comparisons of milk immunoglobulin concentrations among treatment groups were performed using Kruskal-Wallis testing.

C. Results:

General summary: In general, total immunoglobulin was either significantly ($P < 0.05$ for IgA) or tended ($0.05 < P < 0.11$ for $IgG_{4/7}$ and $IgG_{3/5}$) weak positive association between milk and foal serum on day 2, except for IgG_1. For day 32 and the ratio, there was no significant positive association between mare's milk and foal's serum values. For *R. equi*-specific concentrations, all isotypes except IgG_1 ($P = 0.1345$) were significantly ($P < 0.05$) positively correlated with mare milk concentration on Day 2. For $IgG_{4/7}$ and $IgG_{3/5}$, this association was also significant on day 32. There was no significant difference among treatment groups in milk concentrations of *R. equi*-specific antibodies for any isotypes, but there were a couple of the totals that did differ significantly BEFORE adjusting for multiple comparisons.

1. IgA:

a. Total IgA: There was no significant association of IgA on days 2, 31, or the ratio of Day 32:Day 2 (Table O-1; only log-transformed data shown because these best fit the data). There was no difference among treatment groups in mare's milk concentration of total IgA (P = 0.5019; data not shown).

b. *R. equi*-specific IgA: There was modest but significant association of concentration of *R. equi*-specific IgA in milk with serum on day 2, but not on day 32 or for the ratio of Day 32:Day 2 (Table O-2). There was no difference among treatment groups in mare's milk concentration of total IgA (P = 0.5269; data not shown).

2. IgG_1:

a. Total IgG_1: There was no significant association of IgG_1 on Day 2, or for the ratio of Day 32:Day 2 (Table O-3; only log-transformed data shown because these best fit the data). There was a significant NEGATIVE association of mare's milk IgG_1 and foal serum IgG_1 on day 32. There was no difference among treatment groups in mare's milk concentration of total IgG_1 (P = 0.0443; data not shown). When adjusting for multiple comparisons it is not significant.

b. *R. equi*-specific IgG_1: There was no significant association of concentration of *R. equi*-specific IgG_1 in milk with serum on Day 2 or Day 32, and there was a significant correlation between the mare's milk concentration and the ratio of Day 32:Day 2 (Table O-4). There was no difference among treatment groups in mare's milk concentration of total IgA (P = 0.6256; data not shown). When adjusting for multiple comparisons it is not significant.

3. $IgG_{4/7}$:

a. Total $IgG_{4/7}$: There was no significant association of $IgG_{4/7}$ on Day 2, Day 32, or for the ratio of Day 32:Day 2 (Table O-5; only log-transformed data shown). There was no difference among treatment groups in mare's milk concentration of total $IgG_{4/7}$ (P = 0.3157; data not shown).

b. *R. equi*-specific $IgG_{4/7}$: There was significant association of concentration of *R. equi*-specific $IgG_{4/7}$ in milk with serum on Day 2 and Day 32, but there was not a significant correlation between the mare's milk concentration and the ratio of Day 32:Day 2 (Table O-6). There was no difference among treatment groups in mare's milk concentration of total $Ig_{4/7}$ (P = 0.2612; data not shown.

4. $IgG_{3/5}$:

a. Total $IgG_{3/5}$: There was no significant association of $IgG_{3/5}$ on Day 2, Day 32, or for the ratio of Day 32:Day 2 (Table O-5; only log-transformed data shown). There was a significant difference among treatment groups in mare's milk concentration of total $IgG_{3/5}$ (P = 0.0246; data not shown). When adjusting for multiple comparisons it is not significant

b. *R. equi*-specific $IgG_{3/5}$: There was significant association of concentration of *R. equi*-specific $IgG_{3/5}$ in milk with serum on Day 2 and Day 32, but there was not a significant correlation between the mare's milk concentration and the ratio of Day 32:Day 2 (Table O-6). There was no difference among treatment groups in mare's milk concentration of total IgA (P = 0.2612; data not shown).

Table O-1
Association of total IgA in mare's milk with foal serum IgA on day 2, day 32, and the ratio of day 32:2 (log_{10}-transformed data)

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.15	0.0457	0.3063 (-0.1774 to 0.7900)
Day 32	0.04	0.2698	0.2833 (-0.2131 to 0.7798)
Ratio Day 32: Day 2	<0.01	0.9407	0.0230 (-0.5793 to 0.6253)

Table O-2
Association of *R. equi*-specific IgA in mare's milk with foal serum IgA samples on day 2, day 32, and the ratio of day 32:2 (log_{10}-transformed data)

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.41	<0.0001	0.8567 (0.5029 to 1.2105)
Day 32	0.04	0.2388	0.5098 (-0.3220 to 1.3416)
Ratio Day 32: Day 2	0.03	0.3286	-0.3584 (-1.0662 to 0.3494)

Table O-3
Association of total IgG_1 in mare's milk with foal serum IgG_1 on day 2, day 32, and the ratio of day 32:2 (log_{10}-transformed data)

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.03	0.3423	-0.1685 (-0.5111 to 0.1741)
Day 32	0.18	0.0120	-0.3927 (-0.6816 to -0.1038)
Ratio Day 32: Day 2	0.06	0.1808	-0.2242 (-0.5454 to 0.0970)

Table O-4
Association of *R. equi*-specific IgG_1 in mare's milk with foal serum IgG_1 samples on day 2, day 32, and the ratio of day 32:2 (log_{10}-transformed data)

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.07	0.1345	0.2700 (-0.0746 to 0.6146)
Day 32	0.06	0.1495	-0.4859 (-1.1307 to 0.1589)
Ratio Day 32: Day 2	0.15	0.0221	-0.7558 (-1.3716 to -0.1400)

Table O-5
Association of total $IgG_{4/7}$ in mare's milk with foal serum $IgG_{4/7}$ on day 2, day 32, and the ratio of day 32:2 (log_{10}-transformed data)

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.08	0.1050	0.4153 (-0.0727 to 0.9033)
Day 32	0.02	0.3664	0.2526 (-0.2878 to 0.7930)
Ratio Day 32: Day 2	0.04	0.2860	-0.1627 (-0.4567 to 0.1313)

Table O-6
Association of *R. equi*-specific $IgG_{4/7}$ in mare's milk with foal serum $IgG_{4/7}$ samples on day 2, day 32, and the ratio of day 32:2 (log_{10}-transformed data)

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.52	<0.0001	0.7416 (0.4938 to 0.9893)
Day 32	0.29	0.0010	0.6717 (0.3081 to 1.0353)
Ratio Day 32: Day 2	<0.01	0.6248	-0.0699 (-0.3474 to 0.2076)

Table O-7
Association of total $IgG_{3/5}$ in mare's milk with foal serum $IgG_{3/5}$ on day 2, day 32, and the ratio of day 32:2 (log_{10}-transformed data)

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.08	0.0837	0.3558 (-0.0344 to 0.7460)
Day 32	0.03	0.3085	0.2062 (-0.1844 to 0.5968)
Ratio Day 32: Day 2	0.06	0.1591	-0.1513 (-0.0543 to 0.3569)

Table O-8
Association of *R. equi*-specific $IgG_{3/5}$ in mare's milk with foal serum $IgG_{3/5}$ samples on day 2, day 32, and the ratio of day 32:2 (log_{10}-transformed data)

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.44	<0.0001	0.6407 (0.3879 to 0.8935)
Day 32	0.19	0.0097	0.5681 (0.1634 to 0.9728)
Ratio Day 32: Day 2	<0.01	0.6967	-0.0752 (-0.4501 to 0.2997)

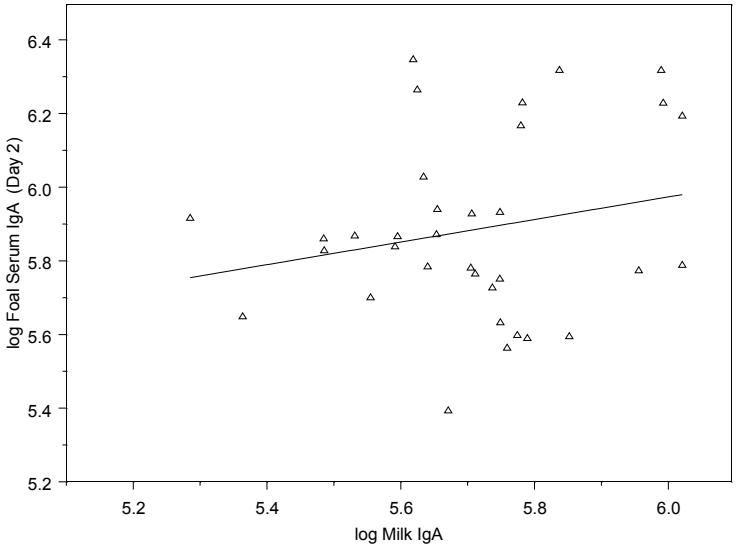


Fig. O-1. IgA (total) on Day 2 in foal serum versus mare's milk. Weak and non-significant association.

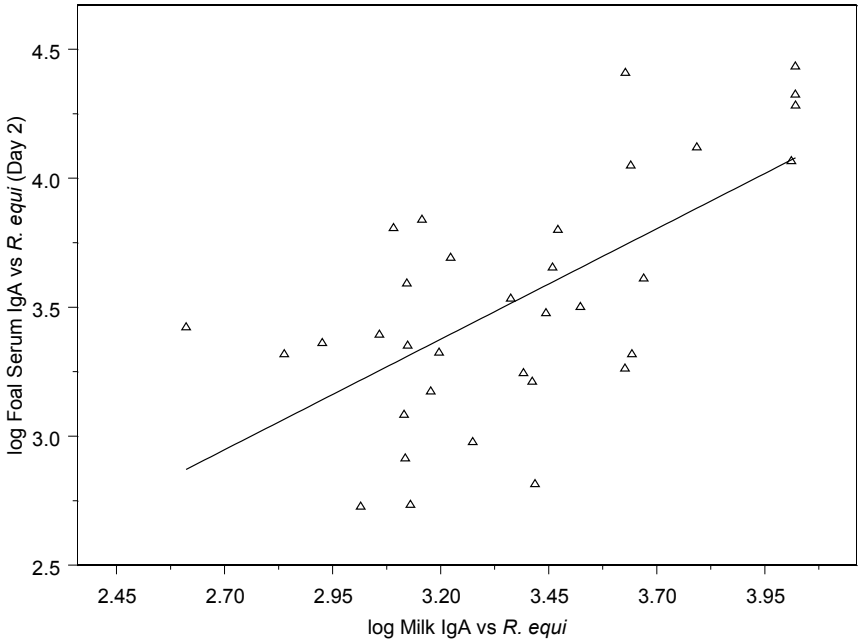


Fig. O-*2.* IgA specific for *R. equi* in foal serum versus mare's milk on Day 2. The association was weak but statistically significant.

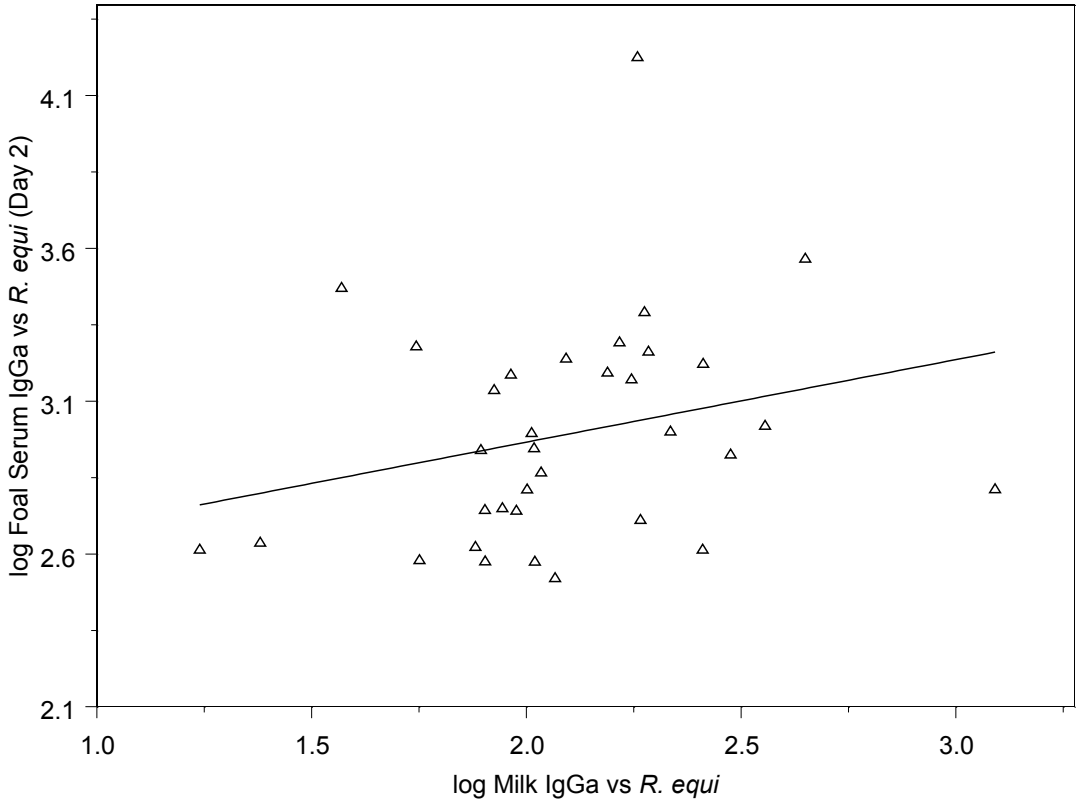


Fig. O-3. IgG $_1$ specific for *R. equi* in foal serum versus mare's milk on Day 2. The association was weak and not statistically significant (P = 0.1345)

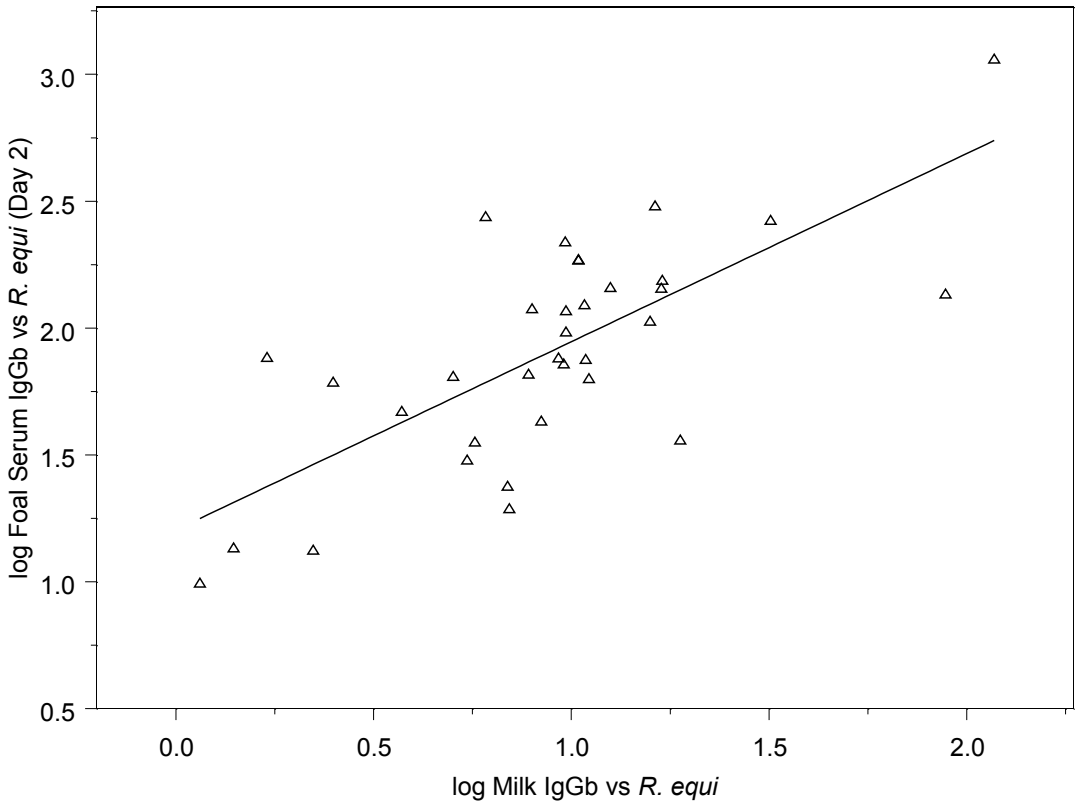


Fig. O-4. $IgG_{4/7}$ specific for *R. equi* in foal serum versus mare's milk on Day 2. The association was weak and not statistically significant ($P < 0.0001$)

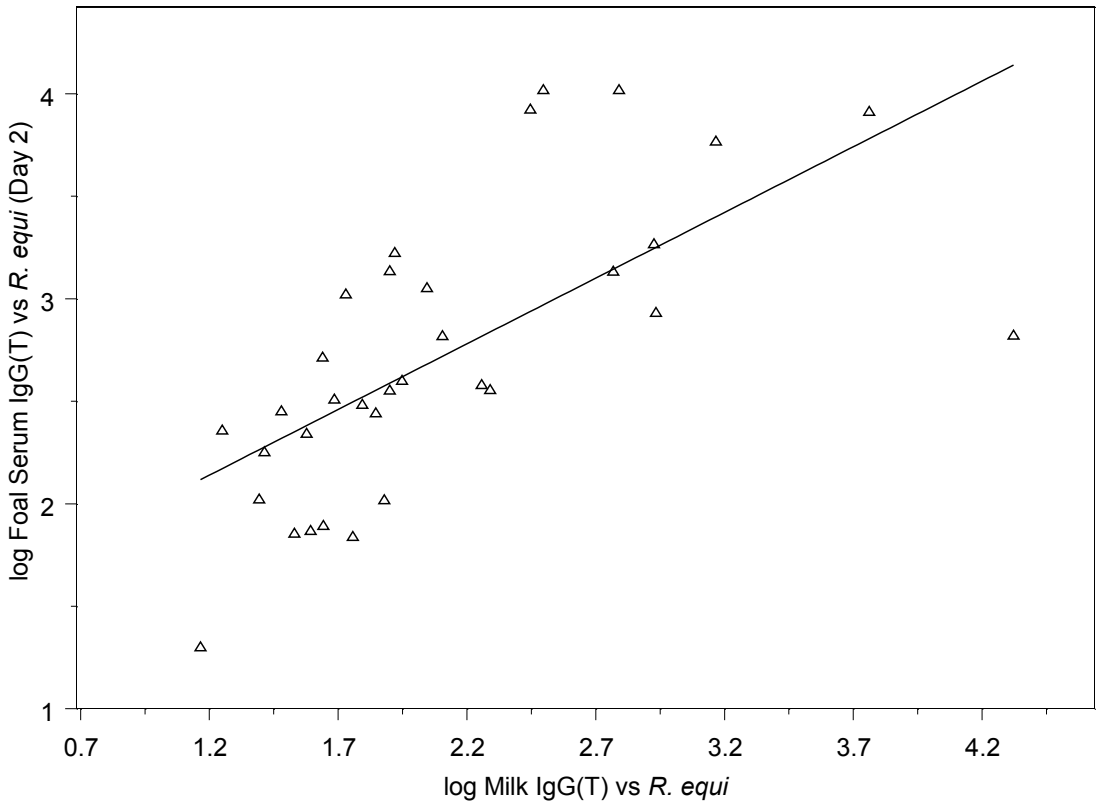


Fig. O-5. $IgG_{3/5}$ specific for *R. equi* in foal serum versus mare's milk on Day 2. The association was weak but statistically significant ($P < 0.0001$)

APPENDIX P

I. Association of Mare Milk IgA and Nasal IgA

A. Data are MFIGA in U:\Infectious Diseases\Requi\Vaccine\Data 2012\Mare Milk and Serum

B. Analysis by linear regression; data log10-transformed as appropriate

C. Results:

1. Total IgA: There was a moderate but significant correlation between mares milk IgA concentration and total nasal IgA detected on day 2 but not on day 32 or for the ratio of day 32 to day 2 (Table P-1; Fig. 1-6). There was no significant effect of treatment group or significant interaction of treatment group and foal sample (data not shown).

Table P-1
Association of total IgA in mare's milk with foal nasal IgA samples on day 2, day 32, and the ratio of day 32:2.

a. Raw data

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.33	0.0004	0.0142 (0.0071 to 0.0212)
Day 32	0.07	0.1414	0.08 (-0.03 to 0.19)
Ratio Day 32: Day 2	0.01	0.5635	0.00 (-0.01 to 0.01)

b. Log_{10}-transformed data

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.27	0.0017	1.14 (0.49 to 1.79)
Day 32	0.15	0.0237	0.96 (0.17 to 1.75)
Ratio Day 32: Day 2	>0.01	0.7974	-0.14 (-1.23 to 0.95)

2. Specific IgA: There was a modest but significant correlation between mares milk IgA concentration and total nasal IgA detected on day 2 but not on day 32 or for the ratio of day 32 to day 2 (Table P-2; Fig. P-7-P-12). There was no significant effect of treatment group or significant interaction of treatment group and foal sample (data not shown).

Table P-2
Association of *R. equi*-specific IgA in mare's milk with foal nasal IgA samples on day 2, day 32, and the ratio of day 32:2.

a. Raw data

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.15	0.0223	0.0054 (0.0011 to 0.0097)
Day 32	>0.01	0.8964	-0.0018 (-0.0292 to 0.0256)
Ratio Day 32: Day 2	0.02	0.4510	-0.0009 (-0.0003 to 0.0012)

b. Log_{10}-transformed data

Foal sample	R^2	*P value*	*Slope (95% CI)*
Day 2	0.12	0.0457	0.58 (0.03 to 1.13)
Day 32	0.04	0.2317	0.45 (- 0.28 to 1.18)
Ratio Day 32: Day 2	>0.01	0.7234	-0.13 (-0.84 to 0.58)

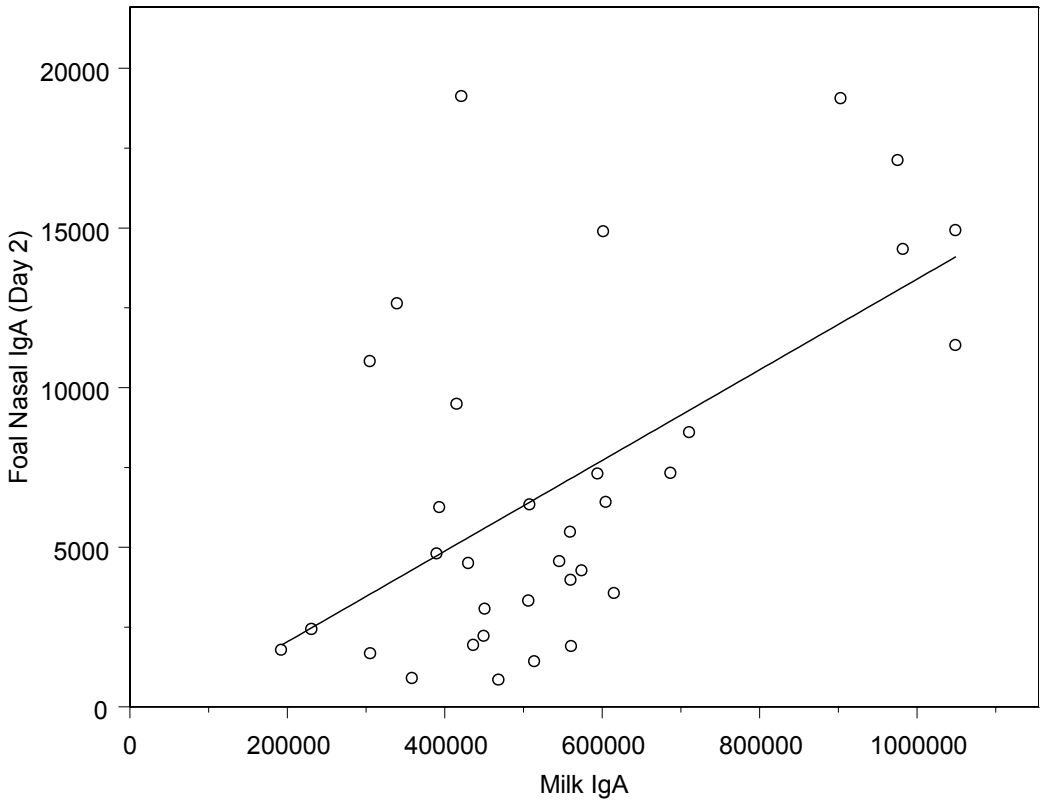


Fig. P-1. Total IgA Nasal vs milk day 2

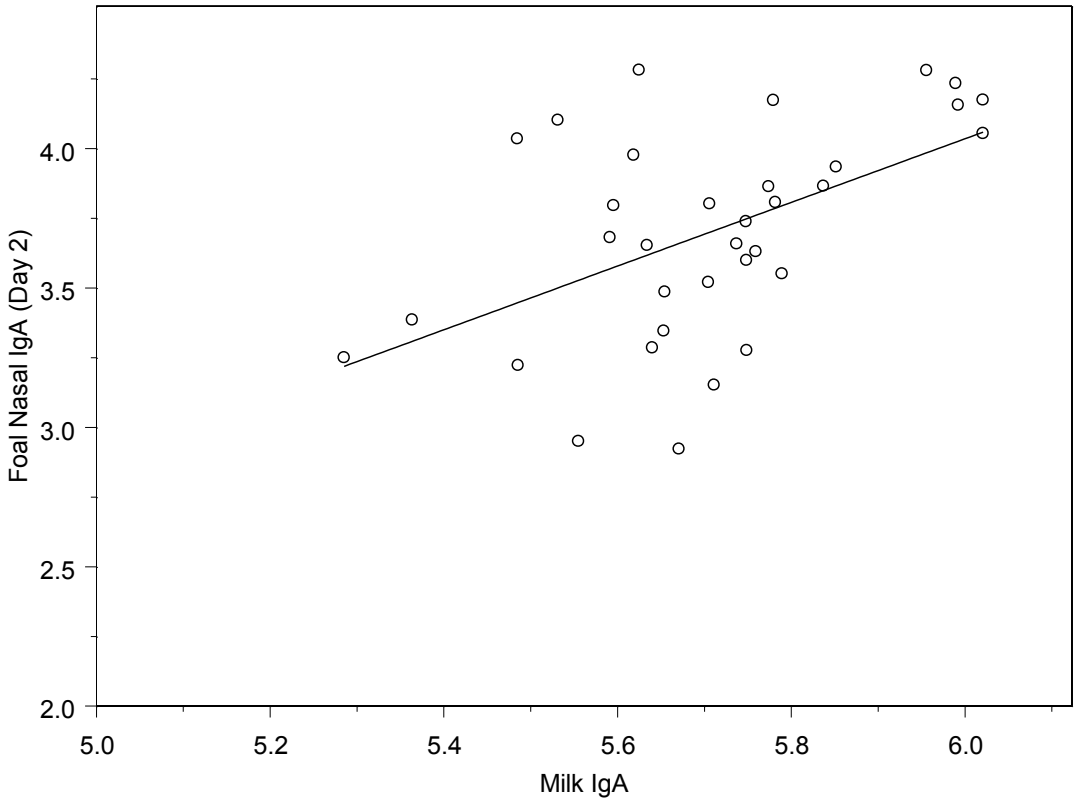


Fig. P-2. Log_{10} total IgA nasal vs milk day 2

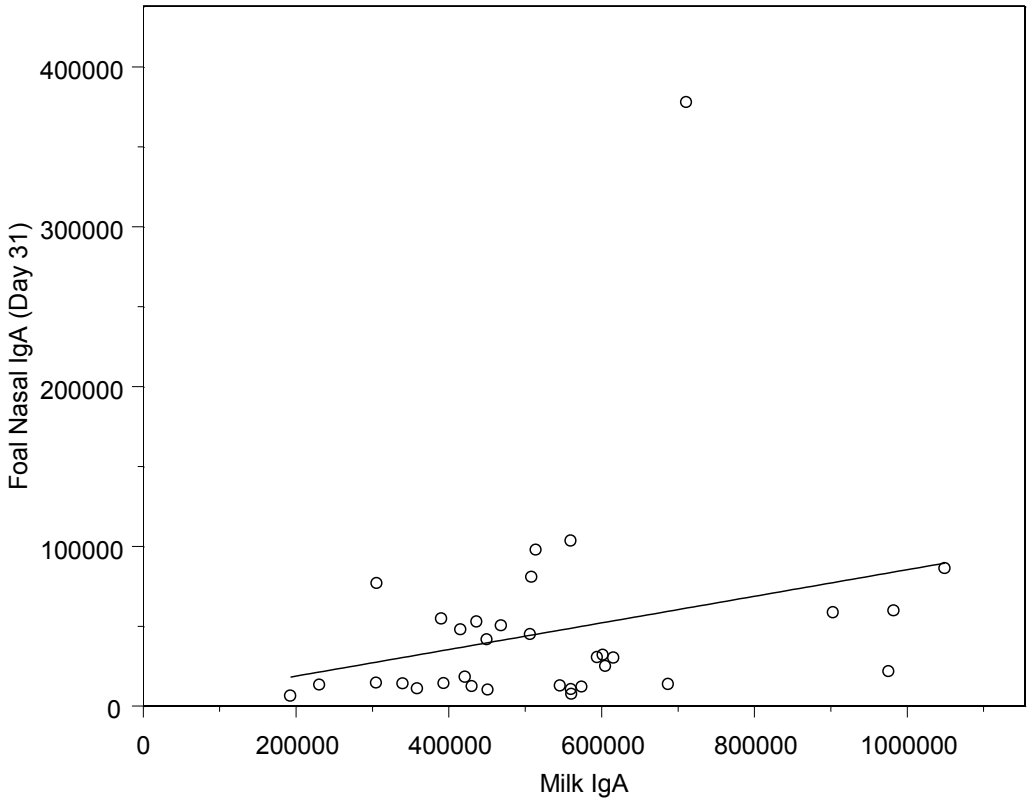


Fig. P-3. Total IgA nasal vs milk day 32

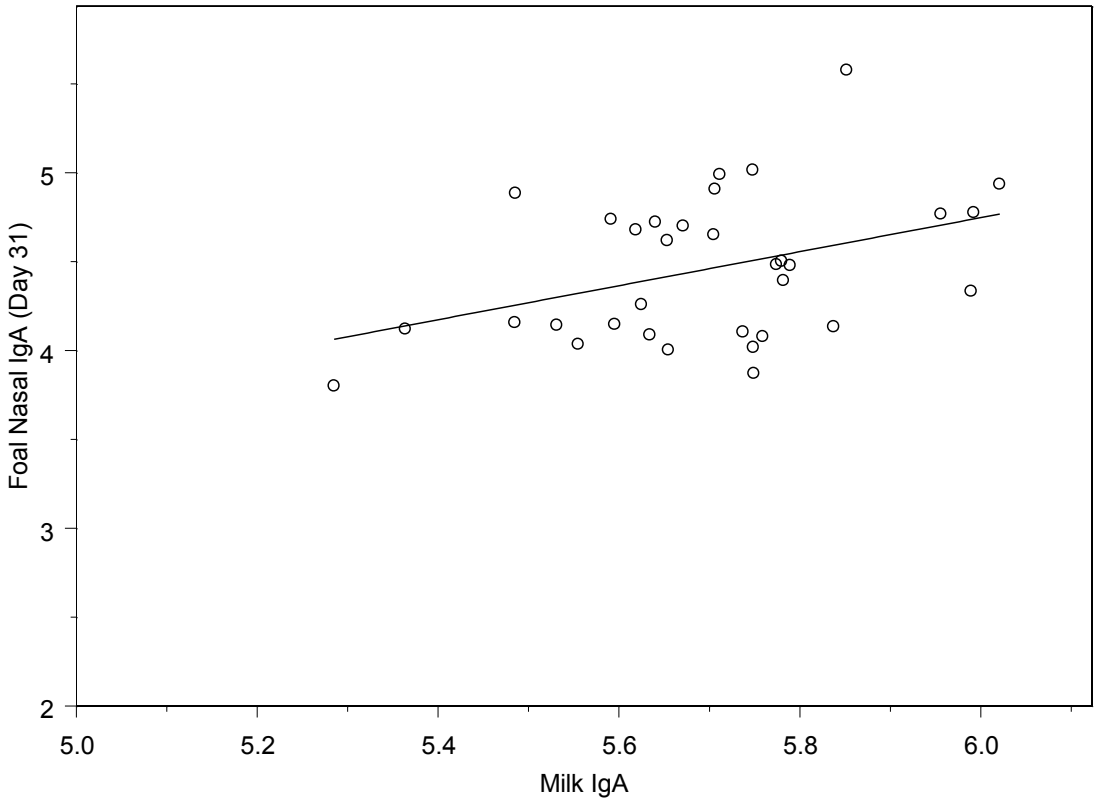


Fig. P-4. Log_{10} total IgA nasal vs milk day 32

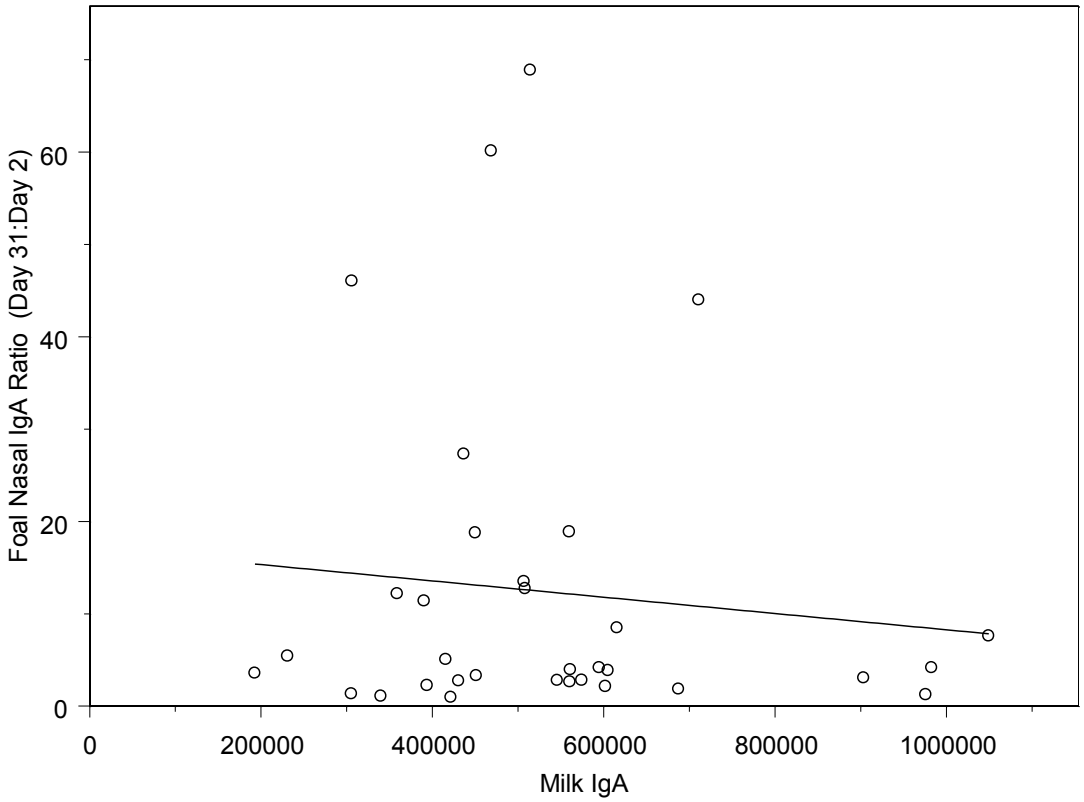


Fig. P-5. Total IgA nasal ratio vs milk

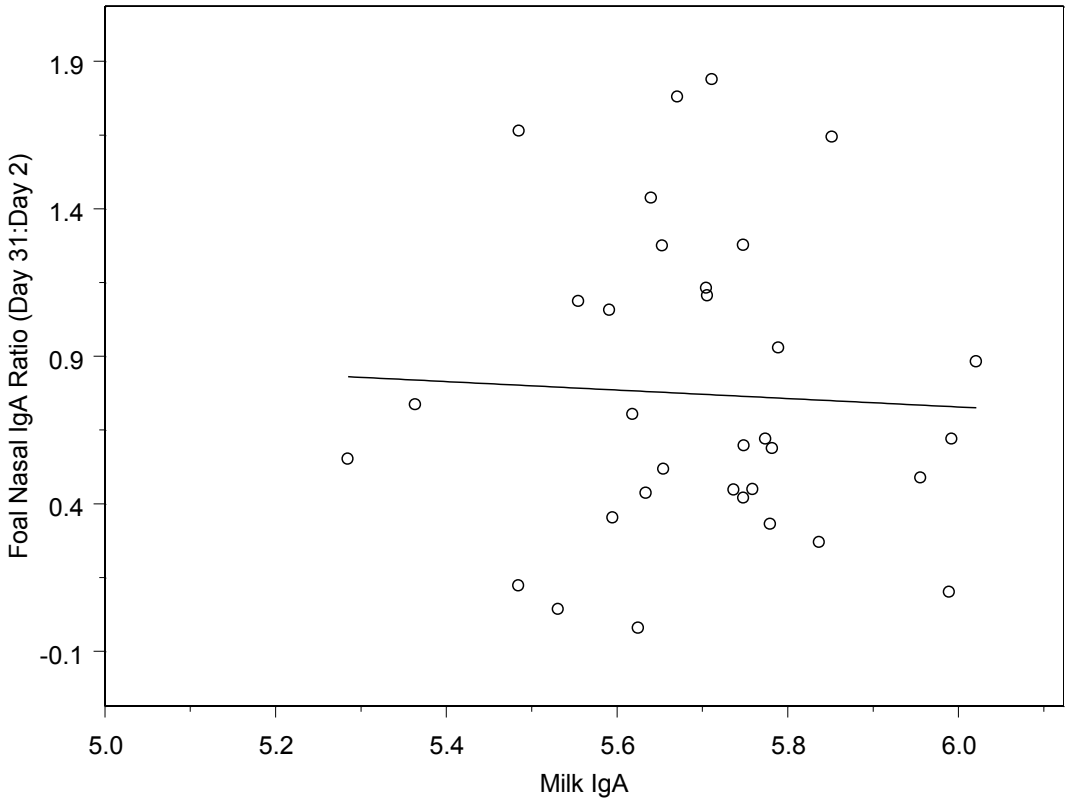


Fig. P-6. Log_{10} total IgA nasal ratio vs milk

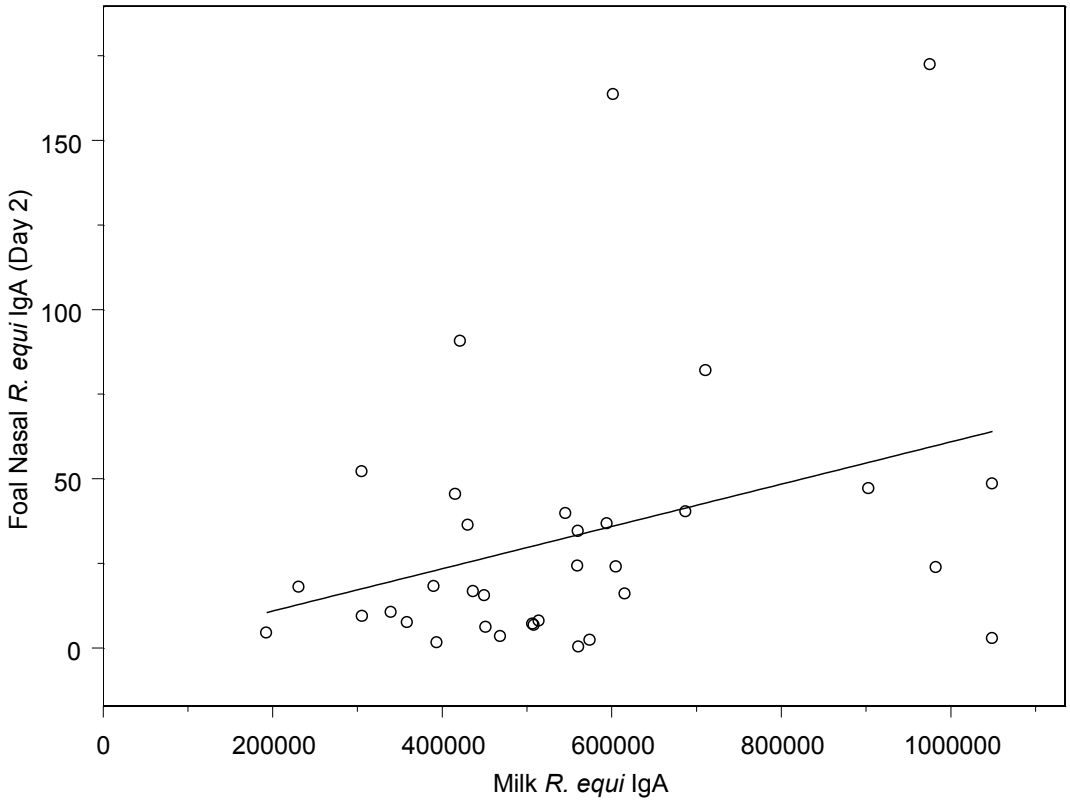


Fig. P-7. Specific IgA day 2 vs milk

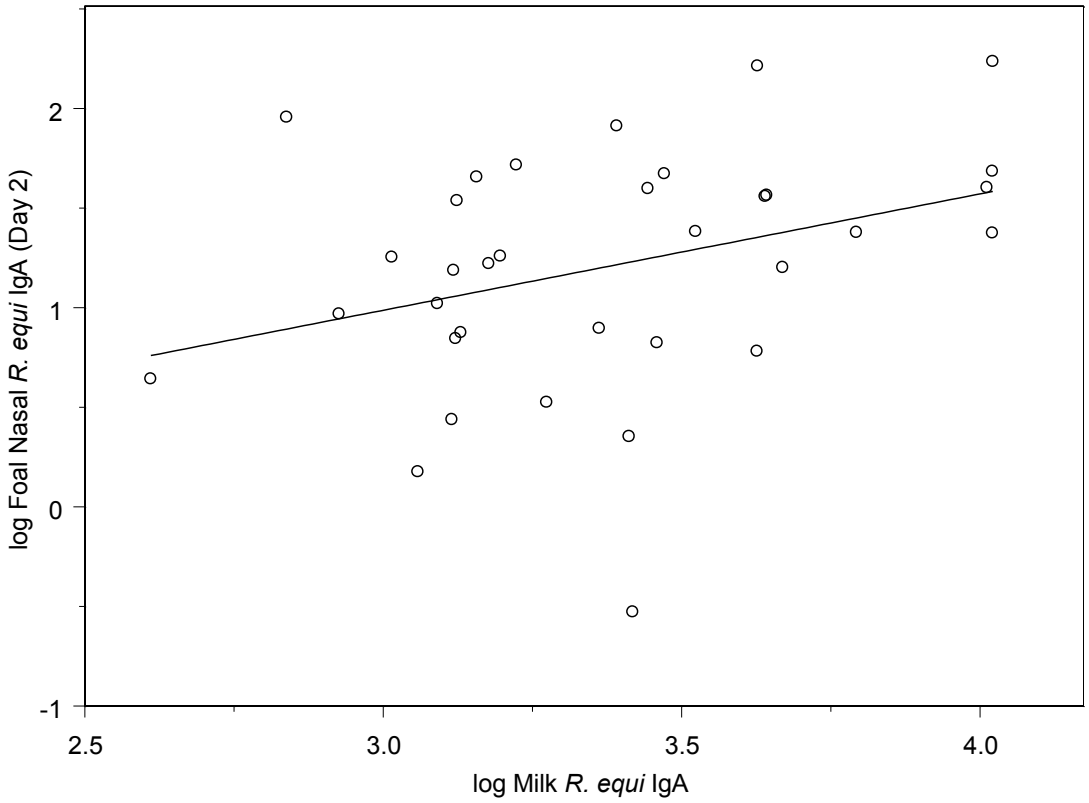


Fig. P-8. Log_{10} specific IgA day 2 vs milk

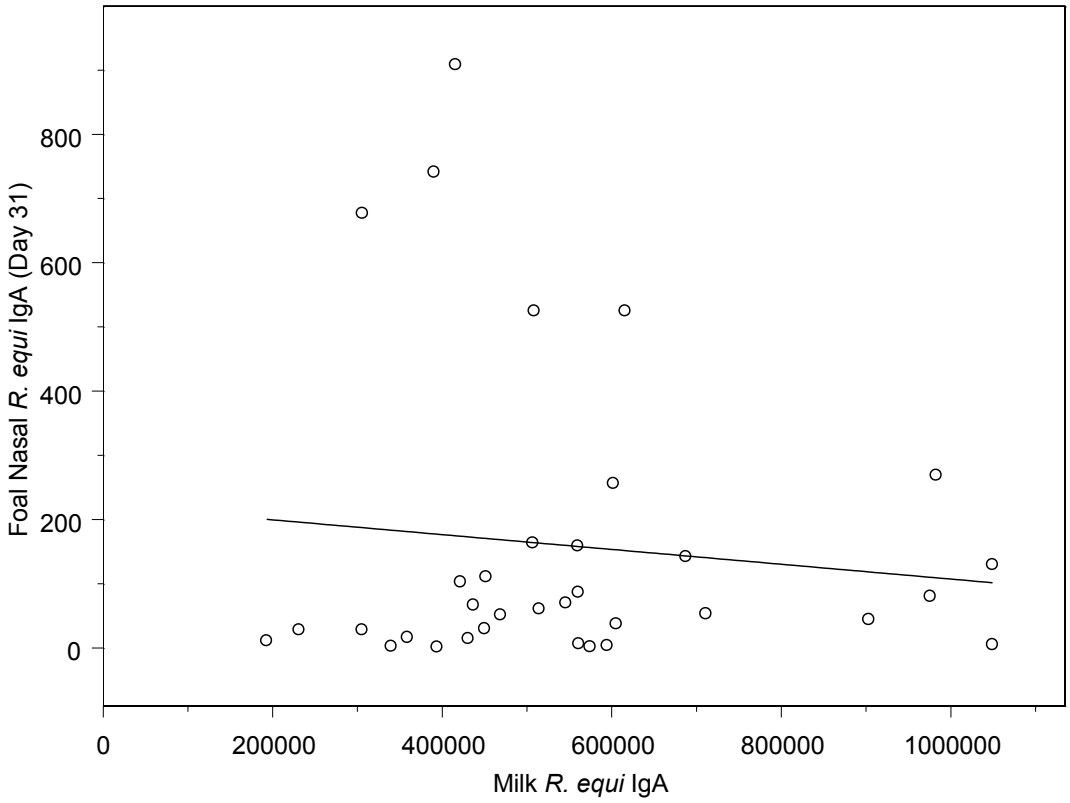


Fig. P-9. Specific IgA day 32 vs milk

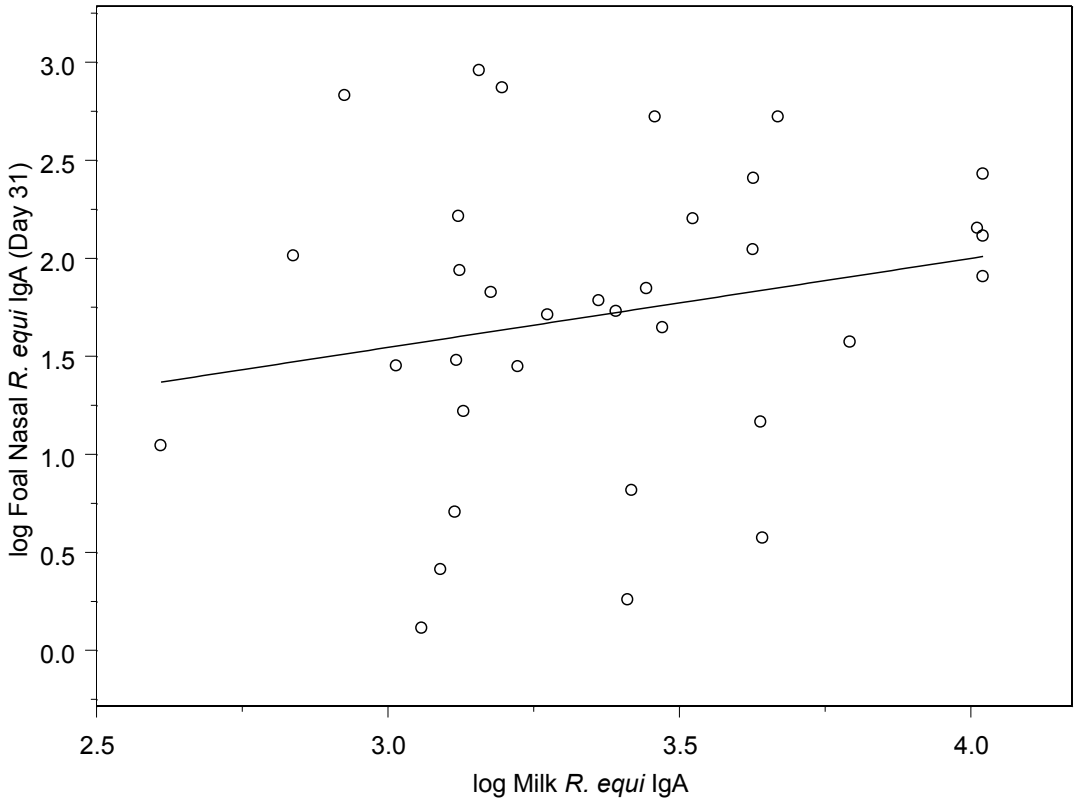


Fig. P-10. Log_{10} specific IgA day 32 vs milk

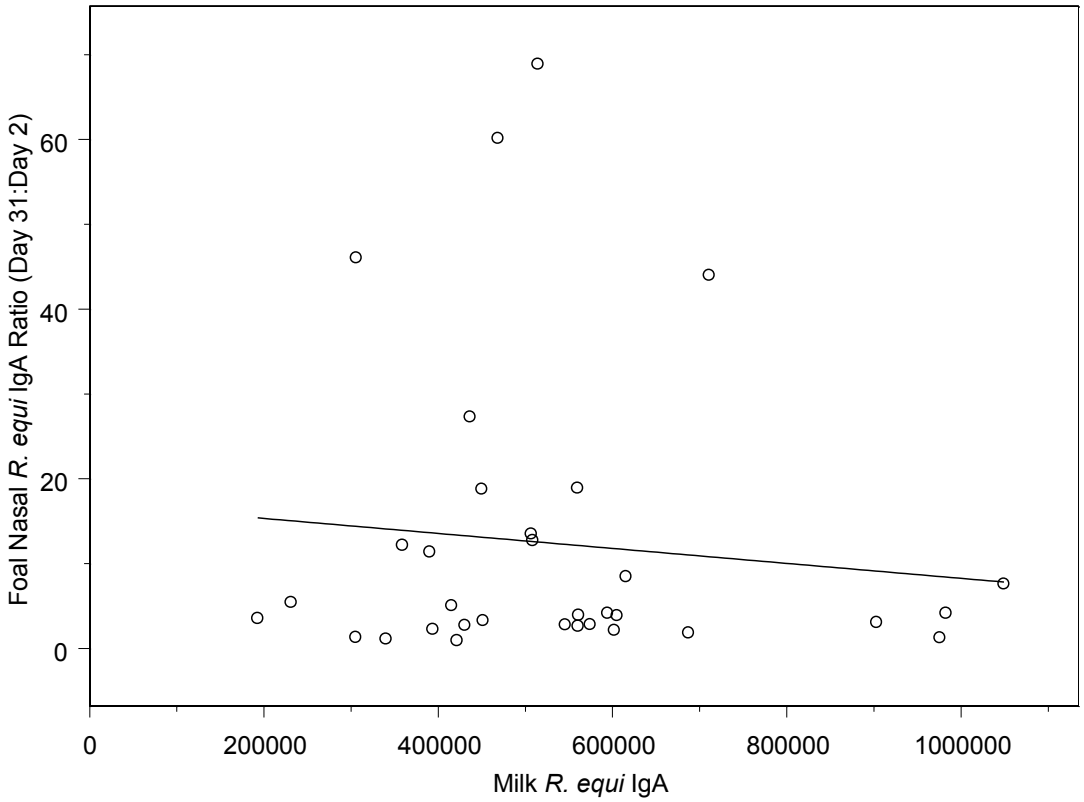


Fig. P-11. Specific IgA day 32:day2 vs milk

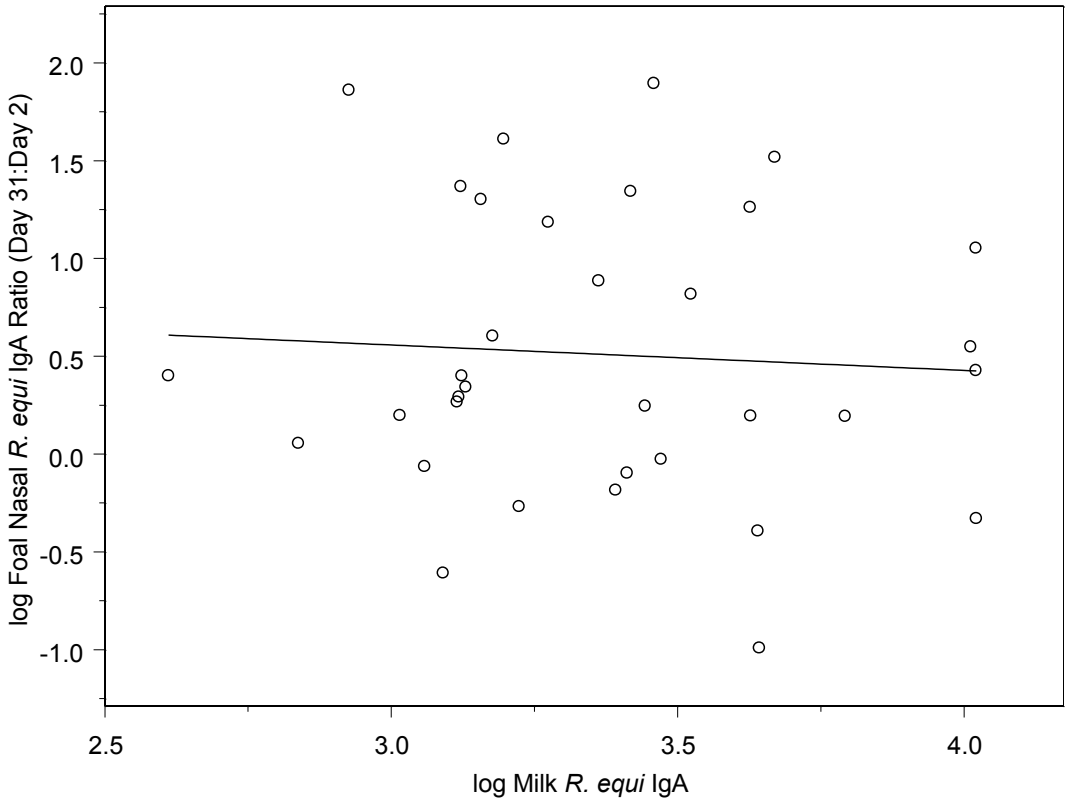


Fig. P-12. Log_{10} specific IgA day 32: day 2 vs milk

APPENDIX Q

Table Q-1
Median and range percentages of sequences represented in the fecal DNA of rectal swab samples from foals (Phylum, class, order, and family).

Microbial Phylum/Class/Order/Family	2-day-old foals (N =	30-day-old foals(N =	P*
Archaea.Euryarchaeota	0% (0%)	0% (0 to 0.6%)	**0.0048**
Methanobacteria	0% (0%)	0% (0 to 0.3%)	**0.0280**
Methanobacteriales	0% (0%)	0% (0 to 0.3%)	0.0924
Methanobacteriacae	0% (0%)	0% (0 to 0.3%)	0.1932
Methanomicrobia	0% (0%)	0% (0 to 0.6%)	0.1341
Methanomicrobiales	0% (0%)	0% (0 to 0.6%)	0.4321
Methanocorpusculaceae	0% (0%)	0% (0 to 0.6%)	0.9089
Bacteria.Acidobacteria	0% (0%)	0% (0 to 0.2%)	0.9515
Acidobacteria	0% (0%)	0% (0 to 0.2%)	1.0000
Acidobacteriales	0% (0%)	0% (0 to 0.2%)	1.0000
Acidobacteriaceae	0% (0%)	0% (0 to 0.2%)	1.0000
Bacteria.Actinobacteria	0.2% (0 to 4.1%)	1.2% (0 to 4.3%)	**0.0048**
Actinobacteria	0.2% (0 to 4.1%)	1.2% (0 to 4.3%)	**0.0280**
Actinomycetales	0.1% (0 to 3.4%)	0.3% (0 to 2.8%)	0.3904
Bifidobacteriales	0% (0 to 0.2%)	0% (0 to 0.2%)	1.0000
Other	0% (0 to 0.7%)	0% (0 to 1.5%)	1.0000
Coriobacteridae (subclass)	0% (0 to 1.5%)	0.2% (0 to 2.7%)	**<0.0001**
Coriobacteriales	0% (0 to 1.5%)	0.2% (0 to 2.7%)	**0.0080**
Rubrobacteridae (subclass)	0% (0 to 0.7%)	0% (0 %)	0.9515
Rubrobacterales	0% (0 to 0.7%)	0% (0 %)	1.0000
Other order	0% (0%)	0% (0 to 0.8%)	0.0578
Other family	0% (0%)	0% (0 to 0.8%)	0.1190
Bacteria.Bacteroidetes	16.7% (0 to 85.5%)	40.6% (0.2 to 87.8%)	**0.0066**
Bacteroidetes	16.7% (0 to 85.4%)	25.3% (0.1 to 80.5%)	0.5376
Bacteroidales	16.7% (0 to 85.4%)	25.3% (0.2 to 80.5%)	0.9515
Bacteroidieacae	16.7% (0 to 85.3%)	5.2% (0 to 53.3%)	1.0000
Porphyromonadaceae	0% (0 to 9.0%)	0.4% (0 to 16.4%)	**0.0080**
Prevotellaceae	0% (0 to 1.5%)	2.8% (0 to 63.1%)	**<0.0001**
Rikenellaceae	0% (0 to 10.2%)	0% (0 to 5.8%)	0.2108
Other	0% (0 to 0.2%)	4.0% (0 to 18.0%)	**<0.0001**

Table Q-1 continued

Flavobacteria	0% (0 to 1.2%)	0% (0 to 2.3%)	0.8167
Flavobacteriales	0% (0 to 1.2%)	0% (0 to 2.3%)	1.0000
Flavobacteriaceae	0% (0 to 1.2%)	0% (0 to 2.3%)	1.0000
Sphingobacteria	0% (0 to 0.2%)	0% (0 to 0.1%)	0.8167
Sphingobacteriales	0% (0 to 0.2%)	0% (0 to 0.1%)	0.9515
Crenotrichaceae	0% (0 to 0.1%)	0% (0%)	1.0000
Flexibacteriaceae	0% (0%)	0% (0 to 0.1%)	1.0000
Sphingobacteriaceae	0% (0 to 0.2%)	0% (0%)	1.0000
Other class	0% (0 to 1.7%)	5.5% (0 to 48.1%)	**<0.0001**
Other order	0% (0 to 1.7%)	5.5% (0 to 48.1%)	**<0.0001**
Other family	0% (0 to 1.7%)	5.5% (0 to 48.1%)	**<0.0001**
Bacteria.Chlamydiae	0% (0%)	0% (0 to 30.1%)	**<0.0001**
Chlamydiae	0% (0%)	0% (0 to 30.1%)	**<0.0001**
Chlamydiales	0% (0%)	0% (0 to 30.1%)	**<0.0001**
Chlamydiaceae	0% (0%)	0.1% (0 to 30.1%)	**<0.0001**
Parachlamydiaceae	0% (0%)	0% (0 to 0.1%)	1.0000
Bacteria.Chloroflexi	0% (0%)	0% (0 to 0.2%)	**0.0441**
Anaerolineae	0% (0%)	0% (0 to 0.2%)	0.2254
Caldilineae	0% (0%)	0% (0 to 0.2%)	0.9515
Caldilineales	0% (0%)	0% (0 to 0.2%)	1.0000
Other	0% (0%)	0% (0 to 0.2%)	0.2635
Other family	0% (0%)	0% (0 to 0.2%)	0.5355
Bacteria.Cyanobacteria	0% (0%)	0% (0 to 0.1%)	0.9515
Cyanobacteria	0% (0%)	0% (0 to 0.1%)	1.0000
Other order	0% (0%)	0% (0 to 0.1%)	1.0000
Other family	0% (0%)	0% (0 to 0.1%)	1.0000
Bacteria.Deferribacteres	0% (0% to 0.2%)	0% (0 to 0.2%)	0.9515
Deferribacteres	0% (0% to 0.2%)	0% (0 to 0.2%)	1.0000
Deferribacterales	0% (0% to 0.2%)	0% (0 to 0.2%)	1.0000
Deferribacteraceae	0% (0%)	0% (0%)	NP
Incertae sedis 3	0% (0 to 0.2%)	0% (0 to 0.2%)	1.0000
Bacteria.Fibrobacteres	0% (0%)	0% (0 to 0.7%)	0.2104
Fibrobacteres	0% (0% to 0.2%)	0% (0 to 0.7%)	0.4698
Fibrobacterales	0% (0% to 0.2%)	0% (0 to 0.7%)	0.9515
Fibrobacteraceae	0% (0% to 0.2%)	0% (0 to 0.7%)	1.0000
Bacteria.Firmicutes	40.4% (5.8 to 69.2%)	23.3% (4.4 to 95.2%)	0.9515
Bacilli	4.8% (0.5 to 32.2%)	2.4% (0.1 to 78.8%)	0.2254
Lactobacillales	4.8% (0.5 to 32.2%)	2.2% (0.1 to 69.8%)	0.6264
Aerococcaceae	0% (0 to 1.6%)	0% (0 to 1.1%)	1.0000
Carnobacteriaceae	0% (0 to 0.2%)	0% (0 to 0.1%)	1.0000
Enterococcaceae	1.2% (0 to 14.5%)	0% (0 to 65.0%)	**0.0080**

Table Q-1 continued

Lactobacillaceae	0% (0 to 7.9%)	0% (0 to 5.6%)	**0.0281**
Leuconostocaceae	0% (0 to 0.2%)	0% (0%)	1.0000
Streptococcaceae	2.1% (0 to 31.2%)	1.6% (0 to 20.8%)	1.0000
Other	0.2% (0 to 1.7%)	0% (0 to 3.8%)	**0.0234**
Bacillales	0% (0 to 0.3%)	0% (0 to 0.8%)	0.9515
Paenibacillaceae	0% (0 to 0.2%)	0% (0 to 0.2%)	1.0000
Staphylococcaceae	0% (0 to 2.5%)	0.1% (0 to 8.2%)	1.0000
Bacillaceae	0% (0 to 0.3%)	0% (0 to 0.1%)	1.0000
Incertae Sedis XI	0% (0 to 0.2%)	0% (0 to 0.1%)	1.0000
Planococcaceae	0% (0%)	0% (0 to 0.2%)	1.0000
Other	0% (0 to 0.2%)	0% (0 to 0.2%)	1.0000
Other order	0% (0 to 0.1%)	0% (0 to 0.5%)	0.9515
Other family	0% (0 to 0.1%)	0% (0 to 0.5%)	1.0000
Clostridia	30.1% (3.4 to 64.5%)	18.8% (3.6 to 82.5%)	0.1314
Clostridiales	30.1% (3.4 to 64.5%)	29.5% (3.4 to 64.5%)	0.9515
Eubacteriaceae	0% (0 to 2.2%)	0% (0 to 1.2%)	0.3839
Lachnospiraceae	3.7% (0 to 55.5%)	5.6% (0.7 to 76.7%)	1.0000
Peptostreptococcaceae	3.4% (0 to 20.1%)	0% (0 to 12.4%)	**<0.0001**
Ruminococcaceae	0.2% (0 to 4.6%)	1.5% (0.1 to 18.5%)	**<0.0001**
Clostridiaceae	7.1% (0.1 to 45.2%)	5.4% (0 to 19.0%)	**0.0080**
Incertae Sedis XI	0% (0 to 1.1%)	1.2% (0 to 14.0%)	**<0.0001**
Incertae Sedis XIII	0% (0 to 0.8%)	0.1% (0 to 5.8%)	**0.0080**
Peptococcaceae	0% (0%)	0% (0 to 2.5%)	**0.0438**
Veillonellaceae	0% (0 to 8.3%)	0.9% (0 to 3.5%)	**<0.0001**
Other	3.1% (0.1 to 13.7%)	2.0% (0 to 16.5%)	1.0000
Other order	0% (0 to 0.4%)	0.2% (0 to 23.5%)	**0.0190**
Other family	0% (0 to 0.4%)	0.2% (0 to 23.5%)	**0.0375**
Erysipelotrichi	0.1% (0 to 1.0%)	0.1% (0 to 2%)	0.5376
Erysipelotrichales	0.1% (0 to 1.0%)	0.1% (0 to 2%)	0.4844
Erysipelotrichiaceae	0.1% (0 to 1.0%)	0.1% (0 to 2%)	1.0000
Other class	0% (0 to 0.5%)	0.3% (0 to 6.5%)	**<0.0001**
Other order	0% (0 to 0.5%)	0.3% (0 to 6.5%)	**<0.0001**
Other family	0% (0 to 0.5%)	0.3% (0 to 6.5%)	**<0.0001**
Bacteria.Fusobacteria	0.8% (0 to 45.5%)	0.8% (0 to 42.5%)	0.9510
Fusobacteria	0.8% (0 to 45.5%)	0.8% (0 to 42.2%)	1.0000
Fusobacteriales	0.8% (0 to 45.5%)	0.8% (0 to 42.2%)	1.0000
Fusobacteriaceae	0.4% (0 to 45.3%)	0.8% (0 to 42.2%)	1.0000
Incertae sedis 11	0% (0 to 0.2%)	0% (0%)	1.0000
Other	0% (0 to 16.1%)	0% (0 to 1.0%)	1.0000
Bacteria.Lentisphaerae	0% (0%)	0% (0%)	NP
Lentisphaerae	0% (0%)	0% (0%)	NP

Table Q-1 continued

Victivallales	0% (0%)	0% (0%)	NP
Victivallaceae	0% (0%)	0% (0%)	NP
Bacteria. Other	0.2% (0 to 8.1%)	4.6% (0.2 to 68.5%)	**<0.0001**
Bacteria. Other Class	0.2% (0 to 8.1%)	4.6% (0.2 to 68.5%)	**<0.0001**
Other Order	0.2% (0 to 8.1%)	4.6% (0.2 to 68.5%)	**<0.0001**
Other family	0.2% (0 to 8.1%)	4.6% (0.2 to 68.5%)	**<0.0001**
Bacteria.Planctomycetes	0% (0 to 0.1%)	0% (0 to 1.4%)	**0.0015**
Planctomycetacia	0% (0 to 0.1%)	0% (0 to 1.4%)	**0.0322**
Planctomycetales	0% (0 to 0.1%)	0% (0 to 1.4%)	**0.0047**
Planctomycetaceae	0% (0 to 0.1%)	0% (0 to 1.4%)	**0.0080**
Bacteria.Proteobacteria	36.3% (0.5 to 85.8%)	2.7% (0 to 40.9%)	**<0.0001**
Alphaproteobacteria	0% (0 to 0.3%)	0% (0 to 0.3%)	0.8167
Caulobacterales	0% (0 to 0.2%)	0% (0)	0.9515
Caulobacteriaceae	0% (0 to 0.2%)	0% (0)	1.0000
Rhizobiales	0% (0 to 0.2%)	0% (0 to 0.2%)	0.9515
Hyphomicrobiaceae	0% (0%)	0% (0 to 0.1%)	1.0000
Methylobacteriaceae	0% (0 to 0.1%)	0% (0 to 0.1%)	1.0000
Other	0% (0 to 0.2%)	0% (0 to 0.3%)	1.0000
Rhodobacteriales	0% (0 to 0.2%)	0% (0 to 0.1%)	0.9515
Rhodobacteriaceae	0% (0 to 0.2%)	0% (0 to 0.1%)	1.0000
Rhodospirales	0% (0%)	0% (0%)	NP
Other	0% (0%)	0% (0%)	NP
Other order	0% (0%)	0% (0 to 0.1%)	0.9515
Other family	0% (0%)	0% (0 to 0.1%)	1.0000
Betaproteobacteria	0% (0 to 0.2%)	0% (0 to 0.5%)	0.4698
Burkholderiales	0% (0 to 0.2%)	0% (0 to 0.5%)	0.9230
Alcaligenaceae	0% (0 to 0.1%)	0% (0 to 0.1%)	1.0000
Comamonadacea	0% (0 to 0.1%)	0% (0 to 0.1%)	1.0000
Other	0% (0 to 0.1%)	0% (0 to 0.5%)	1.0000
Other order	0% (0 to 0.1%)	0% (0 to 0.1%)	0.9515
Other family	0% (0 to 0.1%)	0% (0 to 0.1%)	1.0000
Deltaproteobacteria	0% (0 to 0.4%)	0% (0 to 1.1%)	**0.0084**
Desulfovibrionales	0% (0 to 0.4%)	0% (0 to 0.5%)	**0.0385**
Desulfovibrionaceae	0% (0 to 0.4%)	0% (0 to 0.5%)	0.1136
Other	0% (0 to 0.1%)	0% (0 to 0.2%)	1.0000
Myxococcales	0% (0%)	0% (0 to 1.1%)	0.9515
Nannocystineae	0% (0%)	0% (0 to 1.0%)	1.0000
Other	0% (0%)	0% (0 to 0.1%)	1.0000
Epsilonproteobacteria	0% (0 to 0.1%)	0.3% (0 to 16.4%)	**<0.0001**
Campylobacterales	0% (0 to 0.1%)	0.3% (0 to 16.4%)	**<0.0001**
Campylobacteriaceae	0% (0 to 0.1%)	0.2% (0 to 4.7%)	**<0.0001**

Table Q-1 continued

Helicobacteraceae	0% (%)	0% (0 to 15.9%)	**0.0080**
Gamma proteobacteria	36.3% (0 to 85.8%)	0.5% (0 to 40.9%)	**<0.0001**
Aeromonadales	0% (0 to 3.2%)	0% (0 to 4.2%)	0.9515
Aeromonadaceae	0% (0 to 3.2%)	0% (0%)	0.3234
Succinivibrionaceae	0% (0%)	0% (0 to 4.2%)	**0.0080**
Enterobacteriales	36.2% (0 to 85.8%)	0.1% (0 to 39.8%)	**<0.0001**
Enterobacteriaceae	36.2% (0 to 85.8%)	0.1% (0 to 39.8%)	**<0.0001**
Legionellales	0% (0%)	0% (0 to 0.2%)	0.9515
Coxiellaceae	0% (0%)	0% (0 to 0.1%)	1.0000
Legionellaceae	0% (0%)	0% (0 to 0.2%)	1.0000
Oceanospirillales	0% (0 to 0.1%)	0% (0%)	0.9515
Halomonadaceae	0% (0 to 0.1%)	0% (0%)	1.0000
Pasteurellales	0% (0 to 3.6%)	0% (0 to 1.2%)	0.9515
Pasteurellaceae	0% (0 to 3.6%)	0% (0 to 1.2%)	1.0000
Pseudomonadales	0% (0 to 1.5%)	0% (0 to 1.1%)	0.9515
Moraxellaceae	0% (0 to 0.3%)	0% (0 to 0.7%)	1.0000
Pseudomonadaceae	0% (0 to 1.2%)	0% (0 to 0.4%)	1.0000
Xanthomonadales	0% (0%)	0% (0 to 0.1%)	0.9515
Xanthomonadaceae	0% (0%)	0% (0 to 0.1%)	1.0000
Other order	0% (0 to 0.5%)	0% (0%)	0.3278
Other family	0% (0 to 0.5%)	0% (0%)	1.0000
Other class	0% (0 to 0.2%)	0% (0 to 23.7%)	**0.0099**
Other order	0% (0 to 0.2%)	0% (0 to 23.7%)	**0.0333**
Other family	0% (0 to 0.2%)	0% (0 to 23.7%)	0.9089
Bacteria.Spirochaetes	0% (0%)	0% (0 to 2.1%)	**0.0100**
Spirochaetes	0% (0%)	0% (0 to 2.1%)	**0.0375**
Spirochaetales	0% (0%)	0% (0 to 2.1%)	**0.0360**
Spirochaetaceae	0% (0%)	0% (0 to 2.1%)	0.0720
Other	0% (0%)	0% (0 to 0.2%)	1.0000
Bacteria.TM7	0% (0%)	0% (0 to 1.8%)	**0.0048**
TM7 genera incertae sedis	0% (0%)	0% (0 to 1.8%)	**0.0280**
Other order	0% (0%)	0% (0 to 1.8%)	**0.0156**
Other family	0% (0%)	0% (0 to 1.8%)	**0.0308**
Bacteria.Tenericutes	0% (0%)	0% (0 to 0.1%)	0.9515
Mollicutes	0% (0%)	0% (0 to 0.1%)	1.0000
Anaeroplasmatales	0% (0%)	0% (0 to 0.1%)	1.0000
Anaeroplasmataceae	0% (0%)	0% (0 to 0.1%)	1.0000
Bacteria.Verrucomicrobia	0% (0 to 42.5%)	1.0% (0.4 to 48.7%)	**0.0015**
Verrucomicrobiae	0% (0 to 42.5%)	1.0% (0.4 to 48.7%)	**0.0322**
Verrucomicrobiales	0% (0 to 42.5%)	1.0% (0.4 to 48.7%)	**0.0040**
Other	0% (0%)	0% (0 to 0.2%)	1.0000

Table Q-1 continued

Subdivision 5	0% (0 to 0.2%)	0.3% (0 to 25.4%)	0.0000
Verrucomicrobiaceae	0% (0 to 42.5%)	0.6% (0 to 48.6%)	**0.0158**
Xiphinematobacteriaceae	0% (0%)	0% (0 to 0.2%)	1.0000
Other Kingdom, Other phylum	0% (0 to 1.1%)	0% (0 to 0.3%)	0.9515
Other class	0% (0 to 1.1%)	0% (0 to 0.3%)	1.0000
Other order	0% (0 to 1.1%)	0% (0 to 0.3%)	1.0000
Other family	0% (0 to 1.1%)	0% (0 to 0.3%)	1.0000

Fecal swab samples were collected from 37 Quarter Horse foals on days 2 and 30 of life
*P values represent the results of Wilcoxon sign-rank tests for paired differences, adjusted by the method of Hochberg.
NP = Not Performed

APPENDIX R

Table R-1
Median and range proportion of foals with sequences detected in the fecal DNA of rectal swab samples (Phylum, class, order, and family).

Microbial Family	2-day-old foals	30-day-old foals (N = 37)	P*
Archaea.Euryarchaeota	0% (0/37)	35% (13/37)	**0.0117**
Methanobacteria	0% (0/37)	24% (9/37)	0.0770
Methanobacteriales	0% (0/37)	24% (9/37)	0.1925
Methanobacteriacae	0% (0/37)	24% (9/37)	0.4851
Methanomicrobia	0% (0/37)	16% (6/37)	0.3708
Methanomicrobiales	0% (0/37)	16% (6/37)	0.9064
Methanocorpusculaceae	0% (0/37)	16% (6/37)	1.0000
Bacteria.Acidobacteria	0% (0/37)	3% (1/37)	0.9999
Acidobacteria	0% (0/37)	3% (1/37)	1.0000
Acidobacteriales	0% (0/37)	3% (1/37)	1.0000
Acidobacteriaceae	0% (0/37)	3% (1/37)	1.0000
Bacteria.Actinobacteria	73% (27/37)	97% (36/37)	0.1590
Actinobacteria	73% (27/37)	97% (36/37)	0.1925
Actinomycetales (order)	62% (23/37)	73% (27/37)	1.0000
Bifidobacteriales (order)	3% (1/37)	8% (3/37)	1.0000
Other	14% (5/37)	8% (3/37)	1.0000
Coriobacteridae (subclass)	24% (9/37)	76% (28/37)	**0.0041**
Coriobacteriales	24% (9/37)	76% (28/37)	**0.0221**
Rubrobacteridae (subclass)	3% (1/37)	0% (0/37)	1.0000
Rubrobacterales	3% (1/37)	0% (0/37)	1.0000
Other order	0% (0/37)	27% (10/37)	0.1452
Other family	0% (0/37)	27% (10/37)	0.2944
Bacteria.Bacteroidetes	92% (34/37)	100% (37/37)	0.5152
Bacteroidetes	89% (33/37)	100% (37/37)	1.0000
Bacteroidales	89% (33/37)	100% (37/37)	1.0000
Bacteroidieacae	86% (32/37)	95% (35/37)	1.0000
Porphyromonadaceae	30% (11/37)	89% (33/37)	**<0.0001**
Prevotellaceae	8% (3/37)	95% (35/37)	**<0.0001**
Rikenellaceae	11% (4/37)	49% (18/37)	0.1496
Other	11% (4/37)	95% (35/37)	**<0.0001**

Table R-1 continued

Flavobacteria	16% (6/37)	11% (4/37)	1.0000
Flavobacteriales	16% (6/37)	11% (4/37)	1.0000
Flavobacteriaceae	16% (6/37)	11% (4/37)	1.0000
Sphingobacteria	5% (2/37)	3% (1/37)	1.0000
Sphingobacteriales	5% (2/37)	3% (1/37)	1.0000
Crenotrichaceae	3% (1/37)	0% (0/37)	1.0000
Flexibacteriaceae	0% (0/37)	3% (1/37)	1.0000
Sphingobacteriaceae	3% (1/37)	0% (0/37)	1.0000
Other class	27% (10/37)	95% (35/37)	**<0.0001**
Other order	27% (10/37)	95% (35/37)	**<0.0001**
Other family	27% (10/37)	95% (35/37)	**<0.0001**
Bacteria.Chlamydiae	0% (0/37)	51% (19/37)	**<0.0001**
Chlamydiae	0% (0/37)	51% (19/37)	**<0.0001**
Chlamydiales	0% (0%)	51% (19/37)	**<0.0001**
Chlamydiaceae	0% (0%)	51% (19/37)	**<0.0001**
Parachlamydiaceae	0% (0%)	3% (1/37)	1.0000
Bacteria.Chloroflexi	0% (0/37)	22% (8/37)	0.1463
Anaerolineae	0% (0/37)	22% (8/37)	0.1173
Caldilineae	0% (0/37)	3% (1/37)	1.0000
Caldilineales	0% (0/37)	3% (1/37)	1.0000
Other	0% (0/37)	19% (7/37)	0.7223
Other family	0% (0/37)	19% (7/37)	1.0000
Bacteria.Cyanobacteria	0% (0/37)	3% (1/37)	0.9999
Cyanobacteria	0% (0/37)	3% (1/37)	1.0000
Other order	0% (0/37)	3% (1/37)	1.0000
Other family	0% (0/37)	3% (1/37)	1.0000
Bacteria.Deferribacteres	3% (1/37)	5% (2/37)	0.9999
Deferribacteres	3% (1/37)	5% (2/37)	1.0000
Deferribacterales	3% (1/37)	5% (2/37)	1.0000
Deferribacteraceae	0% (0%)	0% (0%)	NP
Incertae sedis 3	3% (1/37)	5% (2/37)	1.0000
Bacteria.Fibrobacteres	0% (0/37)	14% (5/37)	0.5152
Fibrobacteres	0% (0/37)	14% (5/37)	0.4698
Fibrobacterales	0% (0/37)	14% (5/37)	0.9515
Fibrobacteraceae	0% (0/37)	14% (5/37)	1.0000
Bacteria.Firmicutes	100% (37/37)	100% (37/37)	NP
Bacilli	100% (37/37)	100% (37/37)	NP
Lactobacillales	100% (37/37)	100% (37/37)	NP
Aerococcaceae	27% (10/37)	14% (5/37)	1.0000
Carnobacteriaceae	14% (5/37)	3% (1/37)	1.0000

Table R-1 continued

Enterococcaceae	95% (35/37)	41% (15/37)	**0.0078**
Lactobacillaceae	27% (10/37)	70% (26/37)	0.2944
Leuconostocaceae	8% (3/37)	0% (0/37)	1.0000
Streptococcaceae	97% (36/37)	97%(36/37)	1.0000
Other	78% (29/37)	22% (8/37)	**<0.0001**
Bacillales	46% (17/37)	70% (26/37)	1.0000
Paenibacillaceae	3% (1/37)	5% (2/37)	1.0000
Staphylococcaceae	32% (12/37)	62% (23/37)	1.0000
Bacillaceae	14% (5/37)	14% (5/37)	1.0000
Incertae Sedis XI	11% (4/37)	3% (1/37)	1.0000
Planococcaceae	3% (1/37)	11% (4/37)	1.0000
Other	0% (0/37)	8% (3/37)	1.0000
Other order	30% (11/37)	11% (4/37)	1.0000
Other family	30% (11/37)	11% (4/37)	1.0000
Clostridia	100% (37/37)	100% (37/37)	NP
Clostridiales	100% (37/37)	100% (37/37)	NP
Eubacteriaceae	5% (2/37)	35% (13/37)	0.6076
Lachnospiraceae	89% (33/37)	100% (37/37)	1.0000
Peptostreptococcaceae		43% (16/37)	**0.0150**
Ruminococcaceae	70% (26/37)	100% (37/37)	0.1716
Clostridiaceae	100% (37/37)	78% (29/37)	0.7980
Incertae Sedis XI	11% (4/37)	78% (29/37)	**<0.0001**
Incertae Sedis XIII	5% (2/37)	57% (21/37)	**0.0154**
Peptococcaceae	0% (0/37)	32% (12/37)	0.1065
Veillonellaceae	16% (6/37)	95% (35/37)	**<0.0001**
Other	100% (37/37)	97% (36/37)	1.0000
Other order	32% (12/37)	59% (22/37)	0.8136
Other family	32% (12/37)	59% (22/37)	1.0000
Erysipelotrichi	62% (23/37)	70% (26/37)	1.0000
Erysipelotrichales	62% (23/37)	70% (26/37)	1.0000
Erysipelotrichiaceae	62% (23/37)	70% (26/37)	1.0000
Other class	49% (18/37)	86% (32/37)	**0.0242**
Other order	49% (18/37)	86% (32/37)	0.0594
Other family	49% (18/37)	86% (32/37)	1.0000
Bacteria.Fusobacteria	62% (23/37)	84% (31/37)	0.5152
Fusobacteria	62% (23/37)	84% (31/37)	1.0000
Fusobacteriales	62% (23/37)	84% (31/37)	1.0000
Fusobacteriaceae	62% (23/37)	81% (30/37)	1.0000
Incertae sedis 11	5% (2/37)	0% (0/37)	1.0000
Other	32% (12/37)	43% (16/37)	1.0000

Table R-1 continued

Bacteria.Lentisphaerae	0% (0/37)	0% (0/37)	NP
Lentisphaerae	0% (0/37)	0% (0/37)	NP
Victivallales	0% (0/37)	0% (0/37)	NP
Victivallaceae	0% (0/37)	0% (0/37)	NP
Bacteria.Other	86% (32/37)	100% (37/37)	0.5152
Bacteria. Other Class	86% (32/37)	100% (37/37)	1.0000
Other Order	86% (32/37)	100% (37/37)	1.0000
Other family	86% (32/37)	100% (37/37)	1.0000
Bacteria.Planctomycetes	3% (1/37)	43% (16/37)	**0.0045**
Planctomycetacia	3% (1/37%)	43% (16/37)	**0.0377**
Planctomycetales	3% (1/37)	43% (16/37)	**0.0120**
Planctomycetaceae	3% (1/37)	43% (16/37)	**0.0003**
Bacteria.Proteobacteria	100% (37/37)	97% (36/37)	0.9999
Alphaproteobacteria	11% (4/37)	14% (5/37)	1.0000
Caulobacterales	3% (1/37)	0% (0/37)	1.0000
Caulobacteriaceae	3% (1/37)	0% (0/37)	1.0000
Rhizobiales	5% (2/37)	8% (3/37)	1.0000
Hyphomicrobiaceae	0% (0/37)	3% (1/37)	1.0000
Methylobacteriaceae	3% (1/37)	3% (1/37)	1.0000
Other	3% (1/37)	3% (1/37)	1.0000
Rhodobacteriales	5% (2/37)	3% (1/37)	1.0000
Rhodobacteriaceae	5% (2/37)	3% (1/37)	1.0000
Rhodospirales	0% (0%)	0% (0%)	NP
Other	0% (0%)	0% (0%)	NP
Other order	0% (0%)	3% (1/37)	1.0000
Other family	0% (0%)	3% (1/37)	1.0000
Betaproteobacteria	11% (4/37)	27% (10/37)	1.0000
Burkholderiales	8% (3/37)	24% (9/37%)	1.0000
Alcaligenaceae	3% (1/37)	3% (1/37)	1.0000
Comamonadacea	3% (1/37)	8% (3/37)	1.0000
Other	3% (1/37)	14% (5/37)	1.0000
Other order	5% (2/37)	3% (1/37)	1.0000
Other family	5% (2/37)	3% (1/37)	1.0000
Deltaproteobacteria	5% (2/37)	49% (18/37)	**0.0104**
Desulfovibrionales	5% (2/37)	46% (17/37)	**0.0377**
Desulfovibrionaceae	5% (2/37)	43% (16/37)	0.1496
Other	3% (1/37)	14% (5/37)	1.0000
Myxococcales	0% (0/37)	3% (1/37)	1.0000
Nannocystineae	0% (0/37)	3% (1/37)	1.0000
Other	0% (0/37)	0% (0/37)	NP

Table R-1 continued

Epsilonproteobacteria	3% (1/37)	73% (27/37)	**<0.0001**
Campylobacterales	3% (1/37)	73% (27/37)	**<0.0001**
Campylobacteriaceae	3% (1/37)	65% (24/37)	**<0.0001**
Helicobacteraceae	0% (0/37)	43% (16/37)	**0.0154**
Gamma proteobacteria	97% (36/37)	89% (33/37)	1.0000
Aeromonadales	22% (8/37)	41% (15/37)	1.0000
Aeromonadaceae	22% (8/37)	0% (0/37)	0.7980
Succinivibrionaceae	0% (0/37)	41% (15/37)	**0.0219**
Enterobacteriales	95% (35/37)	62% (23/37)	0.0858
Enterobacteriaceae	95% (35/37)	62% (23/37)	0.2145
Legionellales	0% (0/37)	5% (2/37)	1.0000
Coxiellaceae	0% (0/37)	3% (1/37)	1.0000
Legionellaceae	0% (0/37)	3% (1/37)	1.0000
Oceanospirillales	3% (1/37)	0% (0/37)	1.0000
Halomonadaceae	3% (1/37)	0% (0/37)	1.0000
Pasteurellales	32% (12/37)	38% (14/37)	1.0000
Pasteurellaceae	32% (12/37)	38% (14/37)	1.0000
Pseudomonadales	14% (5/37)	11% (4/37)	1.0000
Moraxellaceae	14% (5/37)	11% (4/37)	1.0000
Pseudomonadaceae	5% (2/37)	3% (1/37)	1.0000
Xanthomonadales	0% (0/37)	3% (1/37)	1.0000
Xanthomonadaceae	0% (0/37)	3% (1/37)	1.0000
Other order	16% (6/37)	0% (0/37)	0.9064
Other family	16% (6/37)	0% (0/37)	1.0000
Other class	5% (2/37)	43% (16/37)	**0.0242**
Other order	5% (2/37)	43% (16/37)	0.0594
Other family	5% (2/37)	43% (16/37)	0.1496
Bacteria.Spirochaetes	0% (0/37)	30% (11/37)	**0.0312**
Spirochaetes	0% (0/37)	30% (11/37)	**0.0439**
Spirochaetales	0% (0/37)	30% (11/37)	0.0910
Spirochaetaceae	0% (0/37)	30% (11/37)	0.1716
Other	0% (0/37)	3% (1/37)	1.0000
Bacteria.TM7	0% (0/37)	35% (13/37)	**0.0117**
TM7 genera incertae sedis	0% (0/37)	35% (13/37)	**0.0218**
Other order	0% (0/37)	35% (13/37)	**0.0351**
Other family	0% (0/37)	35% (13/37)	0.0648
Bacteria.Tenericutes	0% (0/37)	3% (1/37)	1.0000
Mollicutes	0% (0/37)	3% (1/37)	1.0000
Anaeroplasmatales	0% (0/37)	3% (1/37)	1.0000
Anaeroplasmataceae	0% (0/37)	3% (1/37)	1.0000

Table R-1 continued

Bacteria.Verrucomicrobia	24% (9/37)	89% (33/37)	**<0.0001**
Verrucomicrobiae	24% (9/37)	89% (33/37)	**<0.0001**
Verrucomicrobiales	24% (9/37)	89% (33/37)	**<0.0001**
Other	0% (0%)	5% (2/37)	1.0000
Subdivision 5	8% (3/37)	76% (28/37)	**<0.0001**
Verrucomicrobiaceae	16% (6/37)	78% (29/37)	**<0.0001**
Xiphinematobacteriaceae	0% (0%)	3% (1/37)	1.0000
Other Kingdom, Other phylum	19% (7/37)	22% (8/37)	0.9999
Other class	19% (7/37)	22% (8/37)	1.0000
Other order	19% (7/37)	22% (8/37)	1.0000
Other family	19% (7/37)	22% (8/37)	1.0000

Fecal swab samples collected from 37 Quarter Horse foals on days 2 and 30 of life
*P values represent the results of McNemar's test for paired dichotomous data, adjusted by the method of Hochberg
NP = Not Performed

APPENDIX S

The following paper was initially prepared to be sent publication as one single paper, but the main results were added to Chapter II, therefore, this paper was added as an appendix to all results and discussion can be used as future reference.

Selection of electron-beam irradiation dose for inactivation of *Rhodococcus equi*

Overview

Rhodococcus equi is a bacterium commonly isolated from soil that primarily causes pneumonia in foals. The objectives of our study were to evaluate electron-beam (e-beam) irradiation as a method of inactivation of *R. equi* and to establish an e-beam dose that inactivated all microorganisms without disrupting cellular integrity. Two concentrations of *R. equi* (Concentration 1, approximately 1×10^8 colony-forming units/ml [CFU/ml] and concentration 2, approximately 1×10^9 CFU/ml) were irradiated with e-beam doses ranging from 0 to 7 kGy and quantitatively cultured for calculation of the D_{10}-value. Live and heat-inactivated *R. equi* were used as positive and negative controls, respectively. Samples irradiated with selected doses that achieved complete inactivation were evaluated by transmission electron microscopy (TEM) and fluorescence-based viability at 12 hours and 1, 2, and 4 weeks after irradiation. All microorganisms for concentrations 1 and 2 were adequately inactivated at 4 kGy and 5

kGy, respectively, and the D_{10}-value of *R. equi* was estimated to be 0.505 kGy. Ultrastructurally, the overall integrity of the outer bacterial cell wall of all treated groups was preserved. Changes affecting the wall were confined to the layered cell wall of all groups, and were more severe among bacteria of heat-inactivated groups suggesting a more severe compromise of the cell wall integrity. Fluorescence data were similar for concentrations 1 and 2, as well as for live bacteria, indicating an intact the cell wall, whereas fluorescence data for heat-inactivated samples indicated a compromised cell wall. Results of our study suggest that e-beam radiation completely inactivated *R. equi* samples, while retaining their structural integrity. Electron-beam irradiation is an appropriate method of inactivation of *R. equi* for in vitro studies of immune responses to inactivated bacteria and possibly for use as a vaccine in foals against *R. equi* pneumonia.

Keywords: bacteria, ionizing radiation, microbiology, vaccine, pathogen

Introduction

Rhodococcus equi is a facultative intracellular pathogen recognized clinically as a leading cause of severe pneumonia in foals [S-1- S-4]. Although a variety of strategies have been evaluated for vaccination against *R. equi*, including immunization of mares [S-5- S-9] inactivated *R. equi* administered parenterally to foals or mice [S-7, S-10], sub-unit vaccines [S-8, S-9, S-11], DNA vaccines [S-12, S-13], and live, mutant vaccines [S-14, S-15], oral administration of live, virulent *R. equi* is the only vaccination strategy

that has been demonstrated repeatedly to be effective for protecting foals against experimental intrabronchial challenge with virulent *R. equi* [S-16- S-18]. The administration of live, virulent organisms is not considered an acceptable strategy for vaccination of foals. To date, an efficacious vaccine against *R. equi* for foals is lacking and there is no approved vaccine for foals against *R. equi* in North America.

Inactivated bacteria and viruses can elicit protective immune responses against systemic infections, including those of the respiratory tract [S-19- S-22]. Electron-beam (e-beam) irradiation is a technology for microbial inactivation that is currently used for sterilization and pasteurization [S-23- S-26]. Electron-beam irradiation at appropriate doses can be used to inactivate large volumes of microbial cultures or to sterilize materials [S-27- S-29]. Electron-beam inactivation has advantages relative to inactivating bacteria using heat or formalin. Inactivation with either heat or formalin is known to denature proteins, including immunogenic epitopes on the cell surface [S-30, S-31]. More importantly, formalin is widely recognized as resulting in incomplete inactivation of organisms, and has been associated with vaccine-associated disease resulting from inadequate inactivation [S-32]. Thus, there is need for a reliable method of microbial inactivation that will retain the bacterial cell structure as similar as possible to a live organism for use in producing vaccines. We therefore investigated the use e-beam irradiation as a method for microbial inactivation for potential use for making vaccines. Our objective was 2-fold: 1) to select a dose of e-beam radiation that would render *R. equi* completely incapable of replication for 2 different concentrations of this bacterium; and, 2) to study the effects of the minimal dose of irradiation that provided

complete inactivation on the integrity of the *R. equi* bacterial cell wall in both freshly irradiated and refrigerated samples by using transmission electron microscopy (TEM) and a 2-color fluorescence assay, using live (positive control), heat-inactivated (negative control), and electron-beam irradiated *R. equi* at different time-points after exposure to e-beam radiation.

Material and methods

Preparation of bacteria and electron beam irradiation

Blood agar plates were inoculated with *R. equi* strain EIDL 5-331, and incubated at 37^{o}C for 48 hours. One colony from this pure culture was selected and used to inoculate 50 ml of brain-heart infusion (BHI) broth. The flask with inoculated broth was placed on a shaker for 24 h at 37^{o}C. Ten ml of this pure culture were used to inoculate 1000 ml of BHI broth. The flask with inoculated broth was placed on a shaker for 24 h at 37^{o}C. The bacterial suspension was divided into 5 aliquots of 200 ml, centrifuged at 3400 × g (Eppendorf AG, Hamburg, Germany) for 20 min at 4^{o}C, the supernatant discarded, and the pellets resuspended with 100 ml of phosphate-buffered saline (PBS). The resuspended pellet was centrifuged again using the same centrifugation protocol. The supernatant was discarded, and the bacteria were resuspended in sterile 0.9% NaCl solution. The initial concentration of bacteria was determined spectrophotometrically (Genesys™ 20, Thermo Scientific, Waltham, MA, USA). Two approximate concentrations were used, Concentration 1, approximately 1×10^{8} colony-forming units

(CFU), and Concentration 2, approximately 1×10^9 CFU. Twenty-five ml of each concentration were dispensed in a 3- x 7-inch Whirl Pak bag (Uline, Pleasant Prairie, WI, USA), the bag tightly heat-sealed with no headspace, placed inside another Whirl Pak bag, and heat-sealed for a second time. The double-bagged bacteria were then placed inside a 95-kPa transport bag (Therapak, Duarte, CA, USA) and sealed. A total of 8 transport bags were placed inside a transport box (Saf-T-Pak, Glen Burnie, MD, USA) and transported to the National Center for Electron Beam Research at Texas A&M University for irradiation. Although high doses of e-beam radiation can completely inactivate all microorganisms, our objective was to determine the lowest dose able to achieve complete inactivation with least amount of damage to bacteria. Each bag was exposed to an irradiation dose ranging from 0 to 7 kGy (in integer-unit doses) using a 10-MeV, 18-kW linear accelerator. Alanine dosimeters were used to verify the delivered e-beam dose. Twenty-five ml of non-irradiated heat-inactivated bacteria were inactivated for 30 min in a water bath at 85^{o}C, and were used as the negative control. After irradiation, quantitative culture was performed to determine concentration of each irradiated sample, and to calculate the D_{10}-value. Experiments were conducted in triplicates, performed on 3 different days. The D_{10}-value was calculated from the negative inverse of the slope from the linear regression between the dose and the logarithm of the microbial population [S-33].

Cell wall integrity of irradiated R. equi

Bacteria were cultured as described above, and the minimum dose that effectively inactivated all microorganisms for both concentrations was selected based on the results of quantitative culture. Eight bags of each concentration were irradiated with the respective selected doses, and both live and heat-inactivated *R. equi* were prepared as positive and negative controls. Samples were kept at 4^{o}C for 12 h, and 1, 2, and 4 weeks after either irradiation or heat-inactivation. Two methods were used to determine whether the bacterial cell wall was intact. The first method was a fluorescence-based assay (LIVE/DEAD® BacLight™ bacterial viability kit, Molecular Probes, Inc., Eugene, OR, USA), which utilizes a mixture of SYTO® 9 green-fluorescent nucleic acid stain and propidium iodide. The SYTO® 9 generally stains all bacteria in a population, regardless of the integrity of the cell membrane. In contrast, propidium iodide penetrates only bacteria with damaged membranes, resulting in a reduction in the SYTO® 9 stain fluorescence when both dyes are present [S-34]. Preparations of live, heat-inactivated, and e-beam irradiated *R. equi* were evaluated at 12 h, and 1, 2, and 4 weeks after processing according to manufacturer's instructions using a microplate reader (Synergy 2, Biotek, Winooski, VT, USA). We expected to observe that irradiation did not increase propidium iodide staining, indicating that the cell walls of the bacteria remained intact, but replication-incompetent.

In the second experiment, the integrity of the bacterial cell wall of irradiated samples, heat-inactivated, and live *R. equi* was evaluated using TEM within 12 h, and 1, 2, or 4 weeks after processing. Bacterial cells were fixed in 2% glutaraldehyde, 3%

formaldehyde in 0.1 M sodium cacodylate buffer, then post-fixed with 1% osmium tetroxide and 0.5% potassium ferrocyanide, dehydrated in an ascending alcohol series, and embedded in epoxy resin. Ultrathin sections of the cells were examined with an FEI Morgagni 268 transmission electron microscope at an accelerating voltage of 80 kV. Digital images were acquired with a MegaView III camera operated with iTEM software (Olympus Soft Imaging Systems, Germany), then post-processed with Adobe Photoshop. We expected to see that e-beam radiation did not alter cell membranes and that only the nucleoid region would appear different between irradiated and live bacteria.

Data analysis

Data from the quantitative culture (and D_{10}-value calculation) were analyzed using a linear mixed-effects model fit by REML. Analyses were conducted using S-PLUS (Version 8.0; Insightful, Inc.) and R (Version 2.12.1; R Statistical Project). The cell wall integrity data (LIVE/DEAD® BacLight™ bacterial viability kit and TEM) were descriptive only.

Results

Inactivation of R. equi by electron-beam irradiation

Samples from both concentrations of *R. equi* were e-beam irradiated with a dose ranging from 0 to 7 kGy (Fig. S-1A and B). After e-beam irradiation, quantitative culture was performed on all samples (Table S-1, Fig. S-2A and B). For concentration 1, e-beam

irradiation with 4 kGy inactivated all microorganisms (Fig. S-2A), and for concentration 2, the dose needed to inactivate all microorganisms was 5 kGy (Fig. S-2B). Therefore, the doses selected for the membrane integrity studies for concentrations 1 and 2 were, respectively, 4 and 5 kGy.

The D_{10}-value was calculated from the negative inverse of the slope from the linear mixed-effects regression of the irradiation dose on the logarithm of the microbial population [33] The D_{10}-values estimated for *Rhodococcus equi* strain 5-331 in 0.9% NaCl exposed to 10-MeV, 18-kW e-beam irradiation for the 2 concentrations were similar (approximately 0.505 kGy; Table S-2) and did not differ significantly ($P>0.05$).

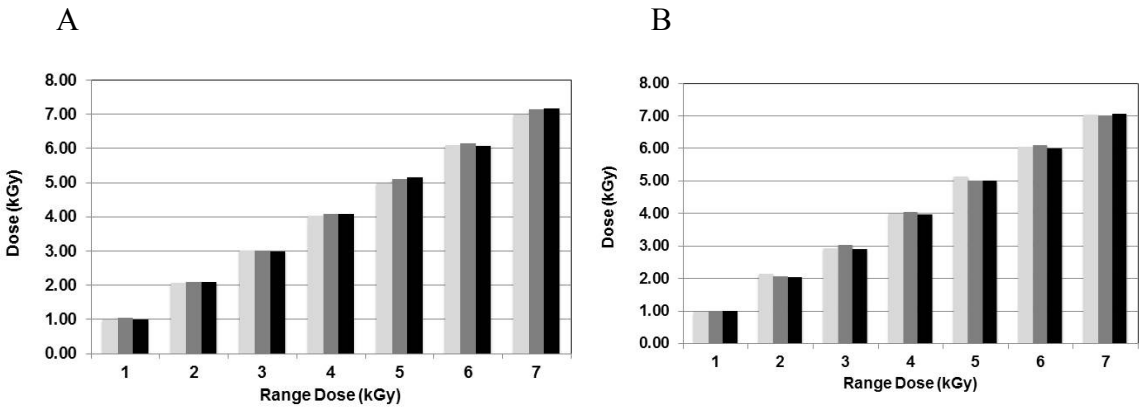


Fig. S-1. Dose-range of electron-beam irradiated *Rhodococcus equi* concentration 1 (A) and 2 (B). Each column for each dose-range represents the actual dose used in each of 3 experiments (1 experiment = 1 study-day). (Light gray: experiment 1, Dark gray: experiment 2, Black: experiment 3).

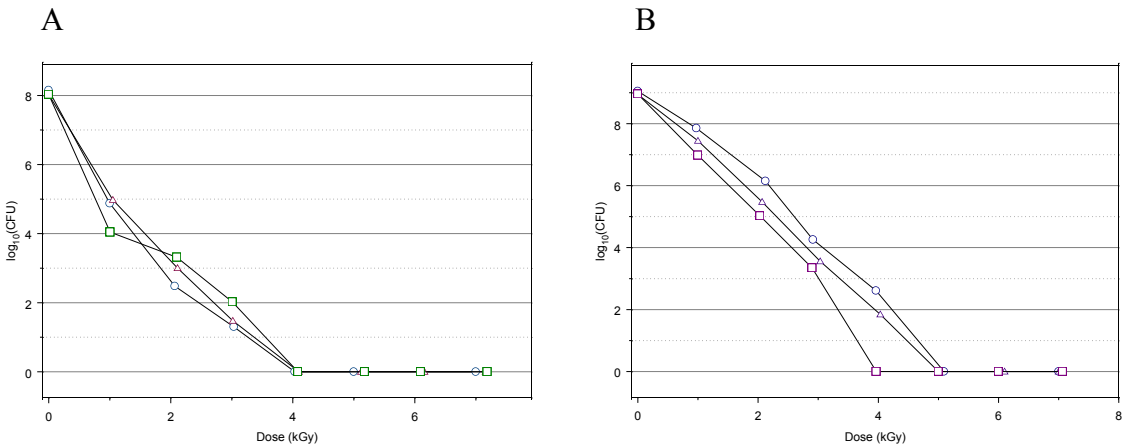


Fig. S-2. Survival curves for *R. equi* samples resuspended in 0.9% NaCl treated with 0 to 7 kGy of e-beam irradiation. Each individual curve represents 1 experiment performed on separate days. Panel A) Concentration 1; Panel B) Concentration 2. Circle: Experiment 1, Triangle: Experiment 2, Square: Experiment 3

Table S-1
Colony-forming units per ml (CFU/ml) of 2 concentrations of *Rhodococcus equi* culture after inactivation with different doses (kGy) of e-beam irradiation on 3 different days

e-beam irradiation dose (kGy)	Experiments concentration 1 (CFU/ml)			Experiments concentration 2 (CFU/ml)		
	1	2	3	1	2	3
0	1.43×10^8	1.06×10^8	1.07×10^8	1.13×10^9	8.70×10^8	8.90×10^8
1	7.50×10^4	5.60×10^4	1.30×10^4	7.10×10^7	2.80×10^7	9.50×10^6
2	3.00×10^2	1.01×10^3	2.05×10^3	1.40×10^6	3.00×10^5	6.60×10^4
3	2.00×10^1	3.00×10^1	1.05×10^2	1.77×10^4	3.60×10^3	1.20×10^3
4	0	0	0	4.05×10^2	7.00×10^1	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0

Table S-2
Mean (95% confidence intervals) D_{10}-values of concentrations 1 and 2 of *Rhodococcus equi* culture after inactivation with e-beam radiation.

Concentration of *R. equi (CFU/ml)*	D_{10}-value (kGy) for *R. equi* in 0.9% NaCl*
1 (approximately 1×10^8)	0.48 (0.37 to 0.69)
2 (approximately 1×10^9)	0.53 (0.47 to 0.61)

Cell wall integrity of R. equi following electron-beam irradiation

The cell integrity was evaluated by 2 methods in the first 24 hours, 1, 2 and 4 weeks after e-beam irradiation. The first method used was the fluorescence-based assay LIVE/DEAD® BacLight™ bacterial viability kit. In all samples, the green/red fluorescence ratio increased with the addition of intact bacteria in all groups with the exception of the heat-inactivated bacteria (Fig. S-3A, B, C and D), indicating that heat-inactivated bacteria had damaged membranes.

The second method of cell integrity evaluation was TEM. Ultrastructurally, the overall integrity of the outer bacterial cell wall of all treated groups was preserved (Fig. S-4A, B, C, and D). Changes affecting the wall were confined to the layered cell wall (LCW) of all groups, and were more severe among bacteria of heat-inactivated groups suggesting a more severe compromise of the cell wall integrity (consistent with the fluorescent-based cell wall integrity assay). The internal cell contents were only morphologically affected in bacteria of the heat-inactivated group. Noticeable changes

included enlarged nuclear areas that were admixed with a filamentous material and inconspicuous glycogen-like deposits (Fig. S-4B).

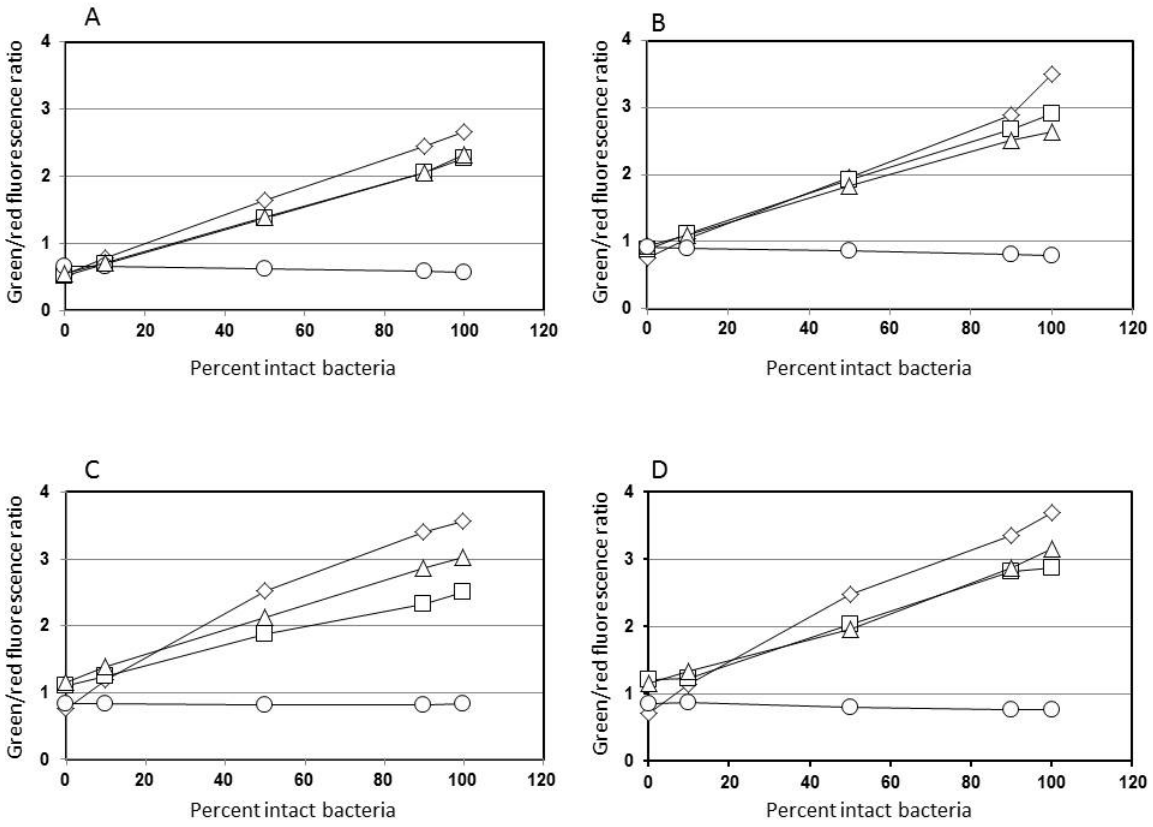


Fig. S-3. Ratio of green/red fluorescence using the fluorescence-based LIVE/DEAD BacLight bacterial viability kit for Concentration 1 (approximately 1×10^8 colony-forming CFU/ml; square) and Concentration 2 (approximately 1×10^9 CFU/ml; triangle) e-beam irradiated, live (diamond shape) and heat-inactivated samples (circle). A) Day 1, B) Week 1, C) Week 2, and D) Week 4 of storage at 4^oC.

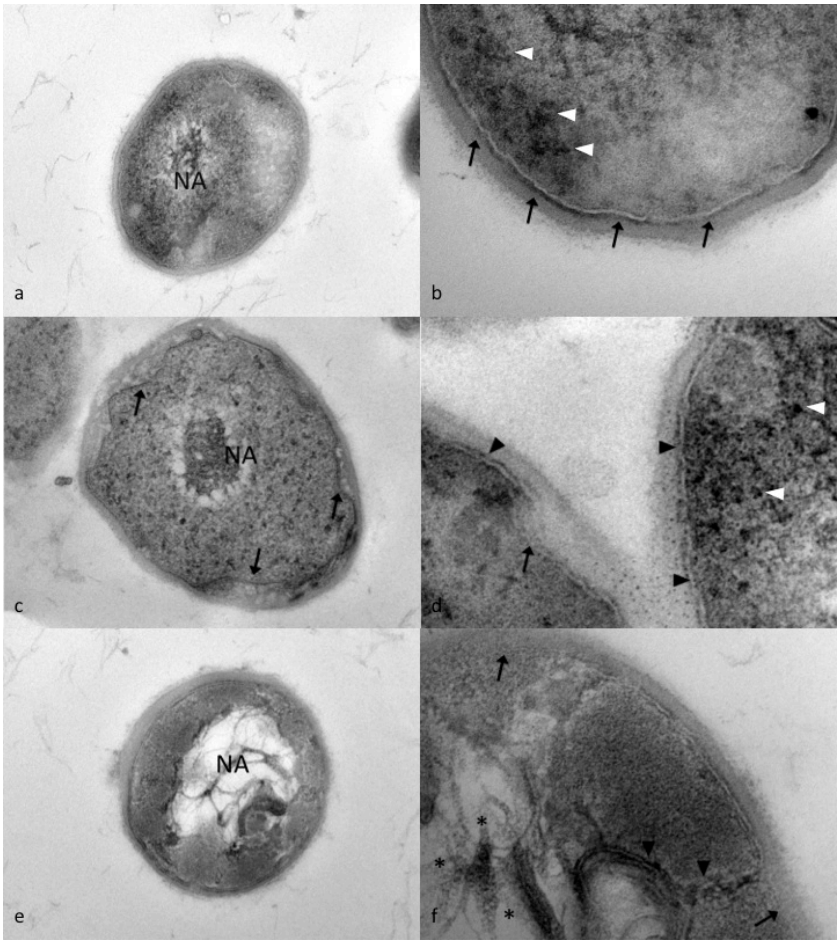


Fig. S-4. Ultrastructure of live, heat-inactivated, and e-beam irradiated *R. equi.* a) Live group, week 4. Normal morphologic appearance of the nuclear area (NA). b) Live group, day 1. Closer magnification of a live bacterium depicting the localization of the layered cell wall (black arrows) and of glycogen-like material (white arrowheads). c) Concentration 2 e-beam irradiated, week 4. Similar morphologic appearance of the NA compared to the live bacterium of image "a". The arrows are indicating invaginations of the layered cell wall. d) Concentration 1 e-beam irradiated, day 1. Closer view of radiated bacteria demonstrating intact layered cell walls (black arrowheads), invaginations of the layered wall (arrow), and preservation of glycogen-like material (white arrowheads). e) Heat-inactivated group, day 1. Nuclear area (NA) markedly vacuolated and has increased electron lucency. f) Heat-inactivated, day 1. Closer magnification of a heat-killed bacterium that presents large areas where the layered cell wall is either not present (arrows) or presents marked invagination/coiling (arrowheads). Note the vacuolated nuclear area (*), and inconspicuous glycogen-like material.

Discussion

The objectives of this study were 2-fold: 1) to select a minimal dose of e-beam irradiation that would prevent replication of any *R. equi* present in 2 different concentrations of *R. equi*; and, 2) to study the effects of irradiation of the lowest doses that rendered *R. equi* replication-incompetent at each concentration on the integrity of the *R. equi* bacterial cell wall in both freshly irradiated and refrigerated samples.

For the initial studies of selection of the dose to completely inhibit replication, a series of irradiation doses that ranged from 0 to 7 kGy, in 1-kGy increments, were used. The doses, as expected, did not vary among the triplicates, as portrayed in Fig. S-1A and 1B. Bacterial survival was inversely related to e-beam irradiation dose (Fig. S-2A and 2B), as previously reported for other microorganisms including Escherichia coli K-12 [S-35], Bacillus atrophaeus [S-36], and avian influenza virus [S-29]. Replication of *R. equi* at both concentrations 1 and 2 was completely inhibited when irradiated with 4 and 5 kGy, respectively (Table S-1, and Fig. S-2A and B). This difference in doses between concentrations was likely attributable to concentration 2 having more bacteria than concentration 1, requiring a higher irradiation dose to achieve complete inhibition of replication. Because we only evaluated integer doses of irradiation, it is possible that inactivation of replication could have been achieved with doses between 3 and 4 kGy and 4 and 5 kGy for concentrations 1 and 2, respectively; from the standpoint of safety, we considered erring on the side of a slightly higher dose to be desirable. Depending on the bacterial growth phase, a higher dose might be needed for complete inhibition of

bacterial replication because bacterial cells in logarithmic growth phase can have multiple copies of their genomes per cell [S-37]. It is also worth considering that bacterial cells were irradiated freshly after overnight culture, and their concentration was estimated based on their optical density (OD) value. Therefore, the estimated concentration of *R. equi* form the OD might be slightly different than their actual concentration determined after 48 hours of culture because of the effects on the OD of the presence of cells that died or were otherwise damaged and not replicating. Nevertheless, because of evidence that the more bacteria in the culture the higher the dose needed for complete inhibition of bacterial replication, we chose to err on the side of a higher dose for inactivating *R. equi*.

We estimate the D_{10}-value for *R. equi* to be 0.505 kGy when resuspended in 0.9% NaCl. The very small difference in D_{10}-values observed for the 2 *R. equi* concentrations studied (Table S-2) was not statistically significant and likely attributable to random variation in the sample and processing of material, such as different starting concentration of bacteria, the amount of headspace with air in the irradiation bag, placement of the sample in the e-beam, etc. The initial CFU counts for the 3 replicates performed on separate days varied (Table S-1), and this variation was attributed to random day-to-day variation. In *E. coli* and *Salmonella typhimurium* LT2 inoculated in fresh produce, the oxygen amount in the headspace, among other factors, affected the D_{10}-value [S-38]. Thus, the small difference in observed D_{10} values could have been caused by such random variations as small differences in starting concentrations of bacteria or air in the headspace. The media in which bacteria are irradiated can

influence their sensitivity to irradiation [S-36, S-38]. Physiologic saline (i.e., NaCl 0.9%) is commonly used as a diluent in medications and vaccines because of its isotonic characteristics. Physiological saline was used as the diluent medium in our experiments because this preparation of inactivated *R. equi* could potentially be used as a vaccine for foals.

After the selection of a dose to completely inhibit replication, the LIVE/DEAD® BacLight™ bacterial viability kit was used to evaluate the integrity of the bacterial cell wall in irradiated and control samples. Fluorescence data from both dyes in each sample (irradiated, live, and heat-inactivated) were used to create a ratio of intact to damaged cells. We observed similar ratios for both concentrations of irradiated bacteria tested and live samples for all time-points (day 1, weeks 1, 2, and 4), whereas the heat-inactivated samples were considered damaged at all time-points. This finding was anticipated because e-beam irradiation was expected to damage the bacterial DNA [S-37], but not the cell wall per se. In Bacillus species spores, e-beam irradiation caused membrane disruption with cytoplasm leakage when high doses (i.e., ≥10.4 kGy) were used [S-39]. In our study, however, we did not evaluate bacterial structure in samples irradiated with > 5 kGy.

We used TEM to evaluate the ultrastructure of irradiated, heat-inactivated, and live *R. equi* samples. The ultrastructure of live *R. equi* cells evaluated with TEM has been described previously [S-40]. In our study, all control and irradiated samples preserved the overall structural integrity of the cell wall, meaning that changes in the cell wall indicative of destruction or perforation of the bacterial cell wall were not observed.

However, morphologic changes affecting ultrastructural components of the cell wall (e.g., the layered cell wall) and internal structures (e.g., nuclear area and glycogen-like deposits) were observed. Although variation in severity of changes involving the ultrastructural components of the cell wall was found between the irradiated and heat-inactivated groups, morphologic changes in internal structures only were detected in the heat-inactivated bacteria. Preservation of the cell wall integrity after e-beam irradiation has also been shown for *Paracoccidioides brasiliensis* [S-39], and membrane damage of *Bacillus* spp. spores was observed only at high doses of e-beam irradiation [S-39]. Although the evaluation of the ultrastructure in our study did not reveal changes in the cell wall indicative of perforation or destruction of the cell in any of the treatment groups, the fluorescence data from the LIVE/DEAD® BacLight™ bacterial viability kit, coupled with the presence of more severe ultrastructural changes affecting the layered cell wall of heat-inactivated bacteria, indicate that the cell wall of heat-inactivated bacteria were more severely compromised relative to the other treatment groups. For this study, we used a heat-inactivation protocol of 85oC for 30 min. Other heat-killing protocols using more prolonged exposure, higher temperatures, or both likely would have caused more pronounced changes in the *R. equi* ultrastructure. Nevertheless, we observed that e-beam inactivation resulted in better maintenance of normal membrane integrity and structure. These findings were considered important because structurally-intact but non-replicating bacteria might be good candidates for mucosal vaccines.

Conclusion

In this study we describe the e-beam irradiation doses required to completely inactivate 2 concentrations of *R. equi* without damage to their ultrastructure when irradiated using a 10-MeV, 18-kW linear accelerator. Electron-beam irradiation is an appropriate method for inactivating *R. equi* for in vitro studies of immune responses to inactivated bacteria as well as for potential use as a vaccine in foals against *R. equi* pneumonia. Studies evaluating the immunogenicity of e-beam inactivated *R. equi* administered in newborn foals are currently being performed by our laboratory.

References

[S-1] Prescott JF. *Rhodococcus equi*: an animal and human pathogen. Clin Microbiol Rev. 1991;4:20-34.

[S-2] Takai S. Epidemiology of *Rhodococcus equi* infections: a review. Vet Microbiol. 1997;56:167-76.

[S-3] Muscatello G, Leadon DP, Klayt M, Ocampo-Sosa A, Lewis DA, Fogarty U, et al. *Rhodococcus equi* infection in foals: the science of 'rattles'. Equine Vet J. 2007;39:470-8.

[S-4] Meijer WG, Prescott JF. *Rhodococcus equi*. Vet Res. 2004;35:383-96.

[S-5] Madigan JE, Hietala S, Muller N. Protection against naturally acquired *Rhodococcus equi* pneumonia in foals by administration of hyperimmune plasma. J Reprod Fertil Suppl. 1991;44:571-8.

[S-6] Martens RJ MJ, Fiske RA. Failure of passive immunisation by colostrum from immunised mares to protect foals against *Rhodococcus equi* pneumonia. Equine Vet J (Suppl) 1991;12:19-22.

[S-7] Varga J, Fodor L, Rusvai M, Soos I, Makrai L. Prevention of *Rhodococcus equi* pneumonia of foals using two different inactivated vaccines. Vet Microbiol. 1997;56:205-12.

[S-8] Becu T, Polledo G, Gaskin JM. Immunoprophylaxis of *Rhodococcus equi* pneumonia in foals. Vet Microbiol. 1997;56:193-204.

[S-9] Cauchard J, Sevin C, Ballet JJ, Taouji S. Foal IgG and opsonizing anti-*Rhodococcus equi* antibodies after immunization of pregnant mares with a protective VapA candidate vaccine. Vet Microbiol. 2004;104:73-81.

[S-10] Takai S, Kobayashi C, Murakami K, Sasaki Y, Tsubaki S. Live virulent *Rhodococcus equi*, rather than killed or avirulent, elicits protective immunity to *R. equi* infection in mice. FEMS Immunol Med Microbiol. 1999;24:1-9.

[S-11] Prescott JF, Nicholson VM, Patterson MC, Zandona Meleiro MC, Caterino de Araujo A, Yager JA, et al. Use of *Rhodococcus equi* virulence-associated protein for immunization of foals against *R. equi* pneumonia. Am J Vet Res. 1997;58:356-9.

[S-12] Lopez AM, Hines MT, Palmer GH, Knowles DP, Alperin DC, Hines SA. Analysis of anamnestic immune responses in adult horses and priming in neonates induced by a DNA vaccine expressing the vapA gene of *Rhodococcus equi*. Vaccine. 2003;21:3815-25.

[S-13] Mealey RH, Stone DM, Hines MT, Alperin DC, Littke MH, Leib SR, et al. Experimental *Rhodococcus equi* and equine infectious anemia virus DNA vaccination in adult and neonatal horses: effect of IL-12, dose, and route. Vaccine. 2007;25:7582-97.

[S-14] Lopez AM, Townsend HG, Allen AL, Hondalus MK. Safety and immunogenicity of a live-attenuated auxotrophic candidate vaccine against the intracellular pathogen *Rhodococcus equi*. Vaccine. 2008;26:998-1009.

[S-15] Pei Y, Nicholson V, Woods K, Prescott JF. Immunization by intrabronchial administration to 1-week-old foals of an unmarked double gene disruption strain of *Rhodococcus equi* strain 103+. Vet Microbiol. 2007;125:100-10.

[S-16] Chirino-Trejo JM, Prescott JF, Yager JA. Protection of foals against experimental *Rhodococcus equi* pneumonia by oral immunization. Can J Vet Res. 1987;51:444-7.

[S-17] Hooper-McGrevy KE, Wilkie BN, Prescott JF. Virulence-associated protein-specific serum immunoglobulin G-isotype expression in young foals protected against *Rhodococcus equi* pneumonia by oral immunization with virulent *R. equi*. Vaccine. 2005;23:5760-7.

[S-18] van der Geize R, Grommen AW, Hessels GI, Jacobs AA, Dijkhuizen L. The steroid catabolic pathway of the intracellular pathogen *Rhodococcus equi* is

important for pathogenesis and a target for vaccine development. PLoS Pathog. 2011;7:e1002181.

[S-19] Garrido JM, Sevilla IA, Beltran-Beck B, Minguijon E, Ballesteros C, Galindo RC, et al. Protection against tuberculosis in Eurasian wild boar vaccinated with heat-inactivated *Mycobacterium bovis*. PLoS One. 2011;6:e24905.

[S-20] Quan FS, Compans RW, Kang SM. Oral vaccination with inactivated influenza vaccine induces cross-protective immunity. Vaccine. 2012;30:180-8.

[S-21] Sealy R, Webby RJ, Crumpton JC, Hurwitz JL. Differential localization and function of antibody-forming cells responsive to inactivated or live-attenuated influenza virus vaccines. Int Immunol. 2012; 25:183-95.

[S-22] Paillot R, Prowse L, Montesso F, Huang CM, Barnes H, Escala J. Whole inactivated equine influenza vaccine: Efficacy against a representative clade 2 equine influenza virus, IFNgamma synthesis and duration of humoral immunity. Vet Microbiol. 2013;162:396-407.

[S-23] Dziedzic-Goclawska A, Kaminski A, Uhrynowska-Tyszkiewicz I, Stachowicz W. Irradiation as a safety procedure in tissue banking. Cell Tissue Bank. 2005;6:201-19.

[S-24] Poon PW, Dubeski P, Kitts DD. Effect of electron beam irradiation on microbial growth, lipid oxidation and color of ground beef patties upon refrigerated storage. Adv Exp Med Biol. 2004;542:101-11.

[S-25] Rivera CS, Blanco D, Marco P, Oria R, Venturini ME. Effects of electron-beam irradiation on the shelf life, microbial populations and sensory characteristics of

summer truffles (*Tuber aestivum*) packaged under modified atmospheres. Food Microbiol. 2011;28:141-8.

[S-26] Cabeza MC, Cambero MI, Nunez M, Medina M, de la Hoz L, Ordonez JA. Lack of growth of *Listeria monocytogenes* and *Staphylococcus aureus* in temperature abuse of E-beam treated ready-to-eat (RTE) cooked ham. Food Microbiol. 2010;27:777-82.

[S-27] Pillai SD, Braby LA. Eletronic Pasteurization. In: Tewari G, Juneja VK, editors. Advances in Thermal and Non-Thermal Food Preservation, Ames, IA: Blackwell Publishing; 2007. doi: 10.1002/9780470277898.ch11.

[S-28] Sommers C, Fan X. Food Irradiation Research and Technology. Ames, IA: Blackwell; 2006.

[S-29] Brahmakshatriya V, Lupiani B, Brinlee JL, Cepeda M, Pillai SD, Reddy SM. Preliminary study for evaluation of avian influenza virus inactivation in contaminated poultry products using electron beam irradiation. Avian Pathol: 2009;38:245-50.

[S-30] Khan FH. The Elements of Immunology. Delhi: Pearson Education; 2009.

[S-31] Werner M, Chott A, Fabiano A, Battifora H. Effect of formalin tissue fixation and processing on immunohistochemistry. Am J Surg Pathol. 2000;24:1016-9.

[S-32] Brown F. Review of accidents caused by incomplete inactivation of viruses. Dev Bio Stand. 1993;81:103-7.

[S-33] Rodriguez O, Castell-Perez ME, Ekpanyaskun N, Moreira RG, Castillo A. Surrogates for validation of electron beam irradiation of foods. Int J Food Microbiol. 2006;110:117-22.

[S-34] Probes M (2004). LIVE/DEAD BacLight bacterial viability kit. Life Technologies. Available from: http://www.lifetechnologies.com/order/catalog/product/L7012?CID=search-product.

[S-35] Grasso EM, Uribe-Rendon RM, Lee K. Inactivation *of Escherichia coli* inoculated onto fresh-cut chopped cabbage using electron-beam processing. J Food Protect. 2011;74:115-8.

[S-36] Helfinstine SL, Vargas-Aburto C, Uribe RM, Woolverton CJ. Inactivation of *Bacillus endospores* in envelopes by electron beam irradiation. Appl Environ Microbiol. 2005;71:7029-32.

[S-37] Pillai SD. Food Irradiation. Preharvest and Postharvest Food Safety: Blackwell Publishing; 2008. p. 375-88.

[S-38] Moreira RG, Puerta-Gomez AF, Kim J, Castell-Perez ME. Factors affecting radiation D-values (D_{10}) of an *Escherichia coli* cocktail and *Salmonella Typhimurium* LT2 inoculated in fresh produce. J Food Sci. 2012;77:E104-11.

[S-39] Demicheli MC, Goes AM, de Andrade AS. Ultrastructural changes in *Paracoccidioides brasiliensis* yeast cells attenuated by gamma irradiation. Mycoses. 2007;50:397-402.

[S-40] Garrison RG, Mirikitani FK, Lane JW. Fine structural studies of *Rhodococcus* species. Microbios. 1983;36:183-90.