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Brucellosis Vaccination: assessing the stability and safety of a Nisin inducible plasmid expressing *Brucella* antigen in *Lactococcos lactis* .

Summary:

Brucellosis is a worldwide bacterial disease that negatively affects the Wyoming bovine ranching industry. Current vaccines against the pathogen do not provide total immunity, and some interfere with diagnostic testing. We propose using live *Lactococcus lactis,* a probiotic bacteria, to express an adhesion protein found in wild type *Brucella abortus*. Experiments showing stability and safety in a plasmid-containing, live probiotic introduce the possibility of a viable vaccine. A stable food safe vector such as this, paves a road to quick, robust immunization. This project is aiming to gain confidence that this strain will have normal growth along with adequate antigen expression, and that antibiotic resistance present in the plasmid remains inside the probiotic.

Knowing how the vector responds to bovine rumen conditions is largely unexplored, and illumination will provide knowledge for the field of probiotic vaccines. Dangerous pathogens acquiring and expressing resistance acquired from the recombinant DNA is of great concern, and the extent of this problem is unknown. The rumen conditions may be unfavorable for the strain compared to the local residents, and various testing must show strain durability and plasmid accountability. Despite the challenges, we are expecting standard growth using this nisin-induced system. We aim to grow the vector-strain in conditions varying in pH, temperature, and microbial community, measuring if it has adequate survival capability. Rumen bacteria and pathogens grown alongside *L. lactis* will be reviewed for plasma expression, hopefully quantifying the rate of transfer. Testing these variables will give understanding of the pressures and limits affecting the vaccine-strain, and if successful, ultimately leading to novel innovation and broad application in vaccination methods. .

Statement of Problem and Significance:

B. abortus is a huge issue in commercial livestock production, that if efforts to eradicate this bacteria were halted, the cost of producing beef and milk would increase by $80 million annually (2). This issue is worsened by the fact that *B. abortus* can infect humans and is found in wild ruminants like elk and bison, but also infects the bovine population causing calves to be aborted or become chronically infected, acting as reservoirs for further herd infection . Even with current vaccination methods for cattle, which are an injection based live attenuated

strain, *B. abortus* is still an issue to livestock production . With these issues apparent, the scientific community is hard at work to find alternatives to vaccination of *B. abortus*. Probiotics are an avenue of investigation as a more widespread and efficient method of antigen delivery. If this work is successful, costs of livestock vaccination could significantly drop and human infections could be a thing of the past.

Introduction:

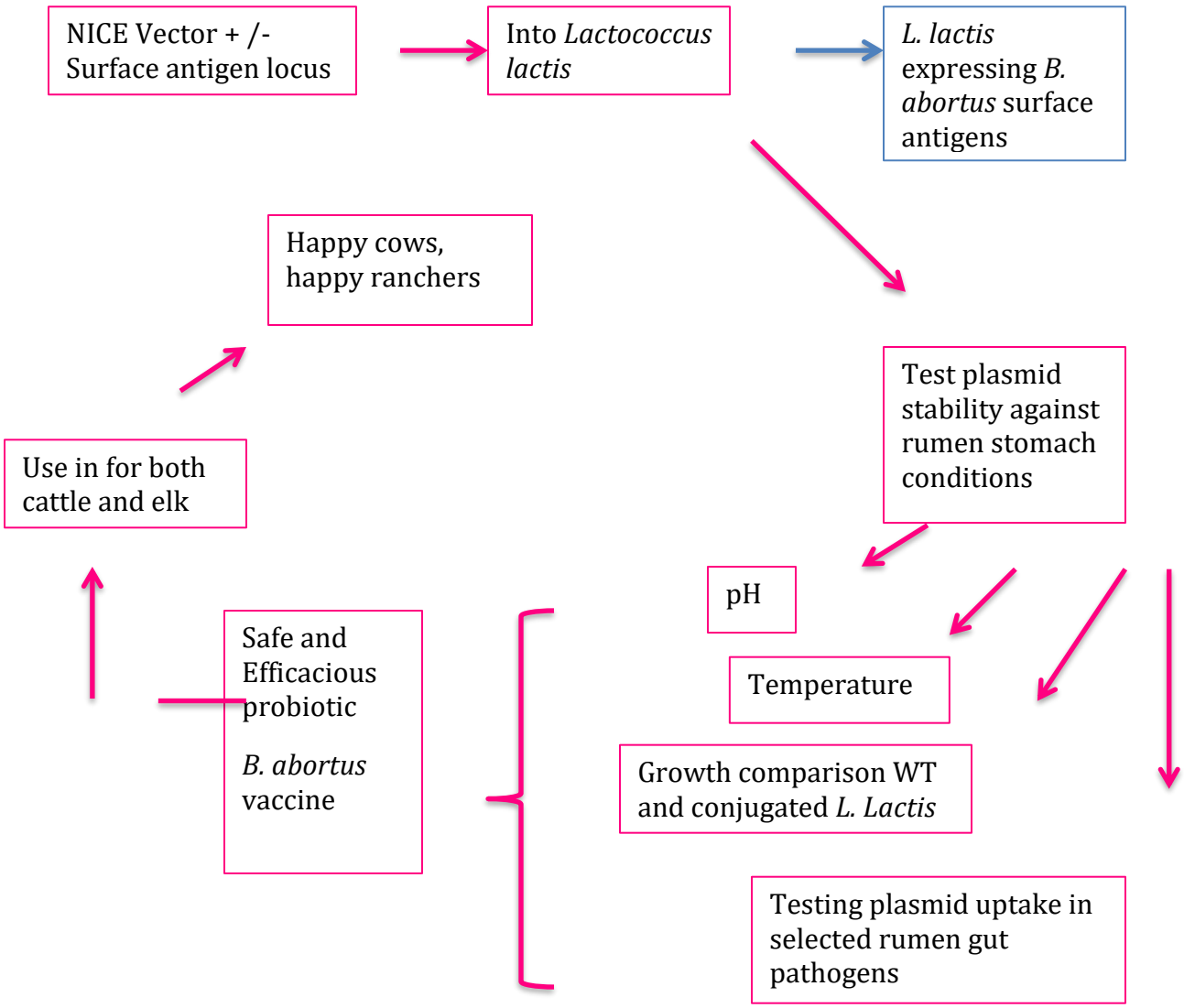
Relevant literature:

Brucellosis, caused by *Brucella abortus* results in spontaneous abortions in domestic animals, as well as wildlife (1). Two animals of importance for our study are domesticated cattle, and wild elk (3). With the prevalence of this pathogen in these species, and their interactions together, the cattle agricultural industry is under economic threat by the loss of fetal calves. Currently one vaccination is on the market for use, a live attenuated strain of *B. abortus* RB51(3). Although this vaccine is on the market for use in the agriculture industry it is only moderately efficacious as a cattle vaccine, and is not applicable to elk, serving as the wild reservoir for this pathogen(3). The elk infected with *B. abortus* develop a chronic infection, which spreads within their own population, and other ungulates that they come in contact with, damaging the populations (3).

Preliminary data:

Our preliminary data is based on the possible immune activating capability of a Type V protein named Hia in *Brucella* Strain RB51, an adhesin homolog of *Heamophilus*. Our group is using this gene inside a nicin induced expression system (NICE) present in the Strain *Lactococcus lactis*.

Conceptual Model:



Justification:

Current methods involve inefficient intravenous procedures, and unnecessary risk to veterinarians administering the vaccine. There have been steps within the research community to advance an alternative vaccine using a non-pathogenic microbe that expresses the *B. abortus* surface antigens. This probiotic vaccine would reside in the gut of the rumens allowing safe protection from *B. abortus.* The live vaccine in the form of *Lactococcos lactis* is a possible route to immunity without the risk of fetal abortion or human infection. From research we are able to gain knowledge of some conditions that will be faced, however there still lies many unknowns. Initially we will be seeking to better understand the growth curve of the transformed *L. lactis* in comparison to the wild type strain. In addition we are attempting to test the transformed *L. lactis* against rumen and gastrointestinal conditions.

Objectives:

• Test the viability of using Lactococcus lactis in the rumen as a vector for Brucellosis vacination.

• Monitor the environmental and generational stability of the expression plasmid in Lactococcus lactis

• Understand the effects the rumen will have on the nicin inducible expression system being used in Lactococcus lactis.

• Evaluate the possibilities of Gram-positive to Gram-negative gene transfer when using a plasmid containing antibiotic resistance.

Hypothesis:

a) We hypothesize that there will be persistent stability of the Nisin Inducible Expression system (NICE) vector plasmid inside the *Lactococcus lactis* and the species itself, over changes in pH, temperature, and growth pressure, similar to what is found in rumen.

b) The stability and expression of the NICE plasmid will not be reduced over generations.

c) Growing common enteric bacteria and pathogens alongside the vector Lactococcus lactis will not result in DNA exchange involving the NICE vector.

Specific Aims:

• Growth in normal conditions (30° Celsius, pH 6.8) will be measured using a spectrophotometer by measuring every 6 hours, plating the culture, and plotting cell count versus OD values to create growth curves.

• Growth of vector strain L. lactis will be measured in ranging temperatures of 30°-40° Celsius, pH values ranging from 5.7-7.3, and varying microbial cultures, all in M17 broth.

• Plasmid stability will be determined by observing growth on chloramphenicol M17 plates.

• Mixed cultures will be screened for gene transfer by plating M17 broth with chloramphenicol, isolating, and testing plasmid presence via cell lysate gel electrophoresis.

Overview:

In our experiments we will first address the stability of the plasmid, expressing the *Brucella* antigen, which must be proven to remain viable in the rumen. This will be tested by growing the transformed strain in specific pH, temperature, and against wild-type and shuttle strains. The plasmid expression and viability will be tested across generations. The plasmid contains an antibiotic resistance gene which could be absorbed by pathogens present in the bovine population. As the growth of the transformed *L. lactis* reaches a later stage, cell death and loss of genetic information into the extracellular fluid is a possibility. Second we will address the safety of the plasmid. We will test the possibility of transformation into other bacterial species by inserting the naked plasmid into broth cultures of the following: *Salmonella enteridis*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Escherichia coli and Bacillus subtilis.* Bacteria that acquire the NICE plasmid will be selected based on growth in chloramphenicol. We will select transformed colonies to extend over generations, testing the stability

Materials and Methods:

i. The laboratory of G.P. Andrews of the University of Wyoming will provide bacterial samples.

ii. *L. lactis* both the wild type and transformed strain will be grown on M17 media that is supplemented with 0.5% glucose and incubated at 30 degrees Celsius(6). We will also be streaking from frozen stalk onto TSA: *Bacilis subtilis, E. coli.* From frozen stalk onto blood agar plates (BPA) we will be streaking *Salmonella enteridis, Yersinia enterocolitica,* and *Campylobacter jejuni (ADD CITATION FOR JEJUN .* All will be grown in their desired 37 degrees Celsius environments.

iii. *L. lactis* will be grown on M17 media, and in M17 broth, which will both be supplemented with 0.5% glucose and incubated at 30 degrees Celsius. We will also streak the previously stated microbes for isolation for use in later tests. We will utilize a pH meter and create an M17 broth and agar that is between the pH of 5.7 and 7.3. We will then grow the *L. lactis w*ild type and transformed strain in the broth and measure titer using a spectometer. We will also use a similar procedure for testing the growth of *L. lactis* between the temperatures of 37.8 and 40 degrees Celsius. PCR will be performed using a standard PCR machine to look for plasmid presence in *L. lactis* after generations have been grown within the specific pH and temperature conditions3. We will also insert the naked plasmid into broth growths of the previously streaked stomach pathogens to test for the uptake of the plasmid. We will also utilize plasmid extraction to determine the plasmids presence within the microbes (5).

iv. Serial subcultures will be performed by inoculating fresh medium from the previous culture. After 5 subcultures, the number of chloramphenicol-resistant CFU will be counted on M17G plates with chloramphenicol.

v. Plasmid presence will be confirmed through plasmid extraction and amplification. We will follow protocol demonstrated by the EDVOTEK Mini-prep isolation of plasmid DNA (7).

Data Collection and Data Analysis

We will initially collect data though the qualitative methods by observing growth, or lack thereof, via media platting and spectrophotometer. We will be analyzing the data using growth curve procedures. OD measurements from the spectrophotometer will be recorded with viable cell counts in a table, these will be plotted against each other forming a growth curve. Images of gel electrophoresis will affirm presence or absence of the plasmid . All data will be input into Microsoft Excel and graphed using Microsoft Excel. We will test the results for statistical evidence ($p = <0.05$) of plasmid uptake, and differing growth under the pressures within the rumen.

Expected Results:

We expect to see the wild type strain have an advantage in growth and replication over the vector strain of L. lactis, under the burden of expression of the NICE.. This result is measured by using growth curves provided by observational methods and quantitative through the use of serial dilutions. The unpredicted result of this experiment is a growth curve showing faster growth of the vector strain. While not likely, this outcome would be welcome in experimentation. This would mean that the vector strain would have a chance in competition with wild strains. We also expect to see retainment of the plasmid to be diminished in non-selective environments, including the in-vitro rumen-like conditions.

Timeline:

Week 0	Streak and isolate *L. lactis* (wild type, and transformed), Make M17 media with 0.5% glucose.
Week 1	Test growth rates and abundance between the *L. lactis* wild type and transformed strain. Make M17 broth with 0.5% glucose. Grow *L. lactis* measuring the growth curve for four days, measuring every 12 hours using a spectrophotometer.
Week 2	Test growth rates of the *L. lactis* wild type and conjugated strain in the different pH, 5.7 and 7.3 by creating pH specific broth cultures. Growing the strains on a four day growth curve. Measuring growth every 12 hours using a spectrophotometer.

Week 3	Test growth rates of the *L. lactis* wild type and conjugated strain incubating in 40 degrees Celsius. Using a four day growth curve, measuring density every 12 hours.
Week 4	Determine the presence of plasmid through five generations of the *L. lactis* growth in M17 broth with 0.5% glucose. Plasmid presence will be confirmed by a plasmid extraction kit.
Week 5	We will insert the naked plasmid into M17 broth with 0.5% glucose of each of the following microbes: *Salmonella enteridis, Yersinia enterocolitica, Campylobacter jejuni, B. subtilis, and E. coli.* Plasmid presence will be determined by streaking the pathogens on M17 0.5% glucose plates with chloramphenicol.
Week 6	Colonies present on the chloramphenicol plates with growth will be run through a plasmid extraction kit to confirm plasmid presence, assuring growth was not a random mutation.
Week 7	Data analysis using Microsoft Excel.

Sources Cited

1. "Brucellosis," Ansci.wisc.edu http://www.ansci.wisc.edu/jjp1/ansci_repro/lab/diseases_2002/brucellosis.html
    a. Was found through a Google search for Brucellosis.
    b. Was used for a lot of the preliminary and background information on the disease.

2. "Facts about Brucellosis," Aphis.usda.gov, www.aphis.usda.gov/animal_health/animal_diseases/brucellosis/downloads/bruc- facts.pdf.
    a. This sources was found using Google.Com. It was written by the USDA and refrences the CDC. This articl has good information that gives basic facts and knowledge about *Brucellosis.* This article was instrumental in giving us a good idea on the impact that *Brucellosis* plays on the agricultural community and economy.

3. J.E. Lowry, D.D. Isaak, J.A. Leonhardt, G. Vernati, J.C. Pate, G.P. Andrews, " Vaccination with *Brucella abortus* Recombinant Induced Antigens reduces Bacterial Load and Promotes Clearance in a Mouse Model for Infection" PLoS ONE, 2011
    a. Accessed reference by searching "G.P. Andrews Brucella" into PubMed.
    b. This paper has been cited 11 times since its publication. It is also a previous study done by one of our partners on this project and gave background information about where this project is coming from.

4. J. Neef, F.J. Milder, D.G.A.M. Koedijk, M. Klaassens, E.C. Heezius, J.A.G. van Strijp, A. Otto, D. Becher, J.M. van Dijl, G. Buist, "Versatile vector suit for the extracytoplasmic production and purification of heterologous His-tagged proteins in *Lactococcus lactis,"* Journal of Applied Genetics and Molecular Biotechnology, 2015(99)9037-9048.
    a. Accessed on PubMed through the search "NICE vector *L. lactis."*
    b. This paper outlined how *L. lactis* was best grown and how the plasmid vectors are inserted and then act once inside *L. lactis.*

5. "M17 Agar." *CM0785, M17 Agar | Oxoid - Product Detail*, 2017. www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=CM0785&org=82&
    a. Was found using a Google search for information on M17 agar.
    b. Was used to better understand the nutrients and ways we will be using this media.

6. "Mini-Prep isolation of plasmid DNA" p. 8-12 http://www.edvotek.com/site/pdf/202.pdf
    a. This resource was found through a simple Google search for the protocol for plasmid extraction. This resource will be used in conjunction along with the Mo Bi Tec resource to find the right system for our needs.

7. Mo BI Tec, "NICE® Expression System for Lactococcus lactis: The effective & easy-to-operate NIsin Controlled gene Expression system" Molecular Biotechnology. November 2015. p. 11-12
    a. This is the protocol for our NICE system and was given to us by Dr. Andrews.

8. Campelo, A. B., Roces, C., Mohedano, M. L., López, P., Rodríguez, A., & Martínez, B. (2014). A bacteriocin gene cluster able to enhance plasmid maintenance in *Lactococcus lactis*. *Microbial Cell Factories*, *13*, 77. http://doi.org/10.1186/1475-2859-13-77
    a. Was found on PubMed through a search for plasmids in *L. lactis.*
    b. Was used to better understand the potential for the stability of the pNZ8121 within our bacteria.