FINAL REPORT
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Livestock feed preservatives based on antioxidant enzymes extracted from animal blood

Principal Investigator(s):

Vladimir Reukov, PhD, Research Assistant Professor
Department of Bioengineering
301 Rhodes Hall
Clemson University
Clemson, SC 29634
Phone: 8646437937
Fax: 8646566644
E-mail: reukov@clemson.edu

Alexey Vertegel, PhD, Associate Professor
Department of Bioengineering
301 Rhodes Hall
Clemson University
Clemson, SC 29634
Phone: 8646560801
Fax: 8646566644
E-mail: vertege@clemson.edu

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Duration of Project: 12 months

Lay Summary: Autooxidation of unsaturated fats is one of the primary mechanisms of quality deterioration in animal feeds and pet food. These alterations in quality are manifested through adverse changes in flavor, color, texture, and nutritive value and there is some concern that toxic compounds are produced during the deterioration process. The pet food industry constantly looks for natural solutions to increase the palatability of livestock feeds and **pet food,** and also studies mechanisms to extend the shelf life of products while maintaining product freshness and quality. Most currently used antioxidants are synthetic chemicals, and there is global concern among regulatory bodies and customers regarding the safety of these compounds (e.g., ethoxyquin). Naturally-derived antioxidants are available (e.g., tocopherols), but are expensive, and are often not as effective as their synthetic counterparts. Thus, there is a clear need for development of novel, inexpensive, and efficient natural antioxidants. Erythrocytes are readily available as a major component of animal blood. Being natural oxygen carriers, they are equipped with highly efficient antioxidant machinery, which utilizes a number of antioxidant enzymes and compounds. Here, we propose to continue development of a cost effective, natural antioxidant derived from animal blood.

During the period of this project we have achieved the following milestones:

- Established a simple protocol to produce an antioxidant enzyme concentrate from animal blood
- We compared the efficacy of our enzyme cocktail to commercially available antioxidants, such as PetOx® (BHA + BHT) and NaturOx® (mixed tocopherols), and completed accelerated shelf-life studies.
- Evaluated the efficacy of our antioxidant cocktail in comparison to the current industry standards, ethoxyquin and BHA/BHT, using food models relevant to those utilized by the industry.
- Performed more detailed efficacy studies with commercial pet food samples, kibbles, turkey protein meal and rendered fat models using FOX-II assay to measure Peroxide Value of the samples, and performed antimicrobial studies.
- Prepared and submitted new AAFCO Ingredient Definition “Erythrocyte Protein Extract (preservative)” for our antioxidant.
- Performed testing for possible viral contamination, including Porcine Epidemic Diarrhea Virus;

We have met with FDA CVM officers in order to determine the required steps to receive an approval for the new ingredient petition. They listed a number of experiments we need to perform in order to satisfy their requirements. So, we propose to focus on the following steps to secure successful approval of the petition: 1) perform shelf live studies according to CVM standards; 2) perform experiments to establish standards for the protein cocktail quality control; 3) prepare and submit new ingredient petition to FDA CVM.

Objective (s):

1. We will perform accelerated shelf life experiments according to the CVM requirements – 2-3 batches of the product, incubated at 3 different temperatures 40°C, 45°C, 54°C for 60, 45, 14 days correspondingly. At each time point, the small sample will be collected and stored at the fridge. After 60 days, when last time point will be completed, all samples will be tested with Cod Oil, oxidized for 24-48hrs at 50°C to ensure minimal variations in level of oxidation. PV, ORAC and TBARS assays will be performed.

2. Perform experiments to establish standards for the protein cocktail quality control – FDA recommended to test our protein control using HPLC, gel chromatography to establish acceptance criteria for the product. We will perform these experiments.
3. Prepare and submit new ingredient petition to FDA CVM

Project Overview:

During the past years our group developed a new, simple, and inexpensive method for the extraction of antioxidant concentrate from animal blood. This method does not require the use of expensive and potentially toxic organic solvents such as chloroform and acetone. The new method only involves one step and utilizes GRAS components. Over the past years, we also compared efficacy of our enzyme concentrate to Ethoxyquin, PetOx (BHA + BHT), NaturOx with a number of food models, and completed shelf-life studies. For assessment of efficacy, we used two techniques – ferrous ion oxidation – xylenol orange assay (FOX), spectrophotometric method for quantifying Peroxide Value (lipid hydroperoxides) in the sample, and thiobarbituric acid reactive substances (TBARS) assay, for detection of aldehydes, the secondary oxidation products that are believed to be responsible for off-flavors associated with rancidity. We also performed long-term shelf-life study at 37°C for 6 months – equivalent to 18 months at room temperature with with pet food "Fresh Pet Select", which containes no preservatives according the label. We also established a protocol for testing for viral contamination.

Experimental Procedures

Aim I: We will perform accelerated shelf life experiments according to the CVM requirements – 2-3 batches of the product, incubated at 3 different temperatures 40°C, 45°C, 54°C for 60, 45, 14 days correspondingly. At each time point, the small sample will be collected and stored at the fridge. After 60 days, when last time point will be completed, all samples will be tested with Cod Oil, oxidized for 24-48hrs at 50°C to ensure minimal variations in level of oxidation. PV, ORAC and TBARS assays will be performed.

Aim II: We will perform experiments to establish standards for the protein cocktail quality control using HPLC, gel chromatography to establish acceptance criteria for the product.

Aim III: We will prepare and submit new ingredient petition to FDA CVM

Materials & Methods:

Peroxide Value (FOX II) assay.

In the ferrous xylenol orange (FOX) method, the sample (1g) is mixed in a test tube with 10 ml isopropanol for peroxide extraction on a vortex mixer for 15 s, then centrifuged at 4000g for 5 minutes. After that 20µl of a sample or a standard was mixed with 200µl of isopropanol and 20

µl of FOX-II reagent were added and absorbance was determined at 560 nm after 5 min of incubation at room temperature. Calibration curve was constructed using 3% H_2O_2 solution.

TBARS assay.

Thiobarbituric Acid(TBA) Reactive Substances assay based on ability of TBA to form a colored adduct after reaction with malonialdehyde (MDA) – a by-product of lipid oxidation. The MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions can be measured colorimetrically at 530-540 nm or fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Samples were extracted using the following procedure – 1 g of sample were vortexed with water, centrifuged at 4000g for 5 minutes, 0.2 ml of supernatant was mixed with TBA reagent and incubated at 90ºC for 5 minutes, cooled in ice and absorbance and fluorescence were measured.

To achieve our aims, we have started with accelerated shelf life studies for the regulatory submission. Fig.1. shows results of 7 days of oxidation of different samples – liquid poultry fat, fish oil, and sunflower oil at 50ºC. Peroxide value was measured using FOX-II.

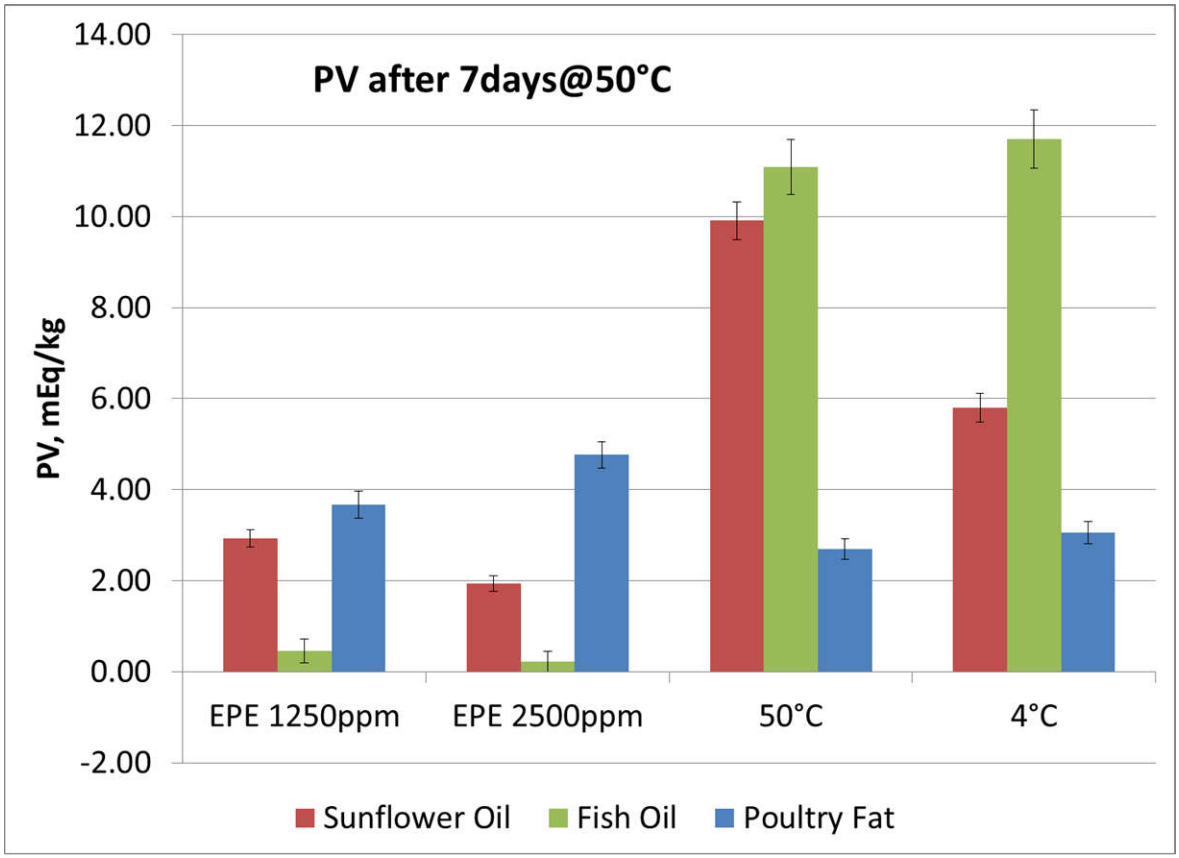


Fig.1. Results of PV measurements after 7 days of incubation at 50°C.

Summary of the acquired data are presented in Figs. 2 and 3.

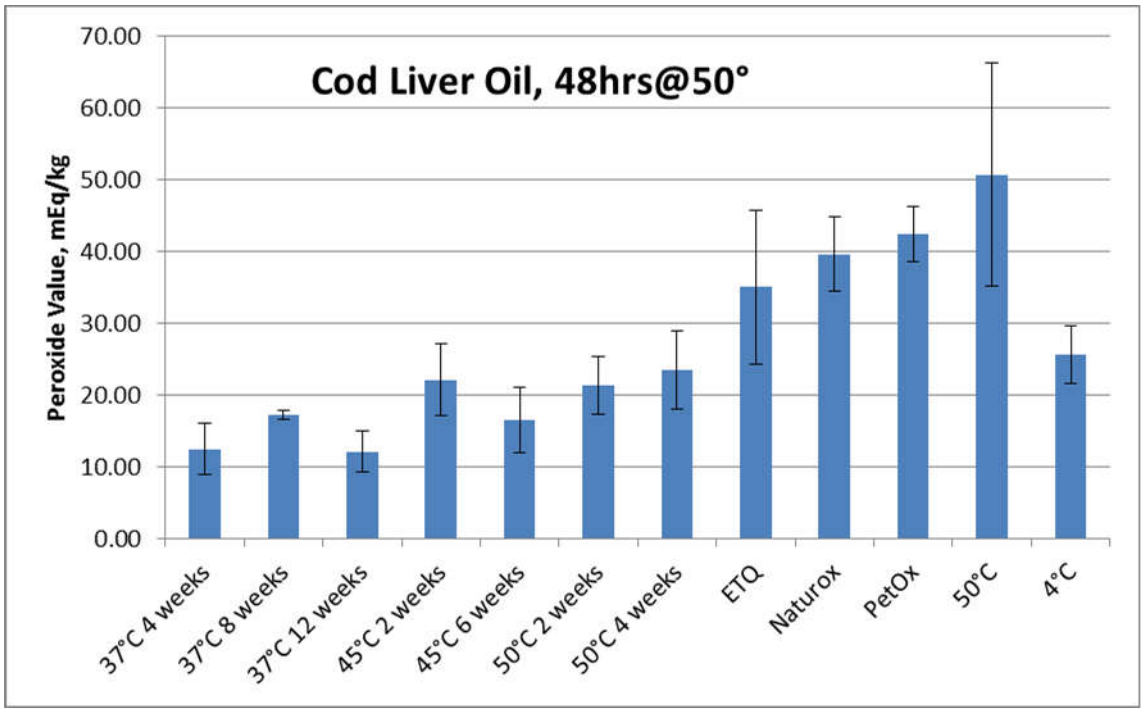


Fig.2. Results of PV measurements after accelerated shelf-life study at 37°, 45°C and 50°C.

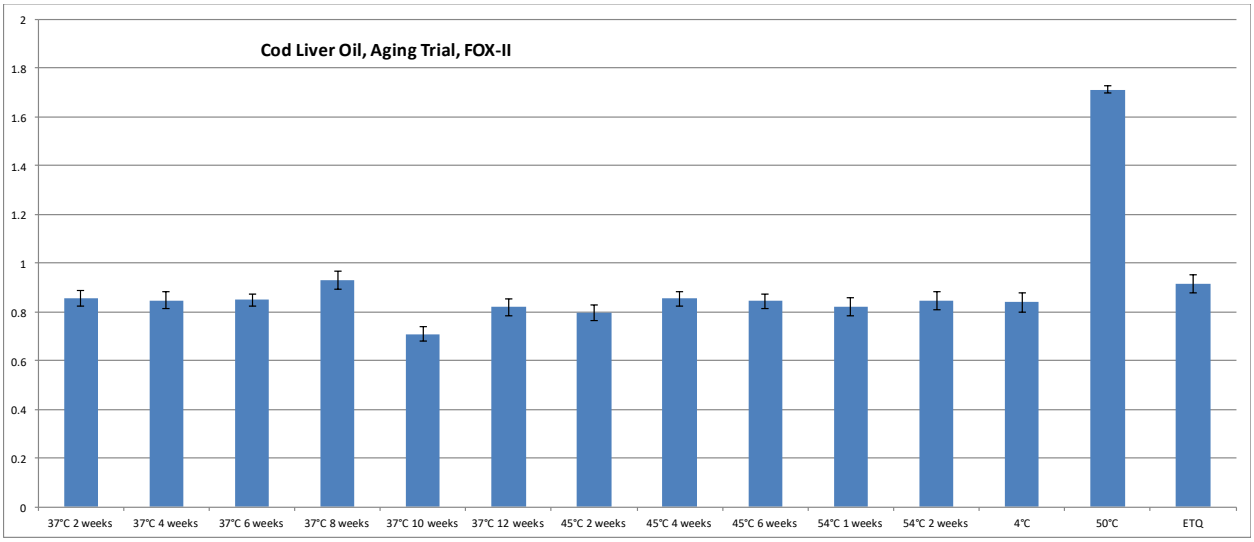


Fig.3. Results of cod oil accelerated oxidation (48hrs) at 50ºC with treatment of Prot-X, compared to ethoxyquin.

We had several meetings with FDA CVM officers to plan a new ingredient submission, and later to update them on our progress. Currently we have completed accelerated shelf life studies (2-month experiment) according to the FDA protocol and currently working on a new ingredient petition.

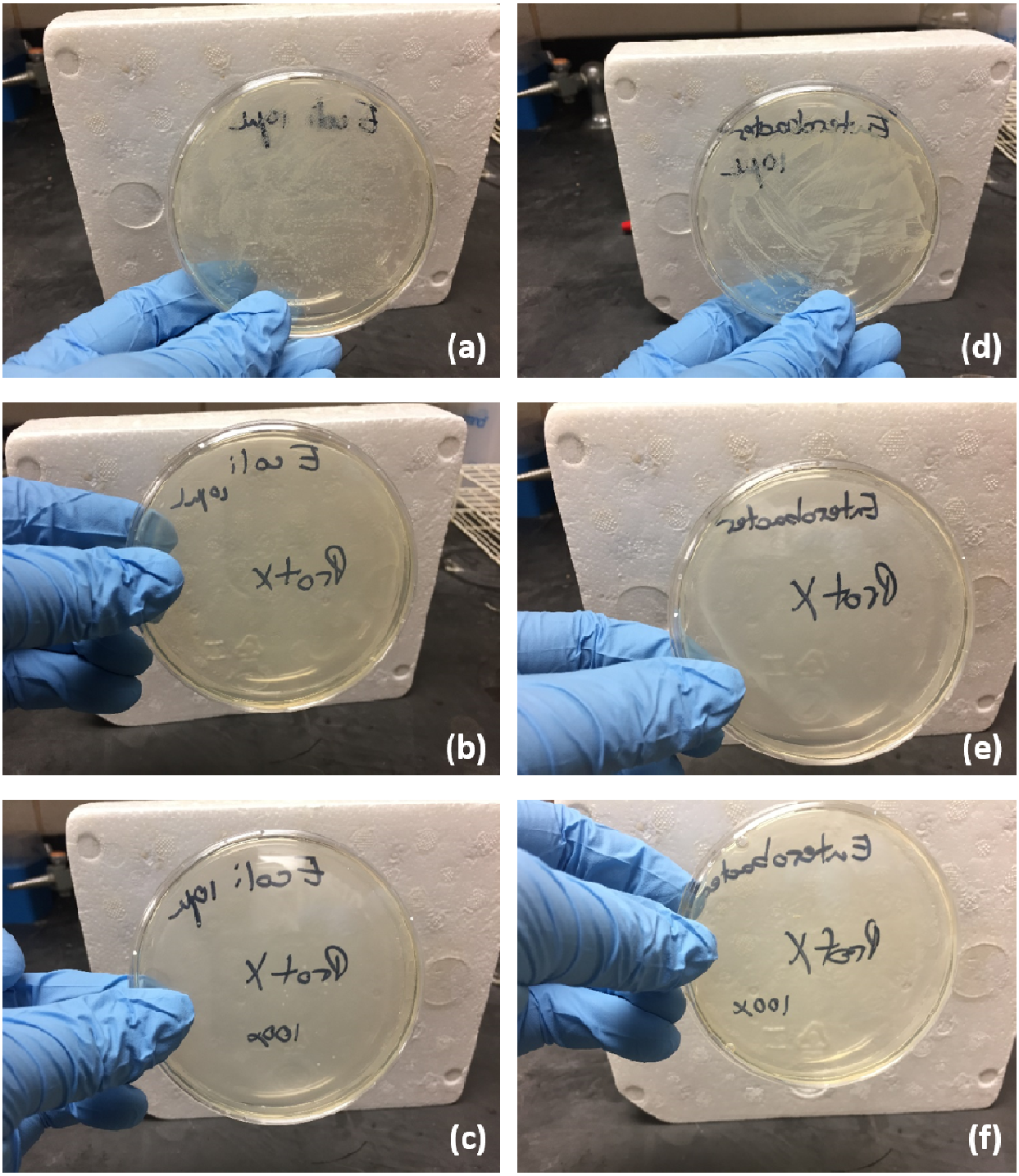
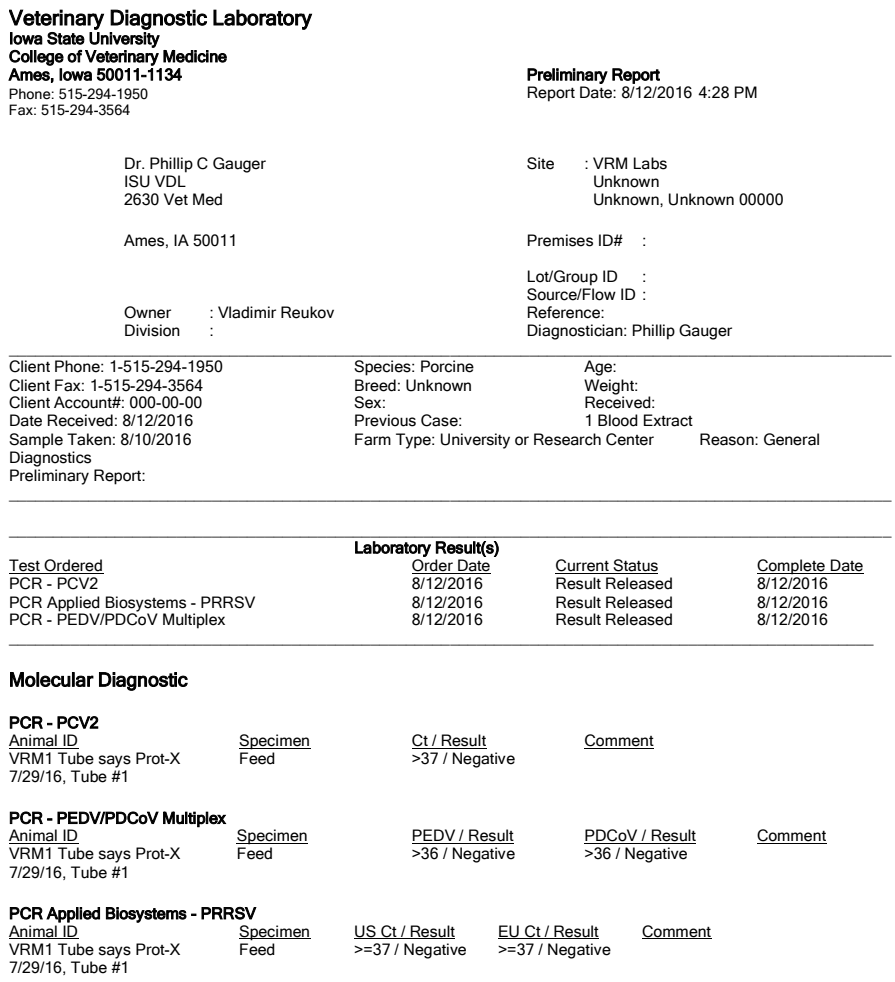


Fig.4. Results of CFU after 24hrs incubation with bacteria.

We also conducted microbiological studies with E. coli and Enterobacter bacteria. Briefly, 1ml of antioxidant either undiluted (Fig.4 (b) and (e)), or diluted 100 times (Fig.4 (c) and (f)), has been inoculated with 10 microliters of bacteria suspension (mid-log phase), and incubated for 24 hours at 37ºC. After incubation, 100 uL of suspension was place on agar plate and cultured for 24 hours at 37ºC in an incubator.

Microbiological and viral contaminations of our product are unlikely though possible, and we are performing analysis of each batches for Porcine epidemic diarrhea virus, Porcine circovirus (PCV2). Porcine reproductive and respiratory syndrome virus (PRRSV). All tests were performed using PCR method at Iowa State University Veterinary Diagnostics laboratory.



Veterinary Diagnostic Laboratory
Iowa State University
College of Veterinary Medicine
Ames, Iowa 50011-1134
Phone: 515-294-1950
Fax: 515-294-3564

Preliminary Report
Report Date: 8/12/2016 4:28 PM

Dr. Phillip C Gauger
ISU VDL
2630 Vet Med

Ames, IA 50011

Owner : Vladimir Reukov
Division :

Site : VRM Labs
Unknown
Unknown, Unknown 00000

Premises ID# :

Lot/Group ID :
Source/Flow ID :
Reference:
Diagnostician: Phillip Gauger

Client Phone: 1-515-294-1950
Client Fax: 1-515-294-3564
Client Account#: 000-00-00
Date Received: 8/12/2016
Sample Taken: 8/10/2016
Diagnostics
Preliminary Report:

Species: Porcine
Breed: Unknown
Sex:
Previous Case:
Farm Type: University or Research Center

Age:
Weight:
Received:
1 Blood Extract
Reason: General

Laboratory Result(s)

Test Ordered	Order Date	Current Status	Complete Date
PCR - PCV2	8/12/2016	Result Released	8/12/2016
PCR Applied Biosystems - PRRSV	8/12/2016	Result Released	8/12/2016
PCR - PEDV/PDCoV Multiplex	8/12/2016	Result Released	8/12/2016

Molecular Diagnostic

PCR - PCV2

Animal ID	Specimen	Ct / Result	Comment
VRM1 Tube says Prot-X 7/29/16, Tube #1	Feed	>37 / Negative	

PCR - PEDV/PDCoV Multiplex

Animal ID	Specimen	PEDV / Result	PDCoV / Result	Comment
VRM1 Tube says Prot-X 7/29/16, Tube #1	Feed	>36 / Negative	>36 / Negative	

PCR Applied Biosystems - PRRSV

Animal ID	Specimen	US Ct / Result	EU Ct / Result	Comment
VRM1 Tube says Prot-X 7/29/16, Tube #1	Feed	>=37 / Negative	>=37 / Negative	

Fig. 5. Sample report of PCR testing for PCV2 and PEDV of our batch performed at the Veterinary Diagnostic Lab at ISU.

SOP for antioxidant activity:

One of the FDA requests was to establish a protocol to validate antioxidant activity in each batch, in order to separate batches with lower activity. After consultations with officers, the following protocol has been developed for antioxidant activity study:

1. **5**0ml of fresh Cod Oil mixed with 0.125% of ProtX from each batch and commercial antioxidants (150ppm ETQ, 200ppm BHA/BHT)
2. Mixed, vortexed for 1 min
3. 1g of samples and controls without treatment placed into 15 mL test tube
4. Oxidized for 24-48 hrs at 50°C
5. 50µL of 8% BHT solution in ethanol added to all samples
6. FOX-II analysis performed according protocol

Summary.

At this moment, we have completed all the experiments requested by the FDA for the new ingredient petition and we are working on finalizing this document. We're planning to have a final review of our data and submission by the end of this year.

Impacts and Significance:

Interest in substituting synthetic food preservatives and antioxidants for substances that can be marketed as natural is increasing. Pet-food and livestock-feed preservatives based on antioxidants extracted from animal tissues carry the potential to have a huge impact on the industry.

Outside funding: In 2014 we were awarded $50k I-CORPs grant from NSF to assess commercial potential for our technology and get entrepreneurial training. We traveled to several major conferences including Iowa Pork Congress, IPPE in Atlanta, and are going to Pet Food Forum. We interviewed more than a hundred companies about their antioxidant needs.
We have applied for 12 grants directly related to ACREC funding: SBIR Phase 0 ($6k) from SC INBRE (awarded, twice); NSF I-CORPs ($50k) - awarded; SCLaunch university start up support grant ($25k) - awarded; Clemson University Research Foundation Technology Maturation Grant -$36k for scaling up – awarded, NSF SBIR Phase I ($175k) – declined, resubmitted, USDA SBIR Phase I ($100k) – declined, resubmitted; two USDA Phase I SBIR grants ($200k total) – both funded in 2016, one USDA SBIR Phase II grant ($600k) - funded in 2017.

Future Work: We plan to meet with FDA CVM officers to present our finalized petition**.**