I. **Project Title**
Effect of prolonged probiotic supplementation in a high-fructose diet in a pig model of non-alcoholic fatty liver disease.

II. **Project Completion Date**
January 2019 (analysis is still ongoing)

III. **Student(s), Department(s), and Major(s)**
(1) Victoria Smith, Animal Sciences, Major: animal science, Minor: microbiology
(2) Megan Melnyk, Biological Sciences, Major: microbiology

IV. **Faculty Advisor and Department**
Dr. Magdalena Maj, Biological Sciences Department

V. **Cooperating Industry, Agency, Non-Profit, or University Organization(s)**
BiOWiSH (supplied probiotics mix)

VI. **Executive Summary**
Non-alcoholic fatty liver disease (NAFLD) is the leading cause of pediatric chronic liver condition, caused by hepatic inflammation, insulin resistance and steatosis. We hypothesize that prolonged intake of diets rich in carbohydrates, specifically fructose, will induce NAFLD in infants, whereas probiotics may ameliorate the symptoms of the disease. Our goal was to establish a diet-induced NALFD in neonatal Iberian piglets within a short period of time (the time period of weeks).

To assess the effect of dietary fructose and fat as well as probiotics in the pathology of the disease, 24 leptin resistant neonate pigs were assigned to 1 of 4 treatment diets for 10 weeks: 1) control (CON), 2) Western diet (WD) (high fructose, high fat), 3) CON + probiotics, 4) WD + probiotics. Animals were fed 40 mL · kg BW⁻¹ at 6-h intervals 4 times per day. Body weight gain was assessed through the study. On week 10, animals were euthanized and liver tissue was collected for gene expression, Western Blot and histology analyses to assess inflammation, activation of de novo lipogenesis pathway, and fat accumulation in liver.
We hypothesized that compared to WD, both CON and WD + probiotic groups will show decreased weight gain, intrahepatic fat accumulation, and activation of de novo lipogenesis pathway in liver, along with an increase in insulin pathway activation and insulin sensitivity in neonatal piglets.

To perform the histological analysis, the liver biopsies were paraﬁn-embedded, cut using a microtome and stained. The hematoxylin and eosin staining conﬁrmed steatosis, ballooning degeneration, and inﬂammation in the liver samples from pigs fed WD and WD + probiotics. There was no steatosis or inﬂammation observed in the liver samples from pigs fed CON and CON + probiotics. Subsequently, the liver tissues were stained with Oil red O, a dye binding to triglycerides and lipids. This revealed a high level of lipid droplets in the liver biopsies from pigs fed WD and WD + probiotics, while there was no positive Oil red O signal observed in the liver biopsies from pigs fed CON and CON + probiotics.

To study the gene expression of inﬂammatory markers, total RNA was isolated from 24 liver biopsies. After conﬁrming RNA integrity and quality, RNA was reverse transcribed to complementary DNA (cDNA). Obtained cDNA samples were analyzed in real-time quantitative polymerase chain reaction (RT-qPCR) using gene speciﬁc primer pairs. There was a signiﬁcant increase in the relative mRNA abundance for several major inﬂammatory markers, such as tumor necrosis factor-alpha (TNF-α), tumor growth factor beta (TGF-β), and interleukin-1 alpha (IL-1α) in the liver tissues from pigs fed WD when compared to CON and CON + probiotics. Surprisingly, all the inﬂammatory markers were also signiﬁcantly increased in the liver tissues from pigs fed WD + probiotics.

To evaluate potential activation of de novo lipogenesis pathway in liver, cDNA samples were analyzed in RT-qPCR using lipogenic-related gene specific primer pairs. There were no signiﬁcant changes in the gene expression for de novo lipogenesis pathway. However, there was a signiﬁcant increase in peroxisome proliferator-activated receptor gamma (PPAR-γ) gene expression in liver from pigs fed WD and WD + probiotics. PPAR-γ is a nuclear receptor known to regulate fatty acid storage, glucose metabolism and has a major role in mitochondrial function.

In summary, we successfully established a Western diet-induced model of non-alcoholic fatty liver disease in neonatal piglets. The probiotic supplementation did not ameliorate the histological features of NALFD nor helped to decrease the liver inﬂammation. Interestingly, we saw a signiﬁcant change in a marker of mitochondrial function. In future, we think it would be worth to investigate in more details mitochondrial story and look how high-fructose diet impacts the mitochondrial function, mitochondrial mass, and activity of mitochondrial enzymes.

VII. Major Accomplishments

(1) We successfully completed 10 week long animal feeding trial and tissue collection. The neonatal piglets were fed every 6 h a liquid milk diet, which was prepared daily. This feeding regime and feed preparation required an intensive labor of graduate student Mrs. Victoria Smith and several undergraduate students (not listed here).

(2) We successfully developed a neonatal pig model of diet-induced non-alcoholic fatty liver disease (NAFLD). Histological analysis conﬁrmed liver steatosis and inﬂammation in the tissues obtained from piglets fed Western diet. There were no pathological changes observed in the liver tissues from piglets fed control diet.

(3) We revealed that probiotics did not ameliorate the symptoms of NAFLD. Histological analysis showed that the liver tissues obtained from animals fed Western diet supplemented with the probiotics still displayed steatosis and inﬂammation.
(4) We presented our results in the CSUPERB symposium in Los Angeles, January 2019.

VIII. Expenditure of Funds

We did not exceed our budget of $5,000. Majority of the funds was used for purchase of necessary laboratory reagents.

IX. Impact on Student Learning

Mrs. Victoria Smith has actively participated in the 10 week long animal feeding trial, tissue collection, various laboratory analysis, and manuscript writing. On a daily basis, Victoria prepared liquid milk diets, fed and weight animals. At the end of the animal study, she coordinated a 4 day long animal tissue collection: Victoria prepared all necessary dissecting tools, explained undergraduate students their roles during the collection, trained an intermittent student in animal dissection, and collected (with help of several undergraduate students) all tissues. Successful completion of these tasks allowed Victoria not only to become proficient in animal dissection and tissue collection, but also helped her improve her organization, communication, and leadership skills. In the laboratory, Victoria has been trained how to use a microtome (a tool used to cut extremely thin slices of tissue), stain the tissues, and take good quality images with a microscope. She developed the protocol for total RNA isolation from liver, including quality control ensuring the integrity and quality of RNA.

Mrs. Megan Melnyk has contributed to the project by carrying out DNA isolation from different parts of the intestinal tract. She worked closely with another grad student in the lab, and together they established a protocol on a DNA isolation from colon, cecum, distal ileum, and proximal jejunum contents. Megan was trained how to set up a PCR reaction, calculate the proper DNA concentration, prepare DNA dilutions, and run the DNA agarose gel electrophoresis. She successfully completed analysis on probiotic detection in the four different parts of the gastrointestinal tract. This training enhanced her laboratory skills, knowledge in the basic molecular biology techniques, original literature search, and poster presentation skills.