PROPOSAL NARRATIVE

I. Project Title

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

II. Abstract

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. To assess the effect of dietary fructose and probiotics in the pathology of the disease, 24 leptin resistant neonate pigs will be assigned to 1 of 4 treatment diets for 10 weeks: 1) control (CON), 2) high fructose (HF), 3) CON + probiotics, 4) HF + probiotics. Animals will be fed 40 mL · kg BW^{-1} at 6-h intervals 4 times per day. Body weight gain and insulin sensitivity (measured by oral glucose tolerance test) will be assessed through the study. On week 10, animals will be euthanized and liver tissue will be collected for Western Blot and histology analyses to assess protein activation of hepatic insulin and de novo lipogenesis pathways, as well as inflammation and fat accumulation in liver. If our hypothesis is true, we expect to show that compared to HFF, both CON and HFF + 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.

III. Introduction

Non-alcoholic fatty liver disease (NAFLD) represents the major cause of pediatric chronic liver pathology in the United States, with prevalence estimates from 0.7% in 2-4 years old infants to 17.3% in adolescents, and up to 38% among those with obesity (1, 2), and is associated with increased risk of dying or requiring liver transplantation in children (3). Previous studies have found an association between high-carbohydrate diets and the prevalence of NAFLD (4, 5, 6), suggesting an important role of carbohydrates in the pathogenesis of the disease. Particularly, the role of high fructose diets in NAFLD has become a critical question because of the fructose-induced increase of hepatic insulin resistance (IR). Although both dietary fructose restriction (7, 8) and administration of probiotic formulas to modulate intestinal microbiota (9, 10) have been shown to reduce liver fat and improve insulin sensitivity in clinical studies, there is no information available on the mechanistic effect of their interaction in the gut-liver axis of children with and without NAFLD. Most fructose mechanistic studies to date have relied in mouse models (11, 12) despite the significant differences between rodents and humans in terms of pathogenesis and progression of NAFLD (13, 14), and their relevance must be tested. Compared to rodents, pigs represent a better translational model of liver disease, because of their metabolism, sedentary habits, and hepatic histological and physiological resemblance with humans (15, 16, 17, 18).

Specifically, the Spanish Iberian breed has recently emerged as an exciting and novel large animal model of metabolic syndrome and NAFLD (19, 20, 21). Iberian pigs possess a fixed mutation in the leptin receptor (LEPR) (22, 23), which has been linked to a leptin-resistant phenotype characterized by lower gene expression of hypothalamic LEPR and signaling proteins (24), hyperleptinemia, hyperphagia, and extreme adipogenesis (22, 25). Consequently, Iberian pigs fed high-fat hypercaloric diets rapidly developed obesity, dyslipidemia, IR, and steatosis, with most symptoms observed in both mature (19) and neonate animals (20, 21).

IV. Objective

To compare the impact of prolonged high fructose intake vs. high fructose supplemented with probiotics vs. low fructose on NAFLD onset in neonate pigs, and to identify the mechanisms involved in the pathogenesis of the disease.

V. Methodology

Animal Care and Experimental Design: Twenty-four piglets will be allowed to suckle their sow from birth to 10 d of age. At 10 d of age, piglets will be randomly assigned to receive 1 of 4 liquid diets during 10 consecutive weeks (n=6 pigs per diet): 1) control (CON), 2) high fructose (HF), 3) CON + probiotics, 4) HF + probiotics. The 4 diets will be formulated to meet the nutrient requirements according to National Research Council 1998. Desired fructose concentrations will be achieved by replacing lactose by fructose while maintaining the amount of carbohydrates constant. Fat levels and ratio of protein to energy and fructose content will be kept constant across the 4 diets. Animals will be fed 40 mL · kg BW^{-1} at 6-h intervals 4 times per d, with the liquid diet delivered over a 20 min period. Body weights will be recorded before the beginning of the study and thereafter at 3-d intervals until the end of the study, and the average daily gain calculated. On week 8, piglets will be anesthetized and indwelling catheters will be surgically inserted into the jugular vein and carotid using sterile techniques (29). Peripheral insulin sensitivity will be assessed in all animals on week 9. On week 10, all animals will be euthanized with an injection of pentobarbital sodium, and tissue from liver, gastrointestinal tract, and muscle will be collected.

Histological Analysis: Tissue from liver, gastrointestinal tract, and muscle will be flash-frozen in liquid nitrogen and stored at −80°C. Frozen tissue will be fixed in 10% buffered formalin, processed, paraffin embedded, and stained in hematoxylin-eosin (H&E). Liver sections will be examined with help of two veterinarians for macro and microvesicular steatosis, inflammation, hepatocellular ballooning, fibrosis, Kupffer cell vacuolization and Kupffer fat accumulation, as well as zonal distribution of each of these parameters.

Insulin Sensitive Assay and Plasma/Serum Analyses: Insulin sensitivity will be measured by an oral glucose tolerance test as previously described (27). Insulin and glucose concentrations will be measured by a porcine insulin radioimmunoassay kit (Millipore, St. Charles, MO, USA) and the glucose oxidase method (Thermo Scientific, Waltham, MA, USA), respectively.

Tissue insulin signaling: The activation and abundance of components of insulin signaling and de novo lipogenesis pathways in liver will be determined by Western blot analysis (28). Tissue samples will be homogenized, and supernatants will be used for either immunoprecipitation with appropriate antibodies, or used directly in Western blots. Abundance of non-phosphorylated signaling proteins will be normalized with β-actin, while phospho-proteins will be normalized using the total protein abundance from the supernatant or immunoprecipitate. Blots will be

developed using an enhanced chemiluminescense kit, visualized using ChemiDocIt and analyzed with LabWorks Image Acquisition and Analysis Software. We will determine abundance and phosphorylation of the insulin receptor (IR), ribosomal protein S6 kinase (S6K1), sterol-regulatory element-binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP).

VI. Timeline

1. Animal feeding study and tissue collection: Feb 1st- May 15th 2018.
2. Western Blot, histology and serum analyses: May 16th – October 31st 2018.
3. Manuscript writing: November 1st – January 31st 2019.

VII. Final Products and Dissemination

Approval of the knowledge generated in this work by the academic community will be evident by the integration of this novel information in the curriculum at Cal Poly, by the publication of peer-reviewed papers and approval of abstracts for presentation in national meetings.

1. Change in knowledge: Accomplishment of the proposal objectives will provide physiologically relevant information on whether hepatic steatosis and liver inflammation in infants can be prevented or decreased by avoiding prolonged consumption of diets with high fructose, or by supplementation of probiotics in the diet. The results will have implications for the nutritional management of infants with and without the liver disease.
2. Scientific publications in Peer-review journals and Conference Proceedings: Results from the studies presented herein will be presented at 2019 Experimental Biology Meeting in Orlando, as well as undergraduate research presentations at Cal Poly and other CSU campuses. These findings will also be submitted for publication in appropriate peer reviewed scientific journals such as American Journal of Physiology and Pediatric Research.
3. Preliminary data for collaborative NIH grant: Results from these studies will also be used to develop a strong NIH proposal to examine the impact of antioxidant supplementation along with probiotics in high-fat high-carbohydrate diets in pediatric and adult NAFLD.

VIII. Budget Justification

a. Operating Expenses ($3,500)

i. Non-computer Supplies and Materials:

1. Diet ingredients: $500
    a. Whey protein, casein, fructose and fat sources to prepare the milk-based diets to feed the animals.
2. Laboratory consumables: $3,000
    a. Porcine insulin radioimmunoassay kit
    b. Glucose Oxidase assay kit
    c. Antibodies for IR, S6K1, SREBP-1c and ChREBP
    d. Histology supplies

Reagents listed under Laboratory consumables are necessary to conduct described experiments, thus meet the objectives of the project.

b. Travel ($1,500)

ii. 2019 Experimental Biology Meeting in Orlando, FL

Purpose of the travel: dissemination of our research findings at professional meeting.

Warren J. Baker Endowment
for Excellence in Project-Based Learning
Robert D. Koob Endowment *for Student Success*

CAL POLY

PROPOSAL BUDGET

Student Applicant(s):		
Faculty Advisor: Magdalena Maj		
Project Title: Effect of prolonged probiotic supplementation in a high-fructose diet in a pig model of non-alcoholic fatty liver disease		**Requested Endowment Funding**
Travel	*subtotal*	**$ 1,500**
Travel: In-state		**$**
Travel: Out-of-state		**$ 1,500**
Travel: International		**$**
Operating Expenses	*subtotal*	**$ 3,500**
Non-computer Supplies & Materials		**$ 3,500**
Computer Supplies & Materials		**$**
Software/Software Licenses		**$**
Printing/Duplication		**$**
Postage/Shipping		**$**
Registration		**$**
Membership Dues & Subscriptions		**$**
Multimedia Services		**$**
Advertising		**$**
Journal Publication Costs		**$**
Contractual Services	*subtotal*	**$ N/A**
Contracted Services		**$**
Equipment Rental/Lease Agreements		**$**
Service/Maintenance Agreements		**$**
	TOTAL	**$ 5,000**

APPENDIX

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