DEVELOPMENT OF A PEDIATRIC MODEL OF NAFLD IN
NEONATAL IBERIAN PIGS

A Thesis
presented to
the Faculty of California Polytechnic State University,
San Luis Obispo

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Agriculture, Specialization in Animal Science

by
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June 2019
ABSTRACT

Development of a Pediatric Model of NAFLD In Neonatal Iberian Pigs

Gabriella Veronica Hernandez

The prevalence of non-alcoholic fatty liver disease (NAFLD) in children has increased over the past decades, creating a need for animal models that recapitulate the features of the pediatric disease. Iberian pigs have a leptin-resistant phenotype characterized by hyperleptinemia, hyperphagia, and extreme adipogenesis. We hypothesized that neonatal Iberian pigs fed a high fat high-fructose (HFF) diet will develop a pattern of liver injury resembling pediatric NAFLD. In addition, we sought to determine if a mixture of probiotics would prevent the disease. Animals were fed 1 of 4 diets containing (g/kg body weight × d) 0 g fructose, 11 g fat and 199 kcal (CON-N; n=8), 22 g fructose, 16 g fat and 300 kcal (HFF2-N; n=8), CON + probiotic (CON-P; n=6), or HFF2 + probiotic (HFF2-P; n=6) every 6 h for 70 d. The probiotic mixture (6.2 × 10^4 cfu/mL) contained *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum* and *Bacillus amyloliquefaciens*. Body weight was recorded every 3 d. Serum markers of liver injury and dyslipidemia were measured on d 40 and 65 at 2 h post feeding. Fasting leptin, insulin, glucose and homeostatic model assessment (HOMA) values were assessed on d 70. Liver and skeletal muscle (longissimus dorsi) were collected on d 70 for histology, triacylglyceride (TAG) quantification, relative gene expression, and Western blot analysis. Metabolomic analysis was performed on liver tissue and plasma. Body weight was not significantly greater in HFF fed pigs compared to CON. Leptin, alanine and aspartate aminotransferases, alkaline phosphatase, lactate
dehydrogenase and total bilirubin were increased \((P \leq 0.001)\), and high and low density lipoproteins decreased \((P \leq 0.05)\) in HFF2-N and HFF2-P. Livers in HFF2-P and HFF2-N had higher relative weight and TAG \((P \leq 0.001)\), micro and macrovesicular steatosis, ballooning degeneration, Mallory-denk bodies, inflammation and necrosis, increased gene expression of TNFα, TGFβ, IL1α and PPARγ \((P \leq 0.001)\), and decreased ChREBP \((P \leq 0.001)\). A probiotic affect was seen as pigs fed CON-P and HFF2-P had higher insulin and HOMA values were increased \((P \leq 0.05)\). Western blot analysis showed dysregulation of autophagy in liver of pigs fed CON-P and HFF2-P, and in skeletal muscle of pigs fed CON-N and HFF2-N. Metabolomic analysis demonstrated dysregulation of one-carbon metabolism, the tricarboxylic acid cycle (TCA), the urea cycle, and amino acid metabolism of pigs fed HFF2 diets compared to CON diets. In conclusion, Iberian pigs fed a HFF diet recapitulate many pediatric NAFLD-associated features, in the absence of obesity and independently of probiotic supplementation, suggesting a potentially suitable model for pediatric NAFLD research. Furthermore, probiotic supplementation did not ameliorate the onset of NAFLD when fed in conjunction with a HFF diet.

Keywords: NAFLD, Model, Pediatric, Iberian Pig, Probiotics
ACKNOWLEDGMENTS

This thesis would not be possible without the numerous people who have provided their support and contributed their time and skills to this project. Specifically, I would like to thank my advisor, Dr. Rodrigo Manjarin, for giving me the opportunity to conduct this research and for working tirelessly to ensure my overall success and education. I would like to thank Dr. Magdalena Maj for teaching me essential lab skills, supervising all of my laboratory work, and supporting the execution of my trials. Also, I would sincerely like to thank the members of my committee, Dr. Daniel Peterson, Dr. Darin Bennett, Dr. Mark Edwards, and Dr. Douglas Burrin for always providing me with guidance, support, and answers to my questions. I would like to thank Dr. Christopher Kitts for donating his time and skills to this research, and for providing a space to conduct our laboratory work. I would also like to express my gratitude to Dr. Michael LaFrano, Robert Fanter, Dr. Jennifer Vanderkelen, Dr. Peggy Rice, and Dr. Daniel Columbus for providing their expertise and support. Additionally, I would like to thank Dr. Kimberly Sprayberry and Dr. Matthew Burd for providing veterinary care. I would like to acknowledge the Cal Poly animal facilities, as well as their managers, Lee Rincker and Beth Reynolds, for giving us a space to conduct our trials and assistance. Importantly, we gratefully thank California State University Agricultural Research Institute, BiOWiSH Technologies™, Acorn Seekers LLC, and Cal Poly STRIDE Seed Funding for funding this research. Finally, I would like to thank my fellow graduate students, Victoria Smith and Morgan Coffin, undergraduates Megan Melnyk, Lauren Wienker, and Brooke Harbottle, and all other students who worked on this project, for donating countless hours to help conduct these trials and laboratory research.
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1. INTRODUCTION

1.1 Prevalence of NAFLD in children

Non-alcoholic fatty liver disease (NAFLD) has rapidly become the most common cause of chronic liver disease worldwide (Kumar & Mohan, 2017). NAFLD is a progressive disease that ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis without the excessive consumption of alcohol. Currently, the vast majority of research has focused in adults; however, NAFLD affects 3-10% of children worldwide (Giorgio et al., 2013). In the United States, NAFLD prevalence is estimated to be 0.7% in 2-4 years old infants to 17.3% in adolescents, and up to 38% among those with obesity (Schwimmer et al., 2006). Alarmingly, these numbers may underestimate the effected number of children affected, as NAFLD is often asymptomatic in pediatric populations (Berardis and Sokal, 2014). In addition, those who are not diagnosed are at risk of developing cirrhosis and ultimately end-stage liver disease, as well as increase their risk of developing other diseases such as cardiovascular disease and atherosclerosis (Patil and Sood, 2017; Sung et al., 2009).

1.2 Pathogenesis and risk factors of NAFLD

Typically NAFLD is thought to occur as a two-hit model, with the ‘first hit’ being steatosis, and the ‘second hits’ being liver injury such as inflammatory cytokines/adipokines, mitochondrial dysfunction, and oxidative stress, which ultimately leads to NASH and fibrosis (Dowman et al., 2010). However, many studies have suggested a multi-hit model that does not occur in a linear fashion, but is a result of complex interactions between several metabolic processes (Polyzos et al., 2009).
Additionally, other metabolic disorders such as obesity, diabetes, hyperdyslipemia, and insulin resistance, have been linked to pediatric NAFLD (Marchesini et al., 2003). However, it remains unclear if metabolic syndrome is necessary for the development of NAFLD, or simply a related consequence of a diet high in carbohydrates and high in fats (Western diet). It is important to note that lean NAFLD can occur in patients, and is often more prevalent in younger patients (Wattacheril and Sanyal, 2016). It is known that diet plays a critical role in the pathogenesis of the disease. Previous studies have found an association Western diet and the prevalence of pediatric NAFLD, which has led to much interest in the roles of fat and sugar (Solga et al., 2004). Specifically, fructose has emerged as a key mediator in NAFLD. As the liver is the primary site for fructose metabolism, fructose has been implicated to play a critical role in the pathogenesis of NAFLD, and is thought to drive the progression of the disease into NASH through the formation of reactive oxygen species, the dysregulation of the insulin signaling pathway, and elevation in the rate of de novo lipogenesis (Lim et al., 2010). Furthermore, fat sources with high amounts of saturated fatty acids are thought to exacerbate the disease by promoting cellular dysfunction and endoplasmic reticulum stress (Leamy et al., 2014). Finally, cholesterol has been shown to increase levels of steatosis in the liver, although the mechanisms are less clear (Tu et al., 2016). For these reasons, this study utilized these nutrients to ensure the development of NAFLD.

Despite the association between the Western diet and NAFLD, there molecular mechanisms involved in the pathogenesis of NAFLD are still not fully understood. Metabolomic profiling has identified choline metabolism as key mediator of the progression of NAFLD, due to its involvement in lipid metabolism, methylation-
dependent biosynthesis of molecules, and mitochondrial bioenergetics (Corbin and Zeisel, 2012). In fact, the use of choline-deficient diets have often been used in order to induce NAFLD and increase the rate of progression (Corbin and Zeisel, 2012). Nevertheless further insight and analysis on choline and other metabolites is needed to deepen the understanding of the mechanistic driving forces of NAFLD.

1.3 Overview of current models of pediatric NAFLD

While many rodent models of NAFLD/NASH exist, few focus on the pathogenesis of diet-induced NAFLD in children, likely due to the speed at which mice reach sexual maturity. However, a study conducted in 21 day-old mice found that after feeding mice a high-fat diet for 6 consecutive weeks, mice had increased hepatic lipid accumulation (Chen et al., 2015). Additionally, a rabbit model of pediatric NAFLD exists, where rabbits aged 4–6 weeks old were fed a high-fat diet with cholesterol supplementation for 8 and 12 weeks, resulting in hepatic steatosis, inflammation, ballooning, and fibrosis (Fu et al., 2009). While these studies successfully developed models that recapitulated the onset of NAFLD and even the progression to NASH, it is important to consider the anatomical and physiological differences between these animals and humans. Pigs provide a more accurate translational model to humans; however, to date pediatric models of NAFLD in swine are sparse. In the past 10 years there have been several models of NAFLD/NASH in multiple swine breeds such as Ossabaw swine, Göttingen minipigs, Bama minipigs, and Iberian pigs (Table 1.1); however, these models focus on the adult pathogenesis of NAFLD (Liang et al., 2015; Schumacher-Petersen et al., 2019; Torres-Rovira et al., 2011; Xia et al., 2014;). Currently, a model of diet-induced NASH exists in juvenile Ossabaw swine, which was aimed to discover the effects of a
Western diet in juvenile populations. In this study 5 week old, female Ossabaw swine were given ad libitum access to a high-fat, high-fructose, high-cholesterol diet for 16 and 36 weeks, resulting in hepatic steatosis, hepatocyte ballooning, inflammation, and fibrosis (Panasevich et al., 2017). While this study provided relevant insight in to the pathogenesis of juvenile NAFLD, the starting age of the swine and the length of the study resulted in a study population that was no longer truly pediatric, since swine reach sexual maturity between 4 and 6 months (Smith and Swindle, 2006). An additional caveat in published models of NAFLD is found in the diet formulation and feeding protocols. The majority of previous NAFLD studies in swine allow ad libitum access to diets. Furthermore, many of these studies also relied on the reduction of choline (Lee et al., 2009; Liang et al., 2015; Raubenheimer et al., 2006; Rinella et al., 2008), as well as the addition of colic acid (Yamada et al., 2017) in order to achieve the development of NAFLD.

Similar to pediatric models, models dedicated to the pathogenesis of NAFLD in non-obese patients pediatric lean NAFLD are lacking. While obesity is undoubtedly a major risk factor for the development of NAFLD, there exists a subpopulation of patients that develop NAFLD without the typical risk factors, such as obesity or metabolic syndrome. Lean NAFLD manifests in the liver similarly non-lean NAFLD, except it develops in patients with body mass indexes lower than 25 kg/m² (Wattacheril and Sanyal, 2016). It is estimated that prevalence of NAFLD occurring in lean subjects is approximately 7-9% (Kumar and Mohan, 2017). In addition, it has been shown that the occurrence of lean NAFLD is associated with younger age (Kumar and Mohan, 2017; Wang et al., 2017). Lean NAFLD may even present a more dangerous form of NAFLD,
as the lack of adequate adipose tissue available to accept fatty acids causes the redistribution of fat to muscle and liver (Wattacheril & Sanyal, 2016). While models of NAFLD in swine typically develop obesity, there have been studies in mice that were able to develop the histological features of NAFLD without significantly increasing the body weight of adults (Matsuzawa et al. 2007; Tu et al. 2017).
Table 1.1: Summary of recent swine models of non-alcoholic fatty liver disease (NAFLD).

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Breed</th>
<th>Age Start</th>
<th>Age End</th>
<th>Feeding</th>
<th>Outcome</th>
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<tbody>
<tr>
<td>Xia et al</td>
<td>2014</td>
<td>Bama Minipig</td>
<td>6 mo</td>
<td>9 mo</td>
<td>Twice daily</td>
<td>Obesity, insulin resistance, steatosis, inflammation</td>
</tr>
<tr>
<td>Liu et al</td>
<td>2007</td>
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<td>3 mo</td>
<td>8 mo</td>
<td>4% BW/d</td>
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</tr>
<tr>
<td>Li et al</td>
<td>2015</td>
<td>Bama Minipig</td>
<td>6 mo</td>
<td>29 mo</td>
<td>3% BW/d</td>
<td>Obesity, insulin resistance</td>
</tr>
<tr>
<td>Yang et al</td>
<td>2015</td>
<td>Bama Minipig</td>
<td>6 mo</td>
<td>29 mo</td>
<td>3% BW/d</td>
<td>Obesity, insulin resistance, steatosis, inflammation, fibrosis</td>
</tr>
<tr>
<td>Schumacher-Petersen et al</td>
<td>2019</td>
<td>Gottingen</td>
<td>6-7 mo</td>
<td>19-20 mo</td>
<td>Ad libitum</td>
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<td>Christoffersen et al</td>
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<td>Gottingen</td>
<td>0</td>
<td>8 wk and 8 mo</td>
<td>Twice daily</td>
<td>Obesity, insulin resistance</td>
</tr>
<tr>
<td>Johansen et al</td>
<td>2001</td>
<td>Gottingen</td>
<td>9-10 mo</td>
<td>11-12 mo</td>
<td>500 g/d</td>
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<td>Christoffersen et al</td>
<td>2013</td>
<td>Gottingen</td>
<td>7 wk</td>
<td>31-34 wk</td>
<td>Twice daily</td>
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<tr>
<td>Mentzel et al</td>
<td>2017</td>
<td>Gottingen</td>
<td>6 wk</td>
<td>19 wk</td>
<td>3x daily</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Xi et al</td>
<td>2004</td>
<td>Guizhou minipigs</td>
<td>3-4 mo</td>
<td>9-10 mo</td>
<td>Twice daily</td>
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</tr>
<tr>
<td>Torres-Rovira et al</td>
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<td>Iberian Pigs</td>
<td>2-3 yr</td>
<td>2-3 yr</td>
<td>Ad libitum</td>
<td>Obesity, insulin resistance</td>
</tr>
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<td>Torres-Rovira et al</td>
<td>2013</td>
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<td>245 d</td>
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<td>Obesity</td>
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<td>Iberian Pigs</td>
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<td>8-10 yr</td>
<td>4 kg/d</td>
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<td>Fisher et al</td>
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<td>35 d</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Li et al</td>
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<td>Lee-Sung minipigs</td>
<td>4 mo</td>
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<td>Twice daily</td>
<td>Obesity, insulin resistance, fibrosis, inflammation</td>
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<tr>
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<td>2017</td>
<td>Micro minim pigs</td>
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<td>5 mo</td>
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<tr>
<td>Panasevich et al</td>
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<td>Ossabaw</td>
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<td>21 and 41 wk</td>
<td>Ad libitum</td>
<td>Obesity, insulin resistance, steatosis, inflammation, ballooning, fibrosis</td>
</tr>
<tr>
<td>Lee et al</td>
<td>2009</td>
<td>Ossabaw</td>
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<td>11-16 mo</td>
<td>Ad libitum</td>
<td>Obesity, insulin resistance, steatosis, ballooning, fibrosis</td>
</tr>
<tr>
<td>Liang et al</td>
<td>2015</td>
<td>Ossabaw</td>
<td>6 mo</td>
<td>8, 10, 12 mo</td>
<td>1000 g/d</td>
<td>Obesity, ballooning (8 wk), fibrosis (16 wk)</td>
</tr>
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<td>Mathan et al</td>
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<td>29-32 wk</td>
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<td>Trask et al</td>
<td>2012</td>
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<td>-</td>
<td>-</td>
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<td>6-8 yr</td>
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<td>Adult</td>
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</tr>
<tr>
<td>Fullenkamp et al</td>
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<td>5-10 mo</td>
<td>11-16 mo</td>
<td>6000 kcal/d</td>
<td>Obesity, insulin resistance</td>
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<td>Ossabaw &amp; Gottingen</td>
<td>0: 2yr G: 6 mo</td>
<td>0: 3 yr G:14-16 mo</td>
<td>Ad libitum</td>
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<td>Ossabaw &amp; Yukatan</td>
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<td>Burgess et al</td>
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<tr>
<td>Lee et al</td>
<td>2009</td>
<td>Yukatan micro pigs</td>
<td>3 mo</td>
<td>10 mo</td>
<td>Ad libitum</td>
<td>Obesity, insulin resistance</td>
</tr>
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</table>
1.4 Histological characteristics and serum markers of pediatric NAFLD

In children and adults, liver biopsy and histological analysis are the most accurate method of diagnosing NAFLD. While the characteristic histological features of NAFLD/NASH, including steatosis, inflammation, ballooning and fibrosis, are the same between adults and children, the manifestation of these features may differ. In both populations, steatosis can be defined as the presence of intracytoplasmic fat, in the form of triacylglycerides, in >5% of hepatocytes (Tiniakos et al., 2010). One of the first distinctions between adults and children in the progression of NAFLD is the distribution of steatosis. In clinical reviews, researchers have found that steatosis in pediatric populations typically begins in periportal zones or has azonal distribution, whereas adults began in perivenular zones (Giorgio et al., 2013; Nobili et al., 2006; Schwimmer et al., 2005;). Additionally, steatosis is primarily macrovacuolar (Berardis and Sokal, 2014). Conversely, NASH is defined by the presence of >5% macrovesicular steatosis, inflammation and hepatic ballooning of any degree (Tiniakos et al., 2010).

Often times indirect measures of NAFLD, such as blood parameters, are used to diagnose NAFLD as an alternative to liver biopsy. Hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the most common serum markers of liver injury, and are typically elevated in adults (Neuschwander-Tetri and Caldwell, 2003; Tiniakos et al., 2010). While serum levels of these aminotransferases may be moderately elevated in children, the often fluctuate, and can even appear to be in normal range in children with NAFLD (Berardis and Sokal, 2014; Giorgio et al., 2013). In pediatric populations, other biochemical abnormalities such as increased levels of gamma-glutamyl transferase (GGT), bilirubin, and albumin have been seen; however,
they typically remain within normal ranges unless the patient is developing more severe forms of NASH or cirrhosis (Giorgio et al., 2013; Roberts, 2007). Pediatric patients with NASH often have an increase in blood levels of pro-inflammatory cytokines such as TNF-α and IL-6, which is thought to be a result of expansion of adipose tissue (Berardis and Sokal, 2014). It is seldom that other blood parameters appear out of range for children, but in adults and models of NAFLD, lactate dehydrogenase (LDH) and blood urea nitrogen (BUN) levels are typically elevated in those with NAFLD (Dhibi et al., 2011; Hashemi Kani et al., 2013).

1.5 Role of inflammatory cytokines and transcription factors in NAFLD

Pro-inflammatory cytokines are known to play a critical role in the pathogenesis of NAFLD and the progression to NASH. In turn, patients who are diagnosed with NAFLD typically have increased levels of cytokines in the blood and liver (Berardis and Sokal, 2014). The result of the increased production of these cytokines is not only to increase local inflammation, but also to promote additional hepatocyte cell injury, leading to the release of damage associated molecular patterns (DAMPs) (Arrese et al., 2016). DAMPs are intracellular molecules that are released or secreted during cellular injury or death, such as high-mobility group box 1, nuclear and mitochondrial DNA, and purine nucleotides (Arrese et al., 2016). For example, tumor necrosis factor alpha (TNFα) not only induces neutrophil infiltration, but also stimulates mitochondrial oxidant production, increasing their sensitivity to undergo apoptosis, and ultimately producing DAMPs. (Braunersreuther et al., 2012; Lee and Friedman, 2011). Additionally, interleukin 1 alpha (IL-1α) has been shown to increase the expression of inflammatory and fibrosis related genes, as well as the expression of adhesion molecules and chemotactic factors, which
promote the progression to NASH (Kamari et al., 2011). By increasing cellular damage and the production of DAMPs, these cytokines further increase hepatocellular injury by activating Kupffer cells via toll-like receptor 4 (TLR4). Upon the ligand binding of DAMPs to TLR4, an intracellular pro-inflammatory signaling cascade is stimulated, and ultimately can lead to the production of more pro-inflammatory cytokines, and the activation of hepatic stellate cells (Kesar and Odin, 2014). In this way, these pro-inflammatory cytokines create a positive feedback loop to perpetuate the occurrence of inflammation and liver injury. Models of NAFLD in juvenile Ossabaw swine had analogous results in liver gene expression, where pigs fed a Western diet also had increased levels of cytokines, as well as TLR4 and ACTA2 (Panasevich et al., 2018).

Fat metabolism is regulated by transcription factors in the liver, such as carbohydrate-response element-binding protein (ChREBP), Sterol regulatory element-binding transcription factor 1 (SREBP1c) and peroxisome proliferator-activated receptor gamma (PPARγ). Typical expression of PPARγ is low in the liver; however, in animal models of fatty liver disease expression of PPARγ is significantly increased (Malaguarnera et al., 2008). This is consistent with other studies that found that increased PPARγ expression was correlated with lipid accumulation in the liver and upregulation of its target gene, CD36 (Inoue et al., 2005). One of the roles of CD36 is to facilitate the transport of long-chain fatty acids into the cell, so it is plausible that the increase in PPARγ expression ultimately leads to increased importation of fatty acids from the HFF diet into hepatocytes, to ultimately be used for TAG synthesis (Rivera et al., 2007). In addition to the diet, many studies examine the role of DNL and its contribution to the development of NAFLD. Previous studies have found that as much as 26% of liver TAG
content was derived from DNL in patients with NAFLD (Tamura and Shimomura, 2005). In mice models, the increase in the rate of DNL can be determined by an increase in regulatory transcription factors such as liver X receptor alpha (LXRα), SREBP-1c, and ChREBP (Cha and Repa, 2006; Tamura and Shimomura, 2005). Interestingly, a study conducted in human NAFLD patients also found that those with NAFLD had decreased ChREBP levels compared to control patients, suggesting that SREBP-1c, not ChREBP, is the transcriptional factor responsible for the transcription of DNL enzymes acetyl-CoA carboxylase alpha (ACACA) and fatty acid synthase (FASN), and that LXRα may not control for the transcription of ChREBP (Higuchi et al., 2008). Because ChREBP is regulated by glucose, it is possible that when insulin resistance occurs in the hepatocytes, it also dysregulates the induction of ChREBP (Cohen et al., 2011; Higuchi et al., 2008).

1.6 Role of one-carbon metabolism and TCA cycle in NAFLD

For detailed information of dysregulation of these pathways in NAFLD, see Fig. 2.5 and Fig. 2.6.
2. A DIET-INDUCED PEDIATRIC MODEL OF NON-ALCOHOLIC FATTY LIVER DISEASE USING NEONATAL IBERIAN PIGS

2.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) represents the emerging cause of pediatric liver disease, affecting approximately 10% of children worldwide (Clemente et al., 2016). In the United States, the prevalence of pediatric NAFLD is estimated to be 0.7% in 2-4 years old infants to 17.3% in adolescents (Schwimmer et al., 2006). The disease is characterized by intrahepatic lipid accumulation without the excessive consumption of alcohol, progressing from the simple steatosis, to nonalcoholic steatohepatitis (NASH), characterized by inflammation and hepatocellular injury, and in advanced cases, cirrhosis and liver failure (Younossi et al., 2016). The increasing presence of pediatric NAFLD and its potential severity in children creates a critical need to further investigate and understand the pathogenesis of NAFLD in pediatric populations. Unfortunately, a translational model does not exist to date that fully recapitulates the characteristics of pediatric liver disease.

Previous studies have identified an association between high-carbohydrate, high-fat diets (Western diet) and the prevalence of pediatric NAFLD, suggesting an important role of fat and sugar intake in the pathogenesis of the disease (Solga et al., 2004). More specifically, consumption of a Western diet is thought to contribute to the pathogenesis of NAFLD by dysregulating the insulin signaling pathway, elevating the rate of de novo lipogenesis and increasing the formation of triacylglycerides (TAG) derived from dietary free fatty acids (Cohen et al., 2011; Lim et al., 2010). Indeed, metabolic syndrome, which is characterized by obesity, diabetes, hyperdyslipemia, and insulin resistance, have been linked to pediatric NAFLD (Marchesini et al., 2003). However, it remains unclear if
metabolic syndrome is necessary for the development of NAFLD, or simply a related consequence of a Western diet. The molecular mechanisms involved in the pathogenesis of NAFLD are still not fully understood. In an attempt to provide insight on the etiology of NAFLD, studies have begun to investigate the metabolomic profile of the liver and blood in cases of NAFLD in order to elucidate metabolic pathways affected by the disease. Of particular interest, one-carbon metabolism has been identified as a key metabolic pathway involved in the pathogenesis of NAFLD. One-carbon metabolism is required for the synthesis of formylated methionyl-tRNA, the catabolism of choline, purines, and histidine, and the interconversion of serine and glycine (Fox and Stover, 2008). In particular, choline metabolism is a key mediator of the progression of NAFLD, due to its involvement in lipid metabolism, methylation-dependent biosynthesis of molecules, and mitochondrial bioenergetics (Corbin and Zeisel, 2012). In addition to one-carbon metabolism, lipidomics and aminomics have revealed key insights into the pathogenesis of NAFLD. Despite these revelations, no pediatric model of NAFLD to date has examined the combination of these metabolic pathways and their interactions with each other in the pathogenesis of the disease.

As the prevalence of NAFLD increases, studies have focused on discovering therapeutic treatments to ameliorate disease progression and potentially reverse the effects of a Western diet. The role of the gut microbiome has become a critical question in the progression of NAFLD, as the liver and gastrointestinal tract are intrinsically linked via portal circulation, and the gut microbiome has been shown to play a role in related factors such as body weight, insulin sensitivity, and glucose metabolism (Brun et al., 2007; Cani and Delzenne, 2007). Subsequently, probiotics have arisen as a potential
therapeutic strategy to manage NAFLD by improving the intestinal barrier, which may inhibit toxins such as lipopolysaccharides (LPS) from entering portal circulation and reaching the liver (Abenavoli et al., 2013). While recent studies have shown that probiotic administration in both pediatric and adult populations can improve the clinical symptoms of NAFLD, conflicting results have also been reported where probiotics were unable to prevent liver steatosis or inflammation in mice with diet-induced NASH (Famouri et al., 2017; Loguercio et al., 2005; Velayudham et al., 2009). It is therefore unclear whether probiotic supplementation presents a successful therapeutic strategy to attenuate the progression of NAFLD. Furthermore, there has been little to no research focused on the use of probiotics to prevent the onset of NAFLD in children.

Finally, most clinical models of NAFLD to date have relied on rodent models, despite differences between rodents and humans in terms of pathogenesis and progression of NAFLD (Larter and Yeh 2008). Pigs represent a more accurate translational model of liver disease, and more specifically, the Iberian pig has recently emerged as a potential research model of NAFLD because of their elevated voluntary feed intake and extreme lipogenic potential (Spurlock and Gabler, 2008; Torres-Rovira et al., 2012). However, a translational model of pediatric liver disease has not been developed to date, as previous NAFLD models used either adults subjects or involved lengthy feeding protocols that extended beyond the infant stage and often provide unrestricted diets (Table 1.1).

Taken together, the objective of this study was to develop a diet-induced, pediatric model of NAFLD in neonatal Iberian pigs by controlled administration of a Western diet, and to investigate molecular mechanisms underlying the pathogenesis of the disease, as well as their interactions. We hypothesized that 13-d old Iberian pigs fed a
high-fat high-fructose (HFF) diet during 10 consecutive weeks would develop a pattern of liver injury resembling pediatric NAFLD. In addition, we sought to determine if supplementation of a commercial probiotic mixture of *Lactobacillus plantarum, Pediococcus acidilactici, Pediococcus pentosaceus, and Bacillus amyloliquefaciens* in a HFF diet will prevent the development of NAFLD.

### 2.2 Material and methods

#### 2.2.1 Animal and experimental design

Experiments were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC #1611) of California State University and conducted in accordance with the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (NRC, 2011).

The following experimental design pertains to Experiment 1. Nine Iberian pigs from the Iberian Pig Research Colony at California Polytechnic State University were weaned at 10 d old and moved into a temperature-controlled room (28°C) with a 12-h/12-h light-dark cycle. Pigs were housed in pairs in $1.524 \times 1.524$ m pens balanced for sex and weight, and bowl-fed ad libitum a commercial milk replacement diet (Soweena® Dry Fat 7-60TM; Merrick Animal Nutrition, Middleton, WI, USA) for 3 d. On d 13, pens were randomly assigned to receive 1 of 2 liquid diets (g · kg body weight (BW)$^{1} \cdot$ d$^{1}$; Table 2.1): 1) control (CON-N; n = 4): 0 g fructose, 11.2 g fat and 199.3 kcal metabolizable energy (ME), and 2) high-fructose high-fat 1 (HFF1-N; n = 5): 10.1 g fructose, 20.6 g fat and 314.8 kcal ME. The CON-N diet was formulated to meet the nutrient requirements of 5-6 kg pigs according to the NRC (2012), whereas HFF1-N diet was formulated to exceed by 60% the ME but to meet the animal protein requirements...
(Tables 2.1 and 2.2). The desire ME in HFF1-N was achieved by increasing both the corn oil (Healthy Brand Oil Corporation, NY, USA) and the animal fat blend (Fat-Pak 80; MilkSpecialities Global, Eden Prairie, MN, USA) content in the CON-N diet, and adding hydrogenated lard (Armour, TX, USA) and fructose (Tate&Lyle, IL, USA), with 25%, 55% and 19% of ME in HFF1-N provided by carbohydrates, fat and protein sources, respectively. The liquid diets were mixed every 3 d using a 24 L food blender (LAR-25LMB; Skyfood, Miami, FL, USA) and tap water, and stored at 4ºC in a commercial refrigerator. Animals were fed 45 mL · kg BW\(^{-1}\) at 6-h intervals 4 times per d starting at 0700, with the liquid diets poured into 2 bowls per pen to decrease competition between the animals. To prevent diarrhea in the piglets, HFF1-N was gradually increased until week 5 of the study by diluting it with CON-N. The first day the experimental diets were fed was considered as d 0 of the study. Body weights were recorded every 3 d, and average daily gain (ADG) was calculated for the 70 d period. Animals were euthanized on d 70 of the study (83 d of age) at 8-h post feeding using an intramuscular injection (4 mg · kg BW\(^{-1}\)) of tiletamine and zolazepam (Telazol®; Zoetis, Parsippany, NJ, USA), followed by an intracardiac injection of pentobarbital sodium (0.4 mL · kg BW\(^{-1}\); Schering-Plough, Union, NJ, USA). Tissue samples from liver and skeletal muscle (i.e. longissimus dorsi; LD) were weighed, frozen immediately in liquid nitrogen and stored at -80ºC, or fixed in 10% neutral buffered formalin until further analysis. Liver, heart and kidneys were removed and weighed (Fig. 2.1D). Quantitative assessment of lean mass was calculated using the hot carcass weight (lb), loin eye area at the 10th rib (square inch) and fat depth over the loin eye (inch), according to the formula 100 × [8.588 +
(0.465 × hot carcass weight) - (21.896 × 10th rib fat depth) + (3.005 × 10th rib loin muscle area))/hot carcass weight, as previously described (Burson, 2010).

The following experimental design pertains to Experiment 2. Twenty four 10-d old Iberian pigs were housed in the same facility and handled as described previously. On d 13, all pigs were randomly distributed to receive 1 of the 4 liquid diets (Table 2.2): 1) CON-N (n = 4): 0 g fructose, 11.2 g fat and 199.3 kcal ME (kg BW\(^{-1}\) · d\(^{-1}\)), 2) CON-P (n = 8): CON + \(6.2 \times 10^4\) cfu · mL\(^{-1}\) (0.2 g · L\(^{-1}\)) probiotics, 3) HFF2-N (n = 6): 21.6 g fructose, 16.2 g fat and 301.0 kcal ME, and 4) HFF2-P (n = 6): HFF2 + \(6.2 \times 10^4\) cfu · mL\(^{-1}\) probiotics. The feeding protocol was as described in Exp. 1, with the HFF2-N and HFF2-P gradually increased until week 5 by diluting diets with CON-N and CON-P, respectively. The CON-N and HFF2-N diets were prepared every 3 d as described in Exp. 1. To prevent bacteria overgrowth, CON-P and HFF2-P diets were mixed daily by adding a commercial mixture of *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum* and *Bacillus amyloliquefaciens* (BiOWiSH® MultiBio 3PS; BiOWiSH Technologies, Cincinnati, OH, USA) to CON-N and HFF2-N using a commercial agitator (Grovhac Inc, Brookfield, WI, USA) at 300 rpm for 2 min. Both CON-N and CON-P diets had the same ingredient composition as described for CON-N in Exp.1, whereas HFF2-N and HFF2-P diets were formulated to exceed by 50% the ME but to meet the pig protein requirements (Tables 2.1 and 2.2). The increase in ME in HFF2-N and HFF2-P diets was achieved by removing the corn oil from the diet, decreasing the animal fat blend and dextrose (Tate & Lyle) content, and adding hydrogenated coconut oil (Tate & Lyle), hydrogenated lard and fructose to the CON-N diet, with 35%, 44% and 19% of ME provided by carbohydrates, fat and protein sources,
respectively. Animal BW, ADG, organ weights and tissue samples were collected as described in Exp. 1.

2.2.2 Probiotic detection and viability

To detect probiotic DNA in the diets, samples randomly collected during the study were diluted at 1:8 ratio in lysogeny broth (LB; Fisher Scientific, Fair Lawn, NJ, USA) and incubated at 37°C for 48 h in an Innova 42 incubator shaker (New Brunswick, Hauppauge, NY, USA). Detection of probiotic DNA was carried out by PCR using bacteria-specific primers for each bacteria (Table A.3) using Taq 2X Master Mix (New England BioLabs Inc, Ipswich, MA, USA) on a T100 thermocycler (Bio-Rad, Hercules, California, USA). Bacterial genomic DNA and nuclease-free water were used as positive and negative controls, respectively. All PCR reactions were performed with the following thermal cycling conditions: 5 min at 95°C, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 68°C for 30 s, and a final extension step at 68°C for 7 min. Product length and yield was analyzed by electrophoresis in 2.5% agarose gels with 1.0 μg/ml of ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). Gels were analyzed using a Gel Doc XR+ system and Image Lab 5.0 software (Bio-Rad). The PCR products were purified from the agarose gel using a NucleoSpin® Extract II kit (Clontech Laboratories, Mountain View, CA, USA) and sequence verified at UC Davis DNA Sequencing Facility (Davis, CA, USA).

To detect probiotic DNA across the gastrointestinal tract, luminal content from proximal jejunum (PJ), distal ileum (DI), colon, cecum, and rectum was flushed with ice-cold 0.9% sterile saline solution into 50 mL sterile tubes (VWR® High-Performance Centrifuge Tubes, VWR, Visalia, CA, USA), flash frozen in liquid N, and stored at -
80°C. Bacterial DNA was isolated in duplicates using a DNeasy PowerLyzer PowerSoil Kit (QIAGEN, Germantown, MD, USA) according to manufacturer’s instructions, and both DNA yield and purity assessed using a NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA, USA). To minimize the risk of contamination, samples from each section and diet were processed on different days. All PCR reactions, and visualization, purification and sequencing of PCR products were carried out as described above. To evaluate probiotic viability in HFF-P and CON-P diets, samples from each diet were collected at 24 h after mixing, aliquoted in triplicates, diluted at 1:100 in 0.1% sterile peptone (Fisher Science, Fair Lawn, NJ, USA) and spread plated with solid glass beads (Fisher Scientific) on Tryptic Soy agar (TSA; BD Difco™, Tryptic Soy Agar, Fisher Scientific) and De Man, Rogosa and Sharpe (MRS) agar (GranuCult™ MRS agar, Millipore Sigma, Burlington, Massachusetts, CA, USA). Plates were incubated at 37°C in aerobic conditions and colonies were counted at 24, 48 and 72 h.

2.2.3 Serum biochemistry

Blood was sampled from the jugular vein at 2 h post-feeding on d 40 and 65. Samples were centrifuged at 2,100 rpm for 15 min at 4°C, and both plasma and serum stored in 1.5 mL tubes at -80°C. Serum lipid and liver biochemistries were analyzed by the Comparative Pathology Laboratory (CPL) at University of California, Davis (Davis, CA, USA) using a Roche Integra 400 Plus (Roche Diagnostics, Pleasanton, CA, USA).

2.2.4 Serum insulin, glucose, leptin, and cytokines

To assess fasting levels of insulin, glucose, and leptin, blood samples were collected at 8 h post-feeding on d 70. Blood was taken from the left ventricle immediately before euthanasia by using a 2-inch 14 G needle, and serum and plasma samples
processed as described above. Insulin, leptin and glucose levels were analyzed by the Animal Health Diagnostic Center (AHDC) at Cornell University (Ithaca, NY, USA) using a human insulin radioimmunoassay (RIA) kit (Millipore Sigma, Burlington, MA, USA), a multi-specie leptin RIA kit (Millipore) and a Cobas c501 (Roche Diagnostics), respectively. Insulin resistance was estimated from fasting glucose and insulin concentrations using the homeostatic model assessment (HOMA), according to the formula: \( \frac{\text{fasting insulin (µU} \cdot \text{mL}^{-1}) \times \text{fasting glucose (mg} \cdot \text{dL}^{-1})}{405}. \) Serum levels of tumor necrosis factor alpha (TNFα), interleukin 1 alpha (IL-1α) and transforming growth factor beta (TGFβ) were analyzed by the Cytokine Core Laboratory (CCL) at University of Maryland (Baltimore, MD, USA) using a pig-specific enzyme-linked immunosorbent assay (ELISA).

### 2.2.5 Triacylglyceride content and histological and proliferative analysis

Total triacylglyceride (TAG) content in liver and LD tissue was determined using the InfinityTM Triglyceride Reagent (ThermoFisher Scientific, Waltham, MA, USA) according to previously described methods (Jouihan 2012). Liver tissue from the left medial lobe was fixed in 10% neutral buffered formalin at room temperature, then transferred to 70% ethanol and submitted to the Comparative Pathology Laboratory (CPL) at the University of California, Davis (Davis, CA, USA). Tissues were embedded into standard paraffin blocks, and 5-µm-thick sections were stained with hematoxylin and eosin (H&E) for histological analysis. Fresh liver and LD samples were also embedded in optimum cutting temperature compound 4583 (Sakura Finetek USA Inc., Torrance, CA, USA), gradually frozen in 2-methylbutane (Sigma-Aldrich) cooled with liquid nitrogen and stored at -80°C. Frozen blocks were cut with a thickness of 5 µm at the Histology
Core at Baylor College of Medicine (BCM; Houston, TX, USA) and stained with Oil Red O. The liver stains were examined by an experienced pathologist (D.I.; CPL) blinded to the treatment groups. Steatosis, ballooning degeneration, Mallory-denk bodies, lobular inflammation, fibrosis and necrosis were semi-quantitatively evaluated for each pig, and a composite lesion score (CLS) was calculated. Zonal distribution of each of these variables was also systematically annotated. Steatohepatitis (NASH) was defined by the presence of macrovesicular steatosis ≥ 1, hepatocellular ballooning ≥ 1, and lobular inflammatory infiltrates ≥ 1, as previously recommended (Wattacheril & Sanyal., 2016).

To determine the number of proliferative cells in pig liver tissue, Ki67 immunostaining was performed on formalin-fixed and paraffin-embedded tissue by the Histology Core at BCM. Sections were pretreated using heat-mediated antigen retrieval with citrate buffer for 20 min, followed by incubation with an anti-Ki67 antibody at 1:1000 for 15 min (Abcam, Cambridge, MA, USA). The Ki67 antibody was detected using the BondTM Polymer Refine Detection Kit (Leica Biosystems, Buffalo Grove, IL, USA) on a Leica Bond-III automated system (Leica Biosystems). Slides were dehydrated through a series of alcohols and xylene, and a coverslip was applied using a permanent mounting media (ThermoFisher Scientific). The immunostained slides were examined under bright field microscopy using an Olympus BX53 microscope fitted with an Olympus DP73 digital camera and cellSens software (Olympus; Center Valley, PA, USA). Each transverse section was divided into 5 quadrants, and 2 fields of view per quadrant were captured with a final magnification of 200 X. The number of Ki67-positive hepatocytes was determined by manual counting in 5 random visual fields (1 field per quadrant) using the multi-point tool in ImageJ software (National Institutes of Health,
Counts were averaged, and the ratio between the labeled Ki67+ (brown-stained) and total hepatocyte count was expressed as percentage for each sample.

2.2.6 Gene expression analysis

Total RNA was extracted from 100 to 150 mg of frozen liver using guanidinium thiocyanate-phenol-chloroform method with TRIzol reagent (ThermoFisher Scientific), and subsequently purified using RNeasy Mini Kit (QIAGEN, Hilden, Germany). Purity and yield of RNA was assessed with a NanoDrop 1000 (ThermoFisher Scientific), whereas integrity was analyzed by electrophoresis in a 1.2% RNA agarose gel with 1.0 μg·ml⁻¹ of ethidium bromide (Sigma-Aldrich). The QuantiTect Reverse Transcription Kit (QIAGEN) was used to reverse transcribed 200 ng of RNA per sample, and resulting cDNA was diluted 1:6 with free RNAse water (Sigma-Aldrich). Quantitative PCR (qPCR) was performed on an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems). All qPCR reactions were performed with the following thermal cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec, and a final dissociation curve (95°C for 15 sec, 60°C for 60 sec, and 95°C for 15 sec). A relative standard curve was used as the qPCR method to measure relative mRNA abundance of candidate and reference genes (Larionov et al., 2005). Duplicate relative amounts from the standard curve for each sample were -log₂-transformed and averaged to improve the normality of the residuals and homogeneity of the variance (Steibel et al., 2009; Manjarin et al., 2011). Normalization of target genes (i.e. ΔCts) was made by dividing the relative quantities of each target gene by the geometric mean of relative quantities of 3 reference genes selected with geNorm software, as previously described
Gene expression results are presented as inverted ΔCts, whereby ΔCts are subtracted from the same positive entire number (i.e. 5 - ΔCt), so higher values correspond to higher relative mRNA abundance (Manjarin et al., 2011). The constant was selected to be an entire number greater than any ΔCt in the data set, so all values presented in the figures are positive.

Primer sequences for reference and target genes are presented as Supplementary Material (Table A.2). Primers were designed to span exon-exon junctions using the PrimerQuest Tool software (Integrated DNA Technologies, Coralville, IA, USA) based on publicly available swine reference sequences deposited in the National Center for Biotechnology Information (NCBI). Primer pairs were tested by PCR using GoTaq Green Master Mix (Promega, Madison, WI, USA) on a T100 thermocycler (Bio-Rad) with the following thermal cycling conditions: 2 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and a final extension step at 72°C for 5 min. Visualization, purification and sequence-verification of the PCR products was performed as described for probiotic primers. Efficiency of the qPCR reaction for each primer pair was calculated from a 2-fold 5-point standard curve according to the formula \( \left( 2^{-1/\text{slope}} - 1 \right) \times 100. \)

2.2.7 Western Blot analysis

Approximately 100 mg of liver and muscle tissues were separately homogenized and sonicated in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP40, and 0.5% of 20% \( \text{(w/v)} \) sodium dodecyl sulfate (SDS) solution) with a mixture of protease inhibitors (500 mM B-glycerophosphate, 10 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 ug leupeptin/mL, and a
protease inhibitor cocktail (P8340-5ML; Sigma Aldrich, St. Louis, MO). Samples were incubated with agitation for 2 h, centrifuged, and supernatant protein content was measured using a BCA assay (Pierce™ BCA Protein Assay Kit, #23225, Thermo Scientific). Thirty five μg of protein extractions from liver and muscle were electrophoretically separated on 7-12% SDS polyacrylamide gels (PAGE; C.B.S Scientific, Del Mar, CA, USA) and transferred to PVDF transfer membranes (Thermo Scientific, Rockford, IL, USA). After blocking with 5% BSA, the membranes were incubated overnight with primary antibodies, followed by 1 h incubation with secondary antibody. Blots were developed using an enhanced chemiluminescence kit (ECL Select Western Blotting Detection Reagent; GE Health Sciences, Buckinghamshire, UK), visualized, and analyzed using a ChemiDoc-It Imaging System® (BIO-RAD, Hercules, CA, USA). For normalization, immunoblots performed with anti-phospho-specific antibodies were exposed to stripping buffer (Pierce Biotechnology, Rockford, IL, USA) and reprobed with non-phospho-specific antibodies against total protein (Cell Signaling Technology, Danvers, MA, USA). Primary antibodies used were against protein kinase B (PKB Ser473 and total; Cell Signaling Technology), microtubule-associated protein 1A/1B light chain 3A (LC3 I/II; Cell Signaling Technology), ubiquitin-binding protein p62 (Cell Signaling Technology), extracellular signal-regulated kinase (ERK Thr 202/204; Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology).

2.2.8 Metabolomics

Targeted metabolomic assays for primary metabolomics, aminomics, and lipidomics were performed on plasma and liver samples using protein precipitation
extraction with ultra-performance liquid chromatography tandem quadrupole mass spectrometry (UPLC-MS) using modified previously published methods (Townsend et al. 2013). Briefly, 25 µL of plasma or 40-60 mg liver samples were added to 1.5 mL tubes before being spiked with 20 µL isotopically-labeled surrogates, followed by 750 µL chilled methanol. Samples were then vortexed 1 min prior to being centrifuged at 2,000 X g for 10 min. The supernatant was transferred to 1.5 mL high performance liquid chromatography (HPLC) amber glass vials, dried by centrifugal vacuum evaporation, and reconstituted in 3:1 methanol:acetonitrile containing 100 nM of 1 cyclohexyl ureido, 3 dodecanoic acid (Sigma-Aldrich, St. Louis, MO, USA). The reconstituted solution was vortexed 1 min and filtered at 0.1 µm by centrifugation at 2,000 g for 10 min through PVDF Durapore membranes (Millipore, Billerica, MA, USA). All UPLC-MS analyses, including primary metabolomics, aminomics, and lipidomics, were conducted on a Waters Acquity I-Class UPLC (Waters, Milford, MA, USA) coupled with an API 4000 QTrap (Sciex, Framingham, MA) using multiple reaction monitoring quantified with AB Sciex MultiQuant version 3.0. For the primary metabolomics assay, metabolites were separated using a 150 X 2.0mm Luna NH2 column (Phenomenex, Torrance, CA) and detected by negative ion mode electrospray ionization (Townsend et al. 2013; Bajad et al. 2006). For the aminomics assay, metabolites were separated using a 150 × 2.1 mm Atlantis HILIC column (Waters, Milford, MA, USA) and detected by positive ion mode electrospray ionization (Townsend et al., 2013; Wang et al., 2011). For the lipidomics assay, metabolites were separated using a 150 × 3.0 mm Prosphere HP C4 column (Grace, Columbia, MD, USA) and detected by positive ion mode electrospray ionization (Townsend et al. 2013; Rhee et al. 2011).
2.2.9 Statistical analysis

Categorical variables were analyzed by ANOVA using a mixed model in SAS 9.2 (PROC MIXED; SAS Institute Inc., Cary, NC, USA) that included diet, probiotic and their interactions as fixed effects, and pen nested in treatment as random effect. A repeated measurement statement was included to analyze the BW of the animals, with the structure of the covariance selected based on smallest Akaike information criterion. Normality of the residuals and presence of outliers were assessed with PROC UNIVARIATE (SAS). Non-normally distributed parameters were power transformed by a parameter $\varphi$ whose optimal value was estimated using the maximum likelihood (ML) method (Piepho 2009). Multiple comparisons were accounted for with Tukey test. Data is presented as least square means ± SE. Significant effects were considered at $P \leq 0.05$. Differences in hepatic histological features between groups were analyzed by Kruskal-Wallis with Bonferroni multiple comparisons test for non-parametric data in SAS (PROC NPAR1WAY and PROC RANK).

Metabolomics data was imported into the Primer-E software (v.6; Primer-E Ltd., Plymouth, United Kingdom), log transformed into a normal distribution approximation and analyzed with a Euclidean distance matrix. A non-parametric multivariate analysis of variance (PERMANOVA; Primer-E Ltd) was used for testing the null hypothesis of no difference between groups, based on the parameters’ permutation of residuals under a reduced model, 9999 permutations and a type III sum of squares. Further multivariate modeling was conducted using partial least squares-discriminant analysis (PLS-DA) with MetaboAnalyst 4.0 (Xia Lab, McGill University, Montreal, Quebec, Canada; Chong et al. 2018; Xia and Wishart 2016), to visualize any clustering effects related to the diet and
probiotics. Data were Pareto scaled and cross validated using leave-one-out-validation (LOOV) to assess model performance using Q2 and R2 metrics. Identification of metabolites differentially expressed between groups was performed by a 2-way ANOVA with the same random and fixed effects described above. The Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) with a cutoff of 5% was used to adjust the reported P-values to control for the rate of false discoveries.

2.3 Results

The effect of CON-N diet on BW, ADG, organ weight, serum parameters, histology, and gene expression was not significantly different between pigs on Exp.1 (n = 4) and Exp. 2 (n = 4), so data for these animals was combined for statistical analysis (CON-N; n = 8).

2.3.1 Experiment 1

Results for Exp. 1 are presented as Supplementary Material and in Table A.1. There were no differences between groups in initial age, BW on d 0 and 65, ADG and fasting leptin levels on d 70. Insulin and glucose were not measured in HFF1-N animals. Compared to CON, HFF1-N increased relative liver weight on d 70 ($P \leq 0.01$; Table A.1), serum levels of alanine aminotransferase (ALT) and total protein on d 40 ($P \leq 0.01$ and $P \leq 0.05$, respectively), and non-esterified fatty acids (NEFA) on d 65 ($P \leq 0.05$). In addition, HFF1-N decreased total cholesterol ($P \leq 0.01$) and low-density lipoproteins (LDL; $P \leq 0.01$) on d 65, and blood urea nitrogen (BUN) on both d 40 and 65 ($P \leq 0.01$). The grade of steatosis was higher in HFF1-N compared to CON-N ($P \leq 0.01$), with all HFF1-N livers showing macrovesicular steatosis with centrilobular distribution. Ballooning degeneration, Mallory-Denk bodies, inflammation, necrosis, and fibrosis were
not observed in CON-N and HFF1-N, whereas CLS was higher in HFF-N than in CON-N ($P \leq 0.05$). None of the CON-N and HFF1-N animals was classified as NASH. Compared to CON, HFF1-N increased gene expression of toll-like receptor 4 (TLR4; $P \leq 0.05$), whereas transforming growth factor beta (TGFβ), tumor necrosis factor alpha (TNFα), interleukin 1α (IL1α), interleukin 1β (IL1β) and smooth muscle alpha (α)-2 actin (ACTA2) did not differ between diets (Fig. A.1).

2.3.2 Probiotic detection and viability

The remainder of the results will pertain to Experiment 2. To facilitate readership through the manuscript, when diet × probiotic interaction (i.e. CON-N, CON-P, HFF2-N and HFF2-P) is not significant only the main effect of diet (i.e. CON vs HFF2) and probiotics (i.e. N vs P) will be reported and discussed. Figure 2.3A presents colony counts for diets at 24, 48 and 72 h after plating in TSA and MRS agar plates. Tryptic soy agar is a general-purpose media, while MRS is selective for *Lactobacillus*. Initial inoculate count was $6.2 \times 10^2$ cfu · mL$^{-1}$ for CON-P and HFF2-P in both agar plates. Compared to CON-P, HFF2-P increased colony counts at 72 h in TSA ($P \leq 0.05$; $15.7 \pm 2.4$ cfu · mL$^{-1}$ vs $10.0 \pm 2.4$ cfu · mL$^{-1}$) but not in MRS ($6.0 \pm 0.9$ cfu · mL$^{-1}$ vs $5.3 \pm 0.9$ cfu · mL$^{-1}$). A small number of background colonies of different size and color than those formed by probiotics were detected in all agar plates due to the preparation of the diets with tap water and in non-sterile conditions. The PCR amplification of DNA isolated from the 4 diets with strain-specific primers for *Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus plantarum* and *Bacillus amyloliquefaciens* yielded products of the expected size and sequence in both HFF2-P and CON-P, whereas CON-N and HFF2-N were negative (Fig. 2.3A). Similarly, PCR products corresponding to *Bacillus*
*amyloliquefaciens* were detected at 8-h post-feeding in contents from PJ, DI, cecum, colon and feces in CON-P and HFF2-P but not in HFF2-N or CON-N diets (Fig. 2.3B). Amplification of PCR products for both Pediococcus strains were detected in PJ, DI, cecum, colon and feces in all diets but CON-N, whereas *Lactobacillus plantarum* was not detected in the small and large intestine contents of any of the pigs (Fig. 2.3B).

**2.3.3 Body weight, body composition, average daily gain, and tissue weights**

Age, initial BW, and average daily gain (ADG) between d 0 and 70 did not differ among the 4 diet groups. Pig BW was higher in CON-P compared to HFF2-N and HFF2-P between d 55 and d 70 ($P \leq 0.05$), and on d 55, 58 and 67 compared to CON-N ($P \leq 0.05$; Fig. 2.1C). Loin muscle area on d 70 was higher in CON compared to HFF2 ($P \leq 0.05$), whereas percentage of lean mass, hot carcass weight and dorsal fat remained constant across treatments (Fig. 2.1E). Relative liver weight (g liver \cdot kg BW$^{-1}$) was higher in HFF2-N compared to CON-N and CON-P ($P \leq 0.001$), and increased further in HFF2-P ($P \leq 0.05$), while relative kidney weight was higher ($P \leq 0.01$) in HFF2 compared to CON (Fig. 2.1D). Relative heart weight did not differ between diets.

**2.3.4 Hormones, glucose and pro-inflammatory cytokines**

Probiotic (P) increased fasting insulin levels and HOMA values ($P \leq 0.05$), and tended to increase glucose levels ($P \leq 0.1$) compared to non-probiotic (NP) animals, whereas leptin was higher ($P \leq 0.05$) in HFF2 compared to CON (Fig. 2.3C). Serum levels of TGF-$\beta$, IL1$\alpha$ and TNF$\alpha$ remained unchanged between groups (Fig. 2.3C).

**2.3.5 Serum biochemistry**

Serum biochemistry is presented in Figure 2.1F, Figure 2.6.B, and Figure 2.7.B. Compared to CON, HFF2 increased on d 40 and 65 the serum levels of alanine
aminotransferase (ALT; \( P \leq 0.001 \)), aspartate aminotransferase (AST; \( P \leq 0.01 \) and \( P \leq 0.001 \)), alkaline phosphatase (ALP; \( P \leq 0.001 \)), gamma glutamyl transferase (GGT; \( P \leq 0.05 \) and \( P \leq 0.01 \)), lactate dehydrogenase (LDH; \( P \leq 0.01 \) and \( P \leq 0.001 \)), whereas total bilirubin increased (\( P \leq 0.01 \)) only on d 65. Likewise, TAG levels increased (\( P \leq 0.05 \)) on d 40 in HFF2 compared to CON. Blood urea nitrogen was also higher (BUN; \( P \leq 0.001 \)) on d 40, and total protein (\( P \leq 0.01 \) and \( P \leq 0.001 \)) and albumin (\( P \leq 0.001 \)) on d 40 and 65 in HFF2 compared to CON. Conversely, on d 40 and 65 of the study HFF2 decreased total cholesterol (\( P \leq 0.001 \) and \( P \leq 0.01 \), respectively), and high (HDL; \( P \leq 0.05 \) and \( P \leq 0.001 \)) and low-density lipoproteins (LDL; \( P \leq 0.001 \) and \( P \leq 0.05 \)) compared to CON, whereas LDL/HDL ratio was lower only on d 40 (\( P \leq 0.05 \)). Serum levels of non-esterified fatty acids (NEFAs) remained constant across groups.

2.3.6 Triacylglyceride content and histological analysis

Compared to CON, HFF2 diet increased TAG content in both liver (\( P \leq 0.001 \); Fig. 2.2D) and LD (\( P \leq 0.001 \)). Steatosis was classified as macrovesicular with centrilobular distribution in CON animals, and macro and microvesicular with periportal or diffuse distribution in the HFF2 group (Table 2.3), and was increased in HFF2 compared to CON (\( P \leq 0.01 \); Fig. 2.2A and F). Ballooning grade was significantly higher in HFF2-N than in CON-N and CON-P (\( P \leq 0.01 \)), and increased further in HFF2-P compared to HFF2-N (\( P \leq 0.05 \)), whereas the score of Mallory-Denk bodies was higher only in HFF2-P compared to CON-N and CON-P (\( P \leq 0.05 \); Fig. 2.2A and Table 2.3). Inflammation and necrosis were increased in HFF2 compared to CON (\( P \leq 0.001 \); Fig. 2.2A and Table 2.3). Composite lesion scores (CLS) were also higher in HFF2 than in CON diets (\( P \leq 0.05 \); Fig. 2.2C and Table 2.3) but fibrosis was not observed in any of the
liver samples (Fig. 2.2F and Table 2.3). Four pigs (66.6%) in HFF2-N and 6 (100%) in HFF2-P developed NASH, whereas none of CON-N and CON-P was classified as NASH due to the absence of hepatocellular ballooning ($P \leq 0.01$; Fig. 2.2B). Finally, Ki67 immunostaining showed an increase in Ki67 proliferative cell index in HFF2 compared to CON pigs ($P \leq 0.001$; Fig. 2.2E and F).

2.3.7 Gene expression and protein analysis

Cyclin-G-associated kinase (GAK), vacuolar protein sorting-associated protein 4A (VPS4A) and porphobilinogen deaminase (HMBS) were selected as reference genes in geNorm. Inclusion of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not necessary as the pairwise variation parameter (V) was already below the suggested cutoff value of 0.15 (Fig 2.4A-B). Gene expression data is presented in Fig. 2.4C-E. Compared to CON, HFF2 increased gene expression of TNFα ($P \leq 0.05$), TGFβ ($P \leq 0.01$), IL1α ($P \leq 0.05$), TLR4 ($P \leq 0.05$) and peroxisome proliferator-activated receptor gamma (PPARγ; $P \leq 0.01$), and decreased carbohydrate-responsive element-binding protein (ChREBP; $P \leq 0.001$). Expression of IL1β, ACTA2, sterol regulatory element-binding protein 1 (SREBP-1c), liver X receptor alpha (LXR-α), fatty acid synthase (FASN), acyl-CoA synthetase long-chain 1 (ACSL-1), and fatty-acid-binding protein (FABP) did not change between groups.

Protein expression levels are represented in Fig. 2.3D. In the liver, probiotic (P) increased protein levels of p62 ($P = 0.002$), LC3I/II ($P \leq 0.001$) and ERK ($P \leq 0.001$), and tended to increase PKB ($P = 0.052$) compared to non-probiotic (NP) animals. Compared to CON, HFF2 had increased protein levels of LC3I/II ($P \leq 0.001$) in muscle. There was no difference in protein levels of PKB or p62 in the muscle.
2.3.8 Metabolomics

Metabolomic data is represented in Fig. 2.5, 2.6 and 2.7. A total of 224 and 218 metabolites were detected in plasma and liver, respectively. The multivariate analysis showed that there was no probiotic effect, therefore, only the main effect of diet (i.e. CON vs HFF2) will be reported and discussed. We subsequently identified 105 and 64 metabolites in liver and plasma, respectively, with significant changes between CON and HFF2 pigs using a false discovery rate (FDR) of $P \leq 0.05$ in a two-way ANOVA. Compared to CON, pigs fed HFF2 diet showed an increase in cholesterol esters (CE) in the liver, while there was a significant decrease in cholesterol in the plasma. However, the major bile salts glycocholate and taurocholate, were significantly increased in both the liver and the plasma. Levels of diacylglycerols (DAG), specifically C36:1 and C36:2, were increased in the HFF2 livers compared to CON, but showed no change in the plasma. In the HFF2 liver, triacylglycerols (TAG) with carbon chain lengths of 52 to 58 were decreased compared to CON, whereas carbon chain lengths of 44 to 50 were increased compared to control, suggesting a preference towards shorter chain TAG. In the plasma, pigs fed HFF2 diets had significantly decreased levels of TAG compared to CON, with an exception of C50:0, C50:1, and C50:2.

Upon examining phospholipids, there was an inconsistent change in levels of sphingomyelin (SM). In the liver, pigs fed HFF2 diets had increased SM with carbon chain lengths of 16 to 18 and 24, and decreased SM with carbon chain lengths of 20 to 23, when compared to CON. Similarly in the plasma, SM with chain lengths of 24 were increased in HFF2 compared to CON, and chain lengths of 15, 20 and 21 were decreased. When looking at glycerophospholipids, we found that levels of phosphatidylcholines
(PC) were decreased in HFF2 livers compared to CON, with an exception of C32:0, C32:1, C38:2, and C38:3. Only phosphatidylethanolamine (PE) C36:0 was significantly different in the liver, where pigs fed HFF2 diets had significantly increased levels compared to CON. In the plasma, there was far fewer significantly changed glycerophospholipids. Compared to CON, pigs fed HFF2 diets had significantly decreased levels of PC 34:2, PC 38:5, and PE 36:1, and significantly increased levels of PC 38:2. In the liver, the levels of glycerophospholipid metabolites, lysophosphotidylcholines (LPC) and lysophosphoethanolamines (LPE), were both significantly elevates in pigs fed HHF2 diets compared to CON. Contrarily, in the plasma pigs fed HFF2 had decreased levels of LPC and no change in LPE compared to control.

Metabolites connected to β-oxidation, such as carnitine and oleyl carnitine, were significantly increased in HFF2 livers compared to CON. Similarly, plasma levels of carnitine and acetyl carnitine were significantly increased in pigs fed HFF2 compared to CON. In the HFF2 liver, certain metabolites pertaining to one carbon metabolism were decreased, such as acetylcholine, 4-pyridoxate, betaine, glycine and choline; whereas others, such as serine, methionine, and homocysteine, were increased compared to CON. Plasma levels of pigs fed HFF2 showed parallel results with significant decreases in 4-pyridoxate, betaine, glycine and choline, and an increase in homocysteine. In HFF2 livers there was a marked decrease in nucleic acids guanine, uridine, xanthine, adenosine, cytosine, xanthosine, and guanosine compared to CON. In the plasma, a decrease in the nucleic acid component deoxycytidine was seen in pigs fed HFF2 compared to CON.

Metabolites involved in the urea cycle and the tricarboxylic acid (TCA) cycle, such as arginine, ornithine, fumarate and malate, were significantly increased in HFF2
livers compared to control. While these metabolites did not show significant changes in the plasma, HFF2 pig had a significant decrease in citrulline and an increase in succinate, both utilized in the urea cycle and the TCA cycle, respectively. Metabolomics showed a significant decrease in creatinine, a biproduct of the urea cycle, and a significant increase in lactate, the alternative route for pyruvate, in the livers of pigs fed HFF2 diets. The decrease in creatinine was also seen in the plasma. Amino acids and their derivatives were significantly altered in pigs fed HFF2 diets, where there was an increase in isoleucine, kynurenic acid, kynurenine, leucine, phenylalanine, phosphotyrosine, and tyrosine, and a decrease in anthranilic acid, glutamine, histamine, and lysine. Similar results were found in the plasma, where pigs fed HFF2 diets had increased levels of kynurenine, phenylalanine, and tryptophan, and decreased levels of glutamine and lysine. Several metabolites unrelated to the previously mentioned pathways were altered in the livers of pigs fed HFF2 diets compared to CON, including a decrease in hippurate and an increase in oxalate and sorbitol. Contrarily, the plasma of HFF2 fed pigs showed a decrease in oxalate, as well as a decrease in serotonin when compared to CON.

2.4 Discussion

2.4.1 Feeding a high-fructose high-fat diet for 10 weeks induced NASH

Non-alcoholic fatty liver disease represents an emerging chronic liver disease in pediatric populations, increasing the risk of liver transplant in infants, as well as the development of long-term complications such as cardiovascular disease, type 2 diabetes and liver-related death at adult age (Berardis and Sokal, 2014). Despite the high prevalence and severe complications, the etiology and pathophysiology of pediatric NAFLD is not fully understood, in part due to the lack of animal models dedicated to the
liver disease in children. Pigs represent an excellent translational model for liver research, due to the similarities between pigs and humans in liver size and structure, gastrointestinal physiology and lipoprotein profiles (Chapman and Goldstein, 1976; Douglas, 1972; Spurlock and Gabler, 2008; Swindle et al., 2012). Particularly, the Iberian and the Ossabaw pig, which is a direct descendant from the Iberian breed, have recently emerged as excellent research models of metabolic syndrome and NAFLD, because of their elevated voluntary feed intake and extreme lipogenic potential (Mayer and Brisbin 1991; Torres-Rovira et al. 2012; Gonzáles-Bulnes et al. 2014; Fernández-Fíga res et al. 2007). However, most diet-induced models of NAFLD in Iberian and Ossabaw pigs, as well as in other breeds such as Göttingen, Bama and Yucatan minipigs, have been recreated only in adult animals, in which feeding starts around 6 months of age (Table 1.1). Further, causative mechanisms such as *ad libitum* feeding, choline/methionine deficient diets or inclusion of sodium cholate were needed to induce NAFLD, decreasing the significance of the translational models to investigate the consequences and molecular mechanisms elicited by specific components in the diet.

Given the data previously reported in pig models and their limitations, the aim of the present study was to develop and characterize the first pig model of pediatric NAFLD in which animals were fed controlled amounts of a “Western diet” that met the essential nutrient requirements while providing a 50% excess in daily recommended caloric intake (National Research Council, 1998). Notably, 100% of the pigs fed the HFF2 diet presented steatosis and increased levels of hepatic enzymes in blood after 5 weeks, and 83% were classified as NASH at 83 d of age. With Iberian pigs reaching maturity at 160-180 d, 11-week-old animals correspond approximately to 8 or 9-year-old children, which
to our knowledge represents the youngest translational age in a diet-induced pig model of pediatric NAFLD (Table 1.1). The fast time course of the disease is likely related to the fixed leptin receptor (LEPR) mutation in the Iberian breed, which similarly to the Zucker fatty rat (Phillips et al., 1996), is associated with a leptin resistance phenotype characterized by hyperphagia, obesity, and elevated levels of circulating leptin in comparison to other porcine breeds (Fernández-Fígares et al., 2007; Munoz et al., 2009).

In addition, feeding diets in liquid form instead of regular chow may have enhanced liver injury by shifting the microbiome (Mastrocola et al., 2018), decreasing the satiety signals (Pan et al., 2011) and/or increasing BW (DiMeglio and Mattes, 2000). Interestingly, HFF1 and HFF2 diets provided the same caloric intake, but only HFF2-fed animals developed histopathological lesions observed in pediatric NASH, including macro and microvesicular steatosis, hepatocellular ballooning, Mallory hyaline, lobular inflammation and necrosis (Schwimmer et al., 2005). While it is not possible to identify the dietary components responsible for the differences in liver damage between diets, the critical role in the progression to NASH of ingredients in HFF2 such as cholesterol and saturated fats has been documented before (Tu et al., 2017; Wang et al., 2017). The effect of HFF2 fat components in promoting hepatic TAG accumulation is further outlined by the observed increase in lipids with shorter carbon chain and lower number of double bonds in the liver of HFF2 pigs, likely attributable to the elevated content in saturated medium-chain fatty acids in the coconut oil.

Although a pattern of hepatic lesions without fibrosis has been observed in up to 30% of children diagnosed with type 1 NASH (Carter-Kent et al., 2009), the lack of perisinusoidal or portal fibrosis in the HFF2 group was unintended and represents a
limitation in our pediatric model, likely due to the short duration of the study. Neonate Iberian pigs were fed for only 10 weeks, whereas fibrotic lesions were first observed at 16 and 32 weeks in previous diet-induced pig models of NASH (Gutiérrez-Vidal et al., 2018; Lee et al., 2009; Li et al., 2016; Liang et al., 2015; Panasevich et al., 2018; Schumacher-Petersen et al., 2019; Yang et al., 2015). Elevated levels of cell proliferation in the liver of HFF2 pigs, as demonstrated by Ki67 staining (Fig. 2.2), may indicate initial progression towards a neoplastic condition associated with NASH. Cirrhotic NASH is known to progress to hepatocellular carcinoma (HCC); HCC incidence with cirrhotic NASH ranges between 2.4-12.8% (Perumpail et al., 2015). Furthermore, there is a growing body of literature demonstrating HCC incidence in non-cirrhotic NASH (Perumpail et al., 2015). The pathogenesis of NASH-HCC is incompletely understood and multifaceted; proposed mechanisms include genetic or epigenetic modifications and alterations in metabolic, immunologic, and endocrine pathways (Kutlu et al., 2018). Many studies demonstrate the role of bile acids (BAs) in hepatocarcinogenesis (Kim et al., 2007; Xie et al., 2016; Yamada et al. 2018; Yoshimoto et al., 2013). Mice lacking expression of farnesoid X receptor (FXR), a BA-activated nuclear receptor that serves as the master regulator of BA homeostasis, spontaneously developed HCC (Kim et al. 2007). Secondary BAs, which are BAs that have been modified by bacterial enzymes in the intestinal lumen, promote proliferation of hepatocytes through multiple mechanisms, including promotion of the mTOR pathway (Yamada et al., 2018) and induction of a HCC-related cellular phenotype (Yoshimoto et al., 2013). Strikingly, HFF2 pigs were demonstrated to have elevated concentrations of primary BAs taurocholate and glycocholate in their livers, elevated total plasma BAs, elevated relative abundance of
taurodeoxycholic acid (TDCA) in the colon, and increased BA synthesis (Smith et al., under review). Deoxycholic acid (DCA), a secondary BA, is able to permeate cell membranes. Thus, it is reasonable to speculate that elevated levels of TCDA in the colon would translate to elevated hepatic exposure to TDCA, due to its ability to pass from the colon into enterohepatic circulation and permeate hepatocellular cell membranes. Taken together, increased exposure to genotoxic BAs may represent one mechanism by which hepatocellular proliferation was promoted in HFF2 pigs.

2.4.2 Hepatic dysregulation of one-carbon metabolism in pediatric NAFLD

While pediatric NAFLD has been associated to obesity, insulin resistance (IR) and type-2 diabetes in children, the primary metabolic dysregulations leading to TAG accumulation in the liver are not well understood, as patients are diagnosed after developing the disease. The prevailing two-hit model of NASH implies IR to promote the initial steatosis, followed by inflammation and fibrosis in response to multiple factors including oxidative stress and mitochondrial dysfunction (Biczo et al., 2018). This study reports for first time in a large animal model of pediatric NASH the development of significant hepatomegaly and over 10-fold increase in hepatic TAG accumulation without changes in BW, IR and systemic inflammation, suggesting a diet-induced metabolic abnormality in the liver at the center of the pediatric disease. To identify potential mechanisms accounting for the HFF2-induced hepatic steatosis, we quantified the expression of different genes involved in fatty acid and cholesterol metabolism in the liver. Consistent with the lack of obesity and IR, expression of enzymes and transcription factors involved in de novo lipogenesis and fatty acid trafficking in the hepatocyte remained unaltered or decreased in response to the HFF2 diet, and only PPARγ, which
has been previously associated with regulation of hepatic fatty acid uptake (Yu et al., 2003), increased in response to the HFF2 diet.

A key finding in our model was the markedly decrease in choline availability in liver and plasma, which in rodent models on non-obese NAFLD has been shown to reduce the substrate for hepatic synthesis of PCs, impairing the assembly of VLDLs and the subsequent fat export from the liver (Itagaki et al., 2013; Rinella et al., 2008). Further supporting the prediction of a blockage in hepatic TAG outflow is the decrease in circulating levels of cholesterol, LDLs and HDLs in HFF2 compared to control pigs, despite the significantly higher fat content in the HFF2 diet. A link between dysfunctional VLDL metabolism and the progression to NASH has also been described in clinical studies in adults (Fujita et al., 2009), and a growing body of literature suggests the development of NAFLD in non-obese patients with lower prevalence of hypertriglyceridemia and IR but increased hepatic inflammation (Dela Cruz et al. 2014; Margariti et al., 2013). Although the current study does not address the origin of choline decline, our diets were formulated to meet the choline requirements in growing pigs (National Research Council, 1998), suggestive of a metabolic rather than a dietary-induced choline deficiency in the NAFLD animals. Within hepatocytes, choline may enter the CDP-choline pathway for PC synthesis or serve as source of methyl groups via oxidation to betaine, which is then used for remethylation of homocysteine to methionine to further generate SAM, the main methyl donor in the liver (Corbin et al., 2012; Obeid, 2013). Consequently, the observed decrease in choline and betaine levels along with higher levels of methionine in HFF2 animals suggest that an excess of methylation activity may have reduced the choline and betaine pool in the liver. Evidence of
decreased choline and betaine availability in the context of hepatic steatosis has been shown in adults with NASH (Sokooian et al., 2017) and patients receiving parenteral nutrition, in which choline replacement led to a reversal of hepatic fat infiltration (Buchman et al., 2001). Similarly, supplementation of choline in a mixture with vitamin E and docosahexaenoic acid improved steatosis in pediatric NASH (Zöhrer et al., 2017).

Albeit increased levels of homocysteine are commonly found in adults with NAFLD (de Carvalho et al., 2013; Dai et al., 2016; Hu et al., 2016), and correlated with the severity of liver damage in children (Pastore et al., 2014), the mechanisms leading to hyperhomocysteinemia are poorly understood. Besides remethylation into methionine, homocysteine is also converted into cysteine via transsulfuration by the enzyme cystathionine β-synthase, which requires both serine and vitamin B6 (Ubbink et al., 1996). The decrease in vitamin B6 catabolic product 4-pirodoxate in liver and plasma of HFF2 pigs, along with the accumulation of serine in liver, is supportive of the idea that impaired catabolism of homocysteine via the transsulfuration pathway may have contributed to the increase in homocysteine and methionine in our animals. Concomitantly, a deficiency in the vitamin B6, which is also crucial coenzyme of serine hydroxymethyltransferase for the synthesis of 5,10-methylenetetrahydrofolate in the folate cycle (Ubbink et al., 1996), may explain the decrease in purines and pyrimidines observed in the HFF2 group. While increased levels of homocysteine are well described in NAFLD patients, conflicting results exist in the literature regarding changes in methionine metabolism in diet-induced NAFLD. Given that an impairment in the methionine adenosyltransferase (MAT) would be expected to increase homocysteine but also to reduce SAM availability in the liver, this pathway is unlikely to have contributed
to methionine elevation in HFF2 pigs. Previous studies in mouse NAFLD models suggest hepatic methionine levels to be both time and disease-dependent, with increased levels associated to early changes in hepatic transsulfuration reactions (Kwon et al., 2009), and a depletion in longer feeding trials in parallel to the progression to NASH (Pacana et al., 2015). Accordingly, the increase of hepatic methionine stores in our study may be a temporary event, caused by either a methionine overload in response to higher homocysteine levels, or a compensatory mechanism to maintain SAM availability under NAFLD conditions.

2.4.3 Nitrogen metabolism is dysregulated in pediatric NAFLD

In addition to the dysregulation of carbon one metabolism, NAFLD is associated to the dysregulation of mitochondrial function (Wei et al., 2008). Specifically, NAFLD has been shown to be linked to the disruption of the urea cycle and the TCA cycle (De Chiara et al. 2018; Wei et al. 2008). In the present study, pigs fed an HFF2 diet significantly altered levels of amino acids and metabolites associated with the urea cycle and the TCA cycle (Figure 2.6). Of particular interest was the decrease in blood levels of BUN and the increase in lactate found in the liver of pigs fed HFF2 diets. Urea is the main biproduct of the urea cycle, and is excreted from the liver through the blood as BUN. Therefore, the decrease in BUN levels is indicative of a urea cycle disorder (Steiner and Cederbaum, 2001). Although ammonia levels were not measured, we can assume that a decrease in the urea cycle would cause an accumulation of ammonia, which have been shown to lead to the progression of NAFLD (De Chiara et al. 2018). Furthermore, the increase in lactate levels suggests a loss of mitochondrial function and ultimately an increased reliance on ‘anaerobic’ metabolism, and has also been associated
with hepatic disorders (Ghosh and Callier, 2012; Nolan et al., 2008). The dysregulation of the TCA cycle and the urea cycle can cause an increase in energy substrates available, which can then be shunted towards the production of TAGs, and ultimately increase steatosis (Kowalski et al., 2016). Overall, pigs fed a HFF2 diet showed an increase in amino acids catabolized through the TCA cycle, which could be due to a build-up if these cycles were deregulated. Additionally, pigs fed HFF2 diets showed decreased loin eye area, as well as decreased activity levels (shown in unpublished data), suggesting an increase in the catabolism of proteins and ultimately the increase in the amino acid pool.

### 2.4.4 Probiotic supplementation did not ameliorate HFF-induced NAFLD

Recently, studies have shown that probiotic administration in both pediatric and adult populations can improve the clinical symptoms of NAFLD (Famouri et al. 2017, Loguercio et al. 2005). In the present study, a probiotic cocktail of *Lactobacillus plantarum*, *Bacillus amyloliquefaciens*, *Pediococcus pentocaceus*, and *Pediococcus acidilactici* was administered to the pigs at $6.2 \times 10^4$ cfu/mL. The colony counts of our probiotic cocktail on MRS and TSA plates demonstrated that probiotics were viable in the milk, and thus had the potential to survive in the digestive tract of the pig. Although there were colony counts in milk samples that were not supplemented with probiotics, due to differences in colony morphology, it was concluded that bacteria came from tap water as opposed to cross contamination. Furthermore, a PCR analysis using probiotic specific primers for all bacteria in the milk validated the primers and supported that no cross contamination had occurred. Finally, PCR analysis of the gut contents suggested that bacterial DNA from *B. amyloliquefaciens*, *P. pentocaceus*, and *P. acidilactici* could be found in all portions of the gut, with an exception of the PJ. Although bands could be
seen in the contents of pigs that were not supplemented with probiotics, the PCR analysis of the milks leads us to believe that this is likely due to endogenous bacteria in the gut.

In rodent models of NAFLD, each of these probiotic species have been shown to have therapeutic effects against NAFLD, including protection against obesity, decreasing steatosis, decreasing liver cytokine levels, and downregulating genes associates with lipid uptake and de novo lipogenesis (Kim et al., 2018; Li et al., 2014; Moon et al., 2014; Shi et al., 2017; Zhao et al., 2012). It is known that lipopolysaccharides (LPS) derived from Gram-negative bacteria in the gut can bind with TLR4 in the liver, which plays a role in liver injury and inflammatory signaling. Probiotics are thought to attenuate NAFLD by improving the intestinal barrier, which may inhibit LPS from entering portal circulation and reaching the liver (Xue et al., 2017). Contrary to these findings, pigs fed HHF2-P did not have a reduction in the clinical symptoms of NAFLD. In fact, pigs fed HFF2-P diet had increased presence of steatosis, Mallory-Denk bodies, and hepatic ballooning. Additionally, probiotic supplementation did not alter the intestinal barrier, as there was no changes in villi length, crypt depth, or gene expression of occludin (Smith et al., under review). Though probiotic supplementation is generally regarded as a successful therapeutic strategy, studies have shown did not prevent liver steatosis or inflammation in mice with diet-induced NASH (Velayudham et al., 2009). While probiotics did not demonstrate therapeutic effects in the clinical symptoms of NAFLD, pigs fed CON-P diets resulted in increased body weight towards to end of the study, when compared with all other treatments. Although this effect is not seen in humans, studies have shown that probiotic supplementation on early weaned pigs can improve piglet growth performance in an industry setting (Dong et al., 2013, Veizaj-Delia et al., 2010). It is important to note
that all of these probiotic findings can only be attributed to this specific probiotic mixture fed in a liquid diet form.

Similar to obesity, insulin resistance is highly correlated to the occurrence of NAFLD. While insulin resistance occurs in the majority of all children diagnosed with NAFLD, there is significantly lower prevalence of insulin resistance in patients with lean NAFLD, as well as models of non-obese NAFLD (Matsuzawa et al. 2007; Roberts, 2007; Schwimmer et al., 2003; Tu et al. 2017; Younossi et al., 2012; Zhang et al., 2016). In the present study resulting in non-obese pigs, consumption of a HFF diet and the development of NASH did not result in insulin resistance based on fasting insulin levels, fasting glucose levels, HOMA values, and protein levels of PKB in the liver and muscle. Surprisingly, fasting insulin levels, HOMA values, and PKB in the liver were all increased in pigs supplemented with probiotics, regardless of diet. Although insulin levels and HOMA values were higher, true insulin resistance was not achieved as PKB would be decreased in the instance of hepatic insulin resistance (Samuel and Shulman, 2016). We speculate that the increased insulin levels may be related to an impairment of insulin clearance pathways. A majority of insulin is cleared in the liver, where it is degraded via autophagy (Yamamoto et al., 2018). Interestingly, we found a probiotic affect in autophagy as LC3 I/II levels were increased in pigs supplemented with probiotics. LC3 I is converted to LC3 II upon the formation of a mature autophagosome, and serves as a recognition site for p62, which delivers its cargo to autophagosomes to be destroyed (Gonzales-Rodriguez et al., 2014). In normal conditions, an activation of the autophagic flux would cause a decrease in the amount of p62, as p62 is destroyed with its cargo upon delivery to the lysosome (Gonzales-Rodriguez et al., 2014). However, if p62
is also increased, this suggests an impairment of the autophagic flux as opposed to an increase in the rate of autophagy, as autophagosomes are not being destroyed. In the present study, there was an increase in p62 in pigs fed CON-P and HFF2-P diets, suggesting an impairment of autophagy due to probiotic supplementation. Additionally, there was an increase in the phosphorylation of ERK in pigs fed CON-P and HFF2-P. Although the results pertaining to autophagy were unexpected, probiotics, including L. plantarum, have been shown to activate ERK pathway in previous studies (Dai et al., 2012). Although ERK is involved in numerous cell processes, increased activation of the ERK pathway has been shown to disrupt autophagy at the maturation phase, resulting in the buildup of defective autophagosomes, as demonstrated in the present study (Corcelle et al., 2006). However, further investigation is necessary to determine the mechanism behind the dysregulation of autophagy as a result of probiotic supplementation. Contrarily, no probiotic effect was seen in the muscle, rather a diet effect where pigs fed HFF diets had higher abundance of LC3 I/II and p62. Impaired autophagy has been seen in both mice and minipig models of diet induced NAFLD, and is thought to further the progression of NAFLD by promoting endoplasmic reticulum stress and the accumulation of lipid droplets in hepatocytes (Gonzales-Rodriguez et al., 2014, Li et al., 2016).
<table>
<thead>
<tr>
<th></th>
<th>CON-N, CON-P</th>
<th>HFF1-N</th>
<th>HFF2-N, HFF2-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed amount (L·kg BW⁻¹·d⁻¹)</td>
<td>0.18</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>DM (g·kg BW⁻¹·d⁻¹)</td>
<td>40.78</td>
<td>60.99</td>
<td>63.92</td>
</tr>
<tr>
<td>CP (g·kg BW⁻¹·d⁻¹)</td>
<td>13.19</td>
<td>13.25</td>
<td>13.09</td>
</tr>
<tr>
<td>ME (kcal·kg BW⁻¹·d⁻¹)</td>
<td>199.26</td>
<td>314.75</td>
<td>300.97</td>
</tr>
<tr>
<td>CHO (g·kg BW⁻¹·d⁻¹)</td>
<td>12.82</td>
<td>23.01</td>
<td>30.05</td>
</tr>
<tr>
<td>EE (g·kg BW⁻¹·d⁻¹)</td>
<td>11.17</td>
<td>20.56</td>
<td>16.24</td>
</tr>
<tr>
<td>Amino Acids (SID, g·kg BW⁻¹·d⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>0.30</td>
<td>0.31</td>
<td>0.30</td>
</tr>
<tr>
<td>His</td>
<td>0.27</td>
<td>0.27</td>
<td>0.26</td>
</tr>
<tr>
<td>Ileu</td>
<td>0.78</td>
<td>0.78</td>
<td>0.77</td>
</tr>
<tr>
<td>Leu</td>
<td>1.44</td>
<td>1.45</td>
<td>1.43</td>
</tr>
<tr>
<td>Lys</td>
<td>1.11</td>
<td>1.11</td>
<td>1.10</td>
</tr>
<tr>
<td>Met</td>
<td>0.26</td>
<td>0.26</td>
<td>0.26</td>
</tr>
<tr>
<td>Cys</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>Phe</td>
<td>0.41</td>
<td>0.41</td>
<td>0.40</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>Thr</td>
<td>0.76</td>
<td>0.76</td>
<td>0.75</td>
</tr>
<tr>
<td>Trp</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Val</td>
<td>0.70</td>
<td>0.70</td>
<td>0.69</td>
</tr>
<tr>
<td>Fatty Acids (SID, g·kg BW⁻¹·d⁻¹)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Caprylic</td>
<td>0.00</td>
<td>0.00</td>
<td>0.48</td>
</tr>
<tr>
<td>Capric</td>
<td>0.00</td>
<td>0.01</td>
<td>1.07</td>
</tr>
<tr>
<td>Lauric</td>
<td>0.00</td>
<td>0.02</td>
<td>3.39</td>
</tr>
<tr>
<td>Myristic</td>
<td>0.07</td>
<td>0.19</td>
<td>1.38</td>
</tr>
<tr>
<td>Palmitic</td>
<td>1.78</td>
<td>3.99</td>
<td>2.58</td>
</tr>
<tr>
<td>Stearic</td>
<td>0.67</td>
<td>1.83</td>
<td>1.28</td>
</tr>
<tr>
<td>Arachidic/eicosanoic</td>
<td>0.01</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Behenic</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>0.14</td>
<td>0.39</td>
<td>0.23</td>
</tr>
<tr>
<td>Oleic</td>
<td>3.48</td>
<td>7.41</td>
<td>3.88</td>
</tr>
<tr>
<td>Gadoleic</td>
<td>0.06</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>Linoleic</td>
<td>3.86</td>
<td>5.25</td>
<td>0.96</td>
</tr>
<tr>
<td>Linolenic</td>
<td>0.04</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>0.02</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Cholesterol (g·kg BW⁻¹·d⁻¹)</td>
<td>0.03</td>
<td>0.04</td>
<td>1.30</td>
</tr>
<tr>
<td>Ca (g·kg BW⁻¹·d⁻¹)</td>
<td>0.62</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>P (g·kg BW⁻¹·d⁻¹)</td>
<td>0.43</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Fructose (g·kg BW⁻¹·d⁻¹)</td>
<td>0.00</td>
<td>10.08</td>
<td>21.60</td>
</tr>
<tr>
<td>Dextrose (g·kg BW⁻¹·d⁻¹)</td>
<td>10.80</td>
<td>10.79</td>
<td>6.30</td>
</tr>
</tbody>
</table>

BW: body weight; DM: dry matter; CP: crude protein; ME: metabolizable energy; CHO: carbohydrates; EE: ether extract; SID: standard ileal digestibility; Ca: calcium; P: phosphorus
Table 2.2: Ingredient composition of control (CON-N; CON-P), high-fructose high-fat 1 (HFF1-N) and high-fructose high-fat 2 (HFF2-N; HFF2-P) diets fed to 10-d old pigs during 10 consecutive weeks.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>CON-N, CON-P</th>
<th>HFF1-N</th>
<th>HFF2-N, HFF2-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein concentrate^1</td>
<td>8.50</td>
<td>8.5</td>
<td>9.00</td>
</tr>
<tr>
<td>Fructose^2</td>
<td>0.00</td>
<td>5.60</td>
<td>12.0</td>
</tr>
<tr>
<td>Dextrose^2</td>
<td>6.00</td>
<td>5.99</td>
<td>3.50</td>
</tr>
<tr>
<td>Fat Pak 80^3</td>
<td>3.20</td>
<td>5.27</td>
<td>1.75</td>
</tr>
<tr>
<td>Hydrogenated lard^4</td>
<td>0.00</td>
<td>3.20</td>
<td>3.10</td>
</tr>
<tr>
<td>Hydrogenated coconut oil^2</td>
<td>0.00</td>
<td>0.00</td>
<td>4.20</td>
</tr>
<tr>
<td>Corn oil^5</td>
<td>3.20</td>
<td>3.68</td>
<td>0.00</td>
</tr>
<tr>
<td>Xanthan gum^6</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Vitamin premix^7,8</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>Mineral premix^7,8</td>
<td>1.20</td>
<td>1.20</td>
<td>1.20</td>
</tr>
<tr>
<td>Cholesterol^7</td>
<td>0.00</td>
<td>0.00</td>
<td>0.70</td>
</tr>
<tr>
<td>Water</td>
<td>77.2</td>
<td>65.8</td>
<td>63.8</td>
</tr>
</tbody>
</table>

^1 80% Whey Protein Concentrate; BFD Nutrition, AZ, USA

^2 Tate & Lyle, IL, USA

^3 Advanced Fat-Pak 80; Milk Specialties Global Animal Nutrition, MN, USA

^4 Armour, TX, USA

^5 Healthy Brand Oil Corporation, NY, USA

^6 NutraBlend, MO, USA

^7 Dyets Inc., Bethlehem, PA, USA.

^8 Provided per kg vitamin premix: vitamin A, 4,409,171 IU; vitamin D 3, 661,376 IU; vitamin E, 17,637 IU; vitamin B 12, 15.4 mg; menadione, 1764 mg, riboflavin, 3307 mg; D-pantothenic acid, 11,023 mg; niacin, 19,841 mg; phytase, 200,000 FTU. Provided per kg mineral premix: Fe, 110,000 mg; Zn, 110,000 mg; Mn, 26,400 mg; Cu, 11,000 mg; I, 200 mg; Se, 200 mg
**Table 2.3:** Histologic features of liver samples in pigs fed Control (CON-N), Control + probiotics (CON-P; 6.2 × 10^4 cfu/mL), high-fructose high-fat 1 (HFF1-N), high-fructose high-fat 2 (HFF2-N), or high-fructose high-fat 2 + probiotics (HFF2-P; 6.2 × 10^4 cfu/mL) during 10 consecutive weeks. Liver tissue was collected on d 70 at 8 h post-feeding.

<table>
<thead>
<tr>
<th>Item</th>
<th>Score/code</th>
<th>CON-N</th>
<th>CON-P</th>
<th>HFF1-N</th>
<th>HFF2-N</th>
<th>HFF2-P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Steatosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>0 (absent)</td>
<td>6 (8)</td>
<td>3 (8)</td>
<td>1 (5)</td>
<td>1 (6)</td>
<td>1 (6)</td>
</tr>
<tr>
<td></td>
<td>1 (&lt; 10%)</td>
<td>2 (8)</td>
<td>5 (8)</td>
<td>4 (5)</td>
<td>1 (6)</td>
<td>1 (6)</td>
</tr>
<tr>
<td></td>
<td>2 (10 - 25%)</td>
<td>4 (5)</td>
<td>4 (6)</td>
<td>4 (6)</td>
<td>1 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (26 - 50%)</td>
<td>4 (6)</td>
<td>4 (6)</td>
<td>4 (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (&gt; 50%)</td>
<td>1 (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type</td>
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<td>2 (8)</td>
<td>5 (8)</td>
<td>5 (5)</td>
<td>6 (6)</td>
<td>6 (6)</td>
</tr>
<tr>
<td></td>
<td>microvesicular</td>
<td>2 (6)</td>
<td>1 (6)</td>
<td>1 (6)</td>
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<td></td>
</tr>
<tr>
<td>Zonal distribution</td>
<td>random</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>centrilobular</td>
<td>2 (8)</td>
<td>5 (8)</td>
<td>5 (5)</td>
<td>6 (6)</td>
<td>6 (6)</td>
</tr>
<tr>
<td></td>
<td>midzonal</td>
<td>2 (6)</td>
<td>1 (6)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>periportal</td>
<td>1 (6)</td>
<td>1 (6)</td>
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</tr>
<tr>
<td></td>
<td>submassive (2 of above)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>diffuse (all zones)</td>
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<td>5 (6)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ballooning degeneration</strong></td>
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<tr>
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<td>8 (8)</td>
<td>8 (8)</td>
<td>5 (5)</td>
<td>2 (6)</td>
<td>4 (6)</td>
</tr>
<tr>
<td></td>
<td>1 (minimal)</td>
<td></td>
<td></td>
<td></td>
<td>4 (6)</td>
<td>4 (6)</td>
</tr>
<tr>
<td></td>
<td>2 (mild)</td>
<td></td>
<td></td>
<td></td>
<td>2 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (moderate)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (severe)</td>
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</tr>
<tr>
<td><strong>Mallory-Denk bodies</strong></td>
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<td>8 (8)</td>
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<td>1 (6)</td>
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<td>4 (severe)</td>
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<td>5 (5)</td>
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<td>5 (5)</td>
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<td></td>
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</tr>
<tr>
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<td>3 (moderate)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>4 (severe)</td>
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<tr>
<td>Zonal distribution</td>
<td>random</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>6 (6)</td>
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<tr>
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<tr>
<td></td>
<td>midzonal</td>
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</tr>
<tr>
<td></td>
<td>periportal</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>submassive (2 of above)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>diffuse (all zones)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Composite lesion scores</strong></td>
<td>0-11</td>
<td>1.12</td>
<td>1.8</td>
<td>2.8</td>
<td>6.5</td>
<td>8.3</td>
</tr>
</tbody>
</table>
**Fig. 2.1 Physical and serum results pertaining to NASH.** Experimental timeline for animal weighing, diet mixing, probiotic addition, blood sampling, euthanasia and tissue collection for experiment 1 and experiment 2 (A). Representative images of pigs fed CON and HFF2 diets on d 70 (B). Body weight between d 0 and 70 (C), representative liver images taken immediately after removal and relative organ weight of liver, heart, and kidney (D), average lean mass composition (%) (E), and average serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) on d 40 and d 65 at 2-h post feeding (F) from pigs fed control (CON-N), control + probiotics (CON-P; 6.2 × 10⁴ cfu/mL), high-fructose high-fat 2 (HFF2-N), and high-fructose high-fat 2 + probiotics (HFF2-P; 6.2 × 10⁴ cfu/mL) diets during 10 weeks. Values are least square means ± SE. *P ≤ 0.05.
**Fig. 2.2 Histological results of the liver.** Average histological scores of steatosis, hepatic ballooning, Mallory-Denk bodies, inflammation, and necrosis (A), occurrence of steatosis with and without NASH (B), averagebox and whisker plots of composite liver score calculated by the addition of all histological scores (C), triacylglycerol (TAG) concentration (D), % Ki-67+ cells/field (E), and representative microphotographs of hematoxylin-eosin (H&E), trichrome, oil red O, and Ki-67 stains (F) in liver tissue from pigs fed control (CON-N), control + probiotics (CON-P; $6.2 \times 10^4$ cfu/mL), high-fructose high-fat 2 (HFF2-N), and high-fructose high-fat 2 + probiotics (HFF2-P; $6.2 \times 10^4$ cfu/mL) diets during 10 weeks. Values are least square means ± SE. a,b,cP ≤ 0.05, d,e,fP ≤ 0.01, g,h,iP ≤ 0.001.
Fig. 2.3 Probiotic detection and effects. In vitro colony count in control (CON-N), control + probiotics (CON-P; 6.2 × 10⁴ cfu/mL), high-fructose high-fat 2 (HFF2-N), and high-fructose high-fat 2 + probiotics (HFF2-P; 6.2 × 10⁴ cfu/mL) diets plated in Tryptic Soy Agar (TSA) and De Man, Rogosa and Sharpe (MRS) at 37°C during 72 h. Values are least square means ± SE. *P ≤ 0.05; **P ≤ 0.01. DNA amplification by PCR of strains of *Bacillus amyloliquefaciens*, *Pediococcus pentocaceus*, *Lactobacillus plantarum* and *Pediococcus acidilactici* in the 4 experimental diets. Amplification by PCR of probiotic strains in proximal jejunum (PJ), distal ileum (DI), cecum, colon and rectum of pigs (A). Potential mechanism for probiotic dysregulation of autophagy and increase in serum concentrations of 8-h post feeding insulin in liver of pigs fed control + probiotics (CON-P; 6.2 × 10⁴ cfu/mL) and high-fructose high-fat 2 + probiotics (HFF2-P; 6.2 × 10⁴ cfu/mL) (B). Averages serum levels of insulin, glucose, homeostatic model assessment (HOMA), leptin, interleukin 1α (IL-1α), tumor necrosis factor alpha (TNFα), and transforming growth factor beta (TGF-β) at 8-h post feeding (C), and representative western blots and relative protein expression of protein kinase B (PKB Ser473), ubiquitin-binding protein p62 (p62), microtubule-associated protein 1A/1B light chain 3A (LC3 I/II), and extracellular signal-regulated kinase (ERK) in liver tissue and longissimus dorsi (D) from pigs fed a control (CON-N), control + probiotics (CON-P; 6.2 × 10⁴ cfu/mL), high-fructose high-fat 2 (HFF2-N), or high-fructose high-fat 2 + probiotics (HFF2-P; 6.2 × 10⁴ cfu/mL) diet during 10 weeks. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize p62 and ERK. Values are least square means ± SE.
Fig. 2.4 Gene expression analysis for reference genes, inflammation and fibrosis genes, and transcription factor and enzyme genes. Average expression stability values (M) of potential reference genes in liver cyclin-G-associated kinase (GAK), vacuolar protein sorting-associated protein 4A (VPS4A), beta-2-microglobulin (B2M), porphobilinogen deaminase (HMBS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT1), and ribosomal protein S18 (RPS18) plotted from least stable (left) to most stable (right). Pairwise variation (Vn/Vn + 1) between the normalization factors NFn and NFn+1 to determine the optimal number of reference genes for normalization (A). Relative mRNA abundance potential reference genes normalized by 3 most stable genes in liver tissue (VPS4A, GAK and HMBS) (B). Relative mRNA abundance of tumor necrosis factor alpha (TNFα), transforming growth factor beta (TGF-β), interleukin 1α (IL-1α), interleukin 1β (IL-1β), toll-like receptor 4 (TLR4) and smooth muscle alpha (α)-2 actin (ACTA2) (C), sterol regulatory element-binding protein 1c (SREBP-1c), carbohydrate-responsive element-binding protein (ChREBP), liver X receptor alpha (LXR-α), peroxisome proliferator-activated receptor gamma (PPARγ), methionine adenosyltransferase 1a (MAT1A), and phosphate cytidylyltransferase 1, choline, alpha (PCYT1A) (D), and fatty acid synthase (FASN), acetyl-coa carboxylase alpha (ACACA), acyl-CoA synthetase long-chain 1 (ACSL-1), fatty-acid-binding protein (FABP), carnitine palmitoyltransferase 1 (CPT1), branched chain keto acid dehydrogenase E1, alpha polypeptide (BCKDHA), and isocitrate dehydrogenase 2 (IDH2) (E) in liver tissue from pigs fed a control (CON-N), control + probiotics (CON-P; 6.2 × 10⁴ cfu/mL), high-
fructose high-fat 2 (HFF2-N), or high-fructose high-fat 2 + probiotics (HFF2-P; $6.2 \times 10^4$ cfu/mL) during 10 weeks. Values are least square means ± SE.
A

LIVER

B

Fold change HFF2 compared to CON

Bile acids
-glycocholate
taurine
-carnitine
-homocysteine
meatiosine
serine
choline
betaine
4-pyridoxolate
acetylcholine
guanidine
orotidine
xanthine
adenosine
cytosine
xanthosine

β-oxidation
-glycocholate
taurine
carnitine
-acetyl-carnitine
homocysteine
methionine
serine
choline
betaine
4-pyridoxolate

One Carbon metabolism
-glycocholate
taurine
carnitine
-acetyl-carnitine
homocysteine
methionine
serine
choline
betaine
4-pyridoxolate

Nucleic acids
-glycocholate
taurine
carnitine
-acetyl-carnitine
homocysteine
methionine
serine
choline
betaine
4-pyridoxolate

Fold change HFF2 compared to CON

Bile acids
-glycocholate
taurine
-carnitine
-homocysteine
meatiosine
serine
choline
betaine
4-pyridoxolate
acetylcholine
guanidine
orotidine
xanthine
adenosine
cytosine
xanthosine

β-oxidation
-glycocholate
taurine
carnitine
-acetyl-carnitine
homocysteine
methionine
serine
choline
betaine
4-pyridoxolate

One Carbon metabolism
-glycocholate
taurine
carnitine
-acetyl-carnitine
homocysteine
methionine
serine
choline
betaine
4-pyridoxolate

Nucleic acids
-glycocholate
taurine
carnitine
-acetyl-carnitine
homocysteine
methionine
serine
choline
betaine
4-pyridoxolate
**Fig. 2.5 One-carbon metabolism metabolomics results.** One-carbon metabolism metabolic pathways in the liver, and relevant metabolites. Red and blue arrows represent relative increase and decrease in concentration of metabolite in pigs fed HFF2 compared to CON, respectively (A). Fold change in HFF2 compared to CON, and heat map representing the changes in metabolites pertaining to bile acids, β-oxidation, one-carbon metabolism, and nucleic acids. Each row shows data for a specific metabolite, and each column shows data from liver or plasma in pigs fed a control (CON-N), control + probiotics (CON-P; 6.2 × 10⁴ cfu/mL), high-fructose high-fat 2 (HFF2-N), or high-fructose high-fat 2 + probiotics (HFF2-P; 6.2 × 10⁴ cfu/mL) during 10 weeks (B). Blue and red represent the row minimum and maximum, respectively.
A

B

Fold change HFF2 compared to CON

0 1 2 3 4 5

Liver

Bilirubin

BUN

Total protein

Albumin

Serum 2 h postfeeding
Fig. 2.6. Tricarboxylic acid cycle (TCA) and urea cycle metabolomic and serum results. Tricarboxylic acid cycle (TCA), urea cycle, and related metabolic pathways, and relevant metabolites. Red and blue arrows represent relative increase and decrease in concentration of metabolite in pigs fed HFF2 compared to CON, respectively (A). Fold change in HFF2 compared to CON, heat map representing the changes in metabolites pertaining to the TCA cycle, the urea cycle, and amino acids, and average serum levels of bilirubin, blood urea nitrogen (BUN), total protein, and albumin on d 40 and d 65 at 2-h post feeding (B) in pigs fed a control (CON-N), control + probiotics (CON-P; 6.2 × 10⁴ cfu/mL), high-fructose high-fat 2 (HFF2-N), or high-fructose high-fat 2 + probiotics (HFF2-P; 6.2 × 10⁴ cfu/mL) during 10 weeks (B). Each row shows data for a specific metabolite, and each column shows data from liver Blue and red represent the row minimum and maximum, respectively.
Fig. 2.7. Lipid metabolism metabolomic and serum results. Fold change in HFF2 compared to CON and heat map representing the changes in phosphatidyl cholines (PC), lysophosphatidyl cholines (LPC), phosphatidylethanolamine (PE), lysophosphoethanolamines (LPE), cholesteryl esters (CE), sphingomyelins (SM), diacylglycerols (DAG), and triacylglycerides (TAG) in liver and plasma (A), and average serum levels of low-density lipoproteins (LDL), high-density lipoproteins (HDL), cholesterol, non-sterified fatty acids (NEFA), and triacylglycerides (TAG) on d 40 and d 65 at 2-h post feeding in pigs fed a control (CON-N), control + probiotics (CON-P; 6.2 × 10⁴ cfu/mL), high-fructose high-fat 2 (HFF2-N), or high-fructose high-fat 2 + probiotics (HFF2-P; 6.2 × 10⁴ cfu/mL) during 10 weeks (B). Each row shows data for a specific metabolite, and each column shows data from liver or plasma. Blue and red represent the row minimum and maximum, respectively. Values are least square means ± SE.
3. CONCLUSION

The primary objective of this thesis was to develop a diet-induced, pediatric model of NAFLD in neonatal Iberian pigs by the controlled administration of a Western diet, and to investigate the molecular mechanisms underlying the pathogenesis of the disease, as well as their interactions with each other. Based on the serum biochemistry and histological analysis, we conclude that we successfully developed the first large animal model of pediatric NAFLD in neonatal pigs. Upon this development, we revealed that related metabolic disorders, such as obesity and insulin resistance, are not necessary for the development of NAFLD. In addition, unlike other swine models, we were able to develop NASH in only 10 weeks, allowing us to remain in the juvenile time period. The metabolomics elucidated several molecular pathways that may contributed to the pathogenesis of NAFLD: 1) dysregulation of one carbon metabolism, which may have impaired the export of fat out of the liver, and 2) dysregulation the TCA and urea cycles in the mitochondria, which may have led to a decrease in BUN, and hence ammonia excretion, as well as accumulation of amino acids in liver and blood.

The second objective of this thesis was to determine if supplementation of a commercial probiotic mixture of Lactobacillus plantarum, Pediococcus acidilactici, Pediococcus pentosaceus, and Bacillus amyloliquefaciens in a HFF diet will prevent the development of NAFLD. Contrary to our hypothesis, no probiotic effect was seen in any of the clinical parameters of NAFLD. In fact, relative liver weight, histological parameters such as Mallory-Denk bodies and hepatic ballooning were significantly more severe in HFF2-P compared to HFF2-N. In addition, Western blot analysis demonstrated that probiotic supplementation led to the dysregulation of autophagy, regardless of diet,
as well as increased insulin levels. Further research is needed to explore the mechanisms that lead to this dysregulation and to assess whether this effect is strain specific or spread out among all the commercial probiotics.

In summary the results of this study represents a novel large animal model of pediatric NAFLD which can potentially be used to further investigate the pathogenesis and etiology of NAFLD.
REFERENCES


fatty acid levels differentially induces oxidative stress and non alcoholic fatty liver disease (NAFLD) in rats. Nutr Metabolism 8: 65.


Pig Model of Atherosclerosis. Archives of Medical Research. 49 (7): 456-470. DOI: 10.1016/j.arcmed.2019.01.007.


58. Li C., Nie S.P., Zhu K.X., Ding Q., Li C., Xiong T., & Xie M.Y. (2014). Lactobacillus plantarum NCU116 improves liver function, oxidative stress and
l lipid metabolism in rats with high fat diet induced non-alcoholic fatty liver disease. Food Funct 5: 3216–3223.


Homocysteine Elevation in Diet-Induced Nonalcoholic Fatty Liver Disease. PloS one, 10(8), e0136822. doi:10.1371/journal.pone.0136822.


APPENDIX

Table A.1: Feed intake, body weight (BW) on d 0 and 70, average daily gain (ADG), relative liver weight and fasting leptin on d 70 in piglets fed either control (CON-N) or high-fructose high-fat 1 (HFF1-N) during 10 weeks. Values are least square means ± SE. \(^{c,d}P \leq 0.01.\)

<table>
<thead>
<tr>
<th>Item</th>
<th>CON-N</th>
<th>HFF1-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nº (pen)</td>
<td>8 (4)</td>
<td>5 (2)</td>
</tr>
<tr>
<td>M/F</td>
<td>4/4</td>
<td>3/2</td>
</tr>
<tr>
<td>Feed intake, L·kg BW(^{-1} \cdot )day(^{-1})</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>BW d0, kg</td>
<td>3.69 ± 0.26</td>
<td>3.92 ± 0.35</td>
</tr>
<tr>
<td>BW d70, kg</td>
<td>22.3 ± 0.70</td>
<td>23.7 ± 0.89</td>
</tr>
<tr>
<td>ADG, g·d(^{-1})</td>
<td>0.27 ± 0.01</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>Relative liver weight, g·kg BW(^{-1})</td>
<td>31.1(^c) ± 1.28</td>
<td>38.0(^d) ± 1.74</td>
</tr>
<tr>
<td>Leptin, ng·mL(^{-1})</td>
<td>5.56 ± 0.71</td>
<td>6.86 ± 0.92</td>
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Table A.2: Target and reference gene primer information for quantitative PCR (qPCR) assays.

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Gene</th>
<th>Protein</th>
<th>Primer Sequence</th>
<th>Band Size</th>
</tr>
</thead>
</table>
| XM_021066227.1   | ACACA | Acetyl-CoA carboxylase 1 | F: CAGAGCTAGGGCTAGGAGGAATA  
|                  |      |         | R: ATCCAGGTGGTGAGGACAG  
|                  |      |         | 94             |
| XM_005671709.3   | ACSL1 | Long-chain-fatty-acid-CoA ligase 1 | F: GACACGGGTGGCTGGCTTTATT  
|                  |      |         | R: GAGTCGCGTCTTGGTGATG  
|                  |      |         | 96             |
| XM_013990488.2   | ACSL5 | Long-chain-fatty-acid-CoA ligase 5 | F: CAGAATACCTGGGCTCTTTC  
|                  |      |         | R: GATGATCCACTCTGGCCTATT  
|                  |      |         | 98             |
| XM_013983522.2   | ACTA2 | Actin, aortic smooth muscle | F: GGGGAAGAGATGACCAGACATATG  
|                  |      |         | R: TACGTCAGAGGTGCTAGAG  
|                  |      |         | 96             |
| XM_021096362.1   | B2M  | Beta-2-microglobulin | F: CACACTAGTTGCACTCTAACC  
|                  |      |         | R: GGGTCTGATCCACCAAGACTTC  
|                  |      |         | 97             |
| XM_013995540.2   | ChREBP | Carbohydrate-responsive element-binding protein | F: AATCTAGGCCTGCTCATCAAA  
|                  |      |         | R: ATGCTGAACCCAGAAGACTTC  
|                  |      |         | 149            |
| XM_021079578.1   | CLDN2 | Claudin-2 | F: CTCCCTCCAAAGGAAGACTATTG  
|                  |      |         | R: GGGACTGGAGATGGCCTTATT  
|                  |      |         | 128            |
| NM_001004046.2   | FABP1 | Fatty acid-binding protein | F: AACAGGGTTGCTGGGAGAA  
|                  |      |         | R: CAGTGTCATGGTGCTATT  
|                  |      |         | 105            |
| NM_001099930.1   | FASN | Fatty acid synthase | F: ACCTCCACTACATGGTCTACA  
|                  |      |         | R: ATGACAAGCTTCCCGTTCTC  
|                  |      |         | 120            |
| XM_021100830.1   | GAK  | Cyclin-G-associated kinase | F: AATCTAGGCCTGCTCATCAAA  
|                  |      |         | R: ATGCTGAACCCAGAAGACTTC  
|                  |      |         | 88             |
| JK786424.1       | GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | F: ACCTCCACTACATGGTCTACA  
|                  |      |         | R: ATGCTGAACCCAGAAGACTTC  
|                  |      |         | 90             |
| XM_021102144.1   | HMBS | Porphobilinogen deaminase | F: CAGGCGCTGAAAGACCTATAC  
|                  |      |         | R: CAGGCTCCTCTCCACCTTTA  
|                  |      |         | 110            |
| XM_021079503.1   | HPRT1 | Hypoxanthine-guanine phosphoribosyltransferase | F: AAATTAGGACAGGACTGAAGG  
|                  |      |         | R: CAGCAGGTGCAAAGAATTACAG  
|                  |      |         | 114            |
| XM_005655198.3   | IL-1A | Interleukin-1 alpha | F: CCTGGAATGAGCAGTTAAT  
|                  |      |         | R: CAGTGCAGGTTTCTGGGATTT  
|                  |      |         | 96             |
| IQ839263.1       | IL-1B | Interleukin 1B | F: AATCTGGGTTCAATCAGGAGAC  
|                  |      |         | R: CATGGTCTGCCACATTCTGTTAT  
|                  |      |         | 99             |
| XM_001929166.6   | JNK1 | Mitogen-activated protein kinase 8 | F: TGAAGGCTCCACCAAAGAAC  
|                  |      |         | R: GTTCTCTCTCCACAGTTACAC  
|                  |      |         | 111            |
| XM_021081446.1   | LXRα | Oxyterols receptor LXR-alpha | F: GGGATGGGTTGATGGCTTCT  
|                  |      |         | R: TTGGCGAAGCTCTCCGATTAT  
|                  |      |         | 118            |
| XM_005672522.3   | OCLN | Occludin | F: GCGAGGAAGTCTAGGGAGATC  
|                  |      |         | R: CACGCTCCACATTTCTGCTT  
|                  |      |         | 96             |
| XM_005669784.3   | PPARG | Peroxisome proliferator-activated receptor gamma | F: CTCCACACTATGAGACATCCC  
|                  |      |         | R: CACTCTTTGGCTAGCTTTGTA  
|                  |      |         | 101            |
| XR_001298240.2   | RPS18 | Ribosomal protein S18 | F: GGATGGGTTGGAAGCTTAACT  
|                  |      |         | R: CTTCACATCCACAGTTTCTCC  
|                  |      |         | 99             |
| XM_021066226.1   | SREBP | Sterol regulatory element-binding protein 1 | F: GACTCTACCCGTTCTTCCAC  
|                  |      |         | R: GGTCCCTTACAGGATTTGCTT  
|                  |      |         | 103            |
| XM_021093503.1   | TGFβ1 | Transforming growth factor beta-1 | F: CAGTGGAGCTTACAGGAAGAAT  
|                  |      |         | R: ACATCAAAAGGACGCAACTCC  
|                  |      |         | 101            |
| NM_001113039.2   | TLR4 | Toll-like receptor 4 | F: CGTGAGGCTGGTCTTTA  
|                  |      |         | R: AGTTAAGAAGCTCAGGTCCTTATC  
|                  |      |         | 106            |
| JF831365.1       | TNFα | Tumor necrosis factor alpha | F: CACTCTAGCTCGAGGCTTTAT  
|                  |      |         | R: GAGACGATGATGCTCCGTTT  
|                  |      |         | 97             |
| XM_021093432.1   | VSPS4A | Vacuolar protein sorting-associated protein 4A | F: CAAAGGCAAGGAGACGTTCA  
|                  |      |         | R: ATGGTGGGCTTCTCCATCAC  
|                  |      |         | 221            |
Table A.3: Primer information for DNA amplification by PCR of strains of *Pediococcus acidilactici*, *Pediococcus pentocaceus*, *Bacillus amyloliquefaciens* and *Lactobacillus plantarum* supplemented in the diet.

<table>
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<th>Bacteria</th>
<th>Primer Sequence</th>
<th>Region</th>
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<td>Pediococcus acidilactici</td>
<td>F: TCTCGCCGATTGAATATC</td>
<td>1695-1712</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>R: TAGGTCCCGCAATTTAAG</td>
<td>1823-1840</td>
<td></td>
</tr>
<tr>
<td>Pediococcus pentocaceus</td>
<td>F: TCACTCTTTACGCCCTTC</td>
<td>1073-1090</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>R: GCGGGAGCATTACTATTT</td>
<td>1254-1271</td>
<td></td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens</td>
<td>F: CCAACATATAAGACCTCTAC</td>
<td>283-302</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>R: TTATTTACATCCATCTAGAC</td>
<td>519-538</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>F: AAATCGTGATCCATTGCC</td>
<td>117022-117040</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>R: CTACTTTTTAGATCGGCC</td>
<td>117159-117176</td>
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</table>
Fig. A.1 Relative mRNA abundance of inflammatory factors. Relative mRNA abundance of tumor necrosis factor alpha (TNFα), transforming growth factor beta (TGF-β), interleukin 1α (IL-1α), toll-like receptor 4 (TLR4) and smooth muscle alpha (α)-2 actin (ACTA2) in liver tissue from pigs fed a Control (CON-N), Control + probiotics (CON-P; 6.2 × 10⁴ cfu/mL), High-fructose high-fat (HFF2-N), or High-fructose high-fat + probiotics (HFF2-P; 6.2 × 10⁴ cfu/mL) during 10 weeks. Values are least square means ± SE.