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Effects of beef fat enriched with trans vaccenic acid and conjugated linoleic acid on glucose homeostasis in diet-induced obese mice

By

YANQING XU

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

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OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2023

Effects of beef fat enriched with trans vaccenic acid and conjugated linoleic acid on glucose homeostasis in diet-induced obese mice

Abstract

Vaccenic acid (VA, trans11-18:1) and cis-9, trans11-conjugated linoleic acid (also known as rumenic acid; RA) have received widespread attention as "beneficial" trans fatty acids due to their putative health benefits, including anti-diabetic properties. Feeding oilseeds (e.g., sunflower seeds or flaxseeds) to cattle given forage-based diets has been shown to enrich their meat and milk fat with VA and RA. However, there is a shortage of studies evaluating the health effects of the resulting enriched fat. The objective of this research was to determine the effects of beef fat enriched with VA and RA on parameters related to glucose homeostasis and liver health in diet-induced obese (DIO) mice. Thirty-six 8-week-old male C57BL/6 mice were divided into nine cages of similar average body weight (four mice per cage). Each cage was randomly assigned for 18 weeks to either a control low-fat diet (CLF; 10% total calories from fat), a control high-fat diet containing lard (CHF), or an enriched-high fat diet containing beef fat enriched with VA and RA (EHF). The enriched beef fat used in EHF was collected from steers fed a diet containing 75% hay and 25% flaxseed-based concentrate. The CHF and EHF were isonitrogenous and isocaloric, providing 45% of total calories from fat. Body weight and food intake were measured weekly throughout the study. Blood triglycerides, total cholesterol, glucose, and insulin concentrations were determined at week 15 using commercial kits. Plasma aspartate aminotransferase (AST) and alanine transaminase (ALT) were analyzed using plasma from week 15. Glucose and insulin tolerance tests (GTT and ITT) were performed on weeks 16 and 17, respectively. After 18 weeks on the dietary treatments, mice were euthanized by cervical dislocation and liver tissue collected to assess liver morphology and histology. Liver triglyceride content was measured with a colorimetric diagnostic kit. The fatty acid composition of liver was determined using gas

chromatography (GC) and hepatic expression of inflammation markers was determined using qPCR. Data were analysed using the mixed models procedure of SAS and differences between means were considered significant at $P < 0.05$ using the Tukey−Kramer multiple comparison test. Compared with CLF, feeding either CHF or EHF resulted in negative metabolic outcomes associated with high-fat diets, including adiposity, impaired insulin sensitivity and glucose tolerance, and hepatic steatosis. However, the EHF diet induced a higher ($P < 0.05$) liver weight, and hepatic triglyceride content and resulted in a lower hepatic n-3 and n-6 Polyunsaturated fatty acids (PUFA) content compared with the CHF group. In parallel with steatosis, plasma levels of ALT and hepatic expression of MCP-1 and F4/80 were highest in the EHF group. In addition, the area under the curve for the GTT in EHF mice was higher ($P < 0.05$) than in the CHF group. Collectively, the findings from this research suggest that feeding beef fat enriched with VA and RA (i.e. EHF) adversely impact glucose tolerance and liver steatosis in diet-induced obese mice. Additional studies are needed to determine if the adverse effects of EHF on glucose tolerance and hepatic lipid accumulation are related to VA and RA or due to other natural trans-fatty acids present in EHF.

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Akt	Protein kinase B (also known as PKB)
ALA	Alpha-linolenic acid
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area under the curve
CD36	Cluster of Differentiation 36
CLA	Conjugated linoleic acid
CPT-1b	Carnitine Palmitoyltransferase 1B
CVD	Cardiovascular disease
DIO	Diet induced obese
DM	Dry matter
F4/80	Adhesion G protein-coupled receptor E1
FA	Fatty acid
GC	Gas chromatography
GPR40	G-protein coupled receptor 40
GSIS	Glucose-stimulated insulin secretion
GTT	Glucose tolerance test
HDL	High density lipoprotein
HFD	High fat diet
HOMA-IR	Homeostatic model assessment for insulin resistance
IL	Interleukin
ITT	Insulin tolerance test
LNA	Linoleic acid
LDL	Low density lipoprotein
MCP-1	Monocyte Chemoattractant Protein 1
MUFA	Monounsaturated fatty acid
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acid
QPCR	Quantitative polymerase chain reaction
RA	Rumenic acid
SCD	Stearoyl-CoA desaturase
SFA	Saturated fatty acid
SREBP-1c	Sterol-regulatory Element Binding Portein-1c
TE	Total energy
TFA	Trans fatty acids
TPA	Trans palmitoleic acid

TVA	Trans vaccenic acid
WAT	White adipose tissue

Chapter 1: Comprehensive Literature Review

Introduction

Trans fatty acids (TFA) are unsaturated fatty acids that contain at least one double bond in the trans configuration (i.e. the two hydrogen atoms are on opposite sides of the carbon-to-carbon double bond), resulting in a straighter shape more similar to saturated fatty acids. Consequently, TFA are less fluid and have a higher melting point than unsaturated fatty acids with cis double bonds, which are the major monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in plants and animals (Bhardwaj et al., 2011)

TFA in foods mainly come from two sources, including partially hydrogenated vegetable oils (i.e. industrial TFA) and ruminant-derived foods such as dairy and beef (i.e. ruminant TFA). Industrial TFA are found in partially hydrogenated vegetable oils generated using hydrogen in the presence of a catalyst (Bhardwaj et al., 2011), while ruminant TFA are made by rumen bacteria using a process called biohydrogenation (Lichtenstein, 2000; Dhaka et al., 2011).

Until recently, industrial TFA were extensively used by the food industry as a replacement for saturated fats. However, in recent years, many countries have banned industrial TFA from the food supply due to their detrimental effects on cardiovascular health (Lichtenstein, 2000; Mozaffarian et al., 2006). Recent government bans on industrial TFA in developed countries including the U.S, Canada and the E.U has left ruminant-derived fats (e.g. dairy, beef, lamb and goat) as the sole source of TFA in the food supply. As such, there is heightened interest in the composition, content, and health effects of ruminant TFA particularly conjugated linoleic acid (CLA) and trans 18:1 isomers (Brouwer et al., 2010; Prada et al., 2022). The overarching goals of this review are to summarize biosynthesis, concentration range and factors affecting the concentration of TFA in

ruminant fats, current intake of ruminant TFA, and to evaluate evidence on their health effects with an emphasis on type 2 diabetes.

Biosynthesis of ruminant TFA

Ruminant TFA are formed via biohydrogenation of unsaturated fatty acids in the rumen. The process of biohydrogenation is performed by rumen bacteria, during which dietary unsaturated fatty acids are converted to saturated fatty acids (Lichtenstein, 2000; Dhaka et al., 2011; Dugan et al., 2018). Dietary unsaturated fatty acids are toxic to rumen microbes, hence they biohydrogenate them to saturated fatty acids which are neutral or less toxic (Jenkins et al., 2008). The biohydrogenation process includes several isomerization and hydrogenation steps which result in the formation of many intermediates including conjugated and non-conjugated trienoic, dienoic and monoenoic trans fatty acids (Jenkins et al., 2008; Vahmani et al., 2015). A small portion of these intermediates passes the rumen and subsequently find their way into tissues and milk after post-ruminal absorption (Lichtenstein, 2000; Dugan et al., 2018).

The predominant fatty acids in ruminant diets include 18:2n-6 (linoleic acid; LNA) and 18:3n-3 (alpha-linolenic acid; ALA) and thus are considered the main substrates for ruminal biohydrogenation. It is estimated that on average, about 80% and 92% of dietary LNA and ALA are biohydrogenated in the rumen (Conte et al., 2017). Several different biohydrogenation pathways have been proposed for LNA and ALA. Several factors including forage-to-concentrate ratio and ruminal passage rate/ residence time can determine the pathway and extent of the biohydrogenation of LNA and ALA (Chilliard et al., 2014; Dugan et al., 2018; Dewanckele et al., 2020).

The main pathways for the biohydrogenation of LNA and ALA have been described by Harfoot and Hazelwood (1997). Pathways for both LNA and ALA are characterized by initial isomerization of the cis double bond at carbon 12 to a trans double bond at carbon 11 resulting in the production of cis9, trans11-18:2, an isomer of CLA (also known as rumenic acid; RA), and cis9, trans11, cis15-18:3, respectively (Figure 1). This is followed by rounds of hydrogenation and isomerization leading to a trans 18:1 isomer (e.g., trans11-18:1) and eventually complete hydrogenation to 18:0 (stearic acid) as the end product (Dewanckele et al., 2020).

It is noteworthy that the abovementioned pathways were elucidated when greater forage-to-grain ratios (forage-based diets) were fed. When feeding grain-based diets (e.g., feedlot diets), isomerization of the cis 9 double bonds for LA shifts towards a trans double bond at carbon 10, with the same happening for ALA shifts, resulting in the production of trans10, cis12-18:2 and trans10, cis12, cis15-18:3, respectively (Alves and Bessa, 2014). These have been referred to as the "trans-10 shifted" biohydrogenation pathways (Figure 1) and result in the accumulation of trans10-18:1 as the main TFA in ruminant products such as beef from feedlot (grain-finished) cattle (Alves and Bessa, 2014; Alves et al., 2021). Whereas trans11-18:1 (trans vaccenic acid; TVA) is typically the main TFA found in milk and meat from ruminants fed forage-based diets (e.g., grass-fed beef) (Jaakamo et al., 2019). Findings from animal model studies suggest that trans10-18:1 may harm health in a similar fashion to industrial TFA (Alves et al., 2021). On the other hand, TVA can be converted to RA (cis9, trans 11-CLA), both of which have been associated with several health benefits such as prevention of cancer, cardiovascular disease, and inflammation as well as improved immune function (Derakhshande-Rishehri et al., 2014; Kim et al., 2016). Consequently, TVA and RA are sometimes referred to as "good" TFA (Diane et al., 2016; Vahmani et al., 2020).

In addition to the main biohydrogenation pathways mentioned above, there are numerous minor pathways active in the rumen resulting in a plethora of biohydrogenation intermediates including, conjugated and non-conjugated trienoic (18:3), dienoic (18:2) and monoenoic (18:1 and 16:1) *TFA isomers* (Vahmani et al., 2020). In fact, milk and meat fats from ruminants (e.g. cattle, sheep and goats) have the most complex fatty acid composition (> 100 different fatty acids) among all edible fats, in part due to the biohydrogenation process.

Types and concentrations of TFA in ruminant fats

The biohydrogenation process results in about 50 different types of TFA including trans-16:1 (trans6- to trans12-16:1), trans-18:1 (trans4- to trans16-18:1), conjugated 18:2 known as CLA (≥ 12 different CLA isomers with *cis*/*trans* or *trans*/*cis* configurations), non-conjugated non-methylene interrupted 18:2 known as atypical dienes (≥ 10 different isomers with *cis*/*trans* or *trans*/*cis* configurations) and conjugated 18:3 (≥ 3 different isomers with *cis*/*trans/trans* or *cis* /*trans*/*cis* configurations). Trans-18:1s are the predominant TFA (70-80% of total TFA), followed by CLA (10-25% of total TFA), atypical dienes (5-15% of total TFA), trans-16:1 (5-10% of total TFA) and conjugated linolenic acid (CLnA, <5% of total TFA) (Table 1). Among individual TFA isomers, VA, RA and trans9-16:1 (trans palmitoleic acid; TPA) have been the most studied isomers in terms of health effects and bioactivity, due to their high prevalence in ruminant foods and commercial availability (i.e. pure fatty acid isomers).

Current intake of ruminant TFA

There have been numerous studies done on the health effects of industrial TFA during the past 4 decades. These studies have consistently found that industrial TFA have been associated with an increased risk of cardiovascular disease, mainly by lowering HDL and raising LDL levels (Stender

et al., 2008; Brouwer et al., 2010; Radtke et al., 2017; Oteng et al., 2019). In the mid-1990s, many developed countries including the EU made recommendations to limit the intake of industrial TFA to 1% of the daily energy intake (Wanders et al., 2017). According to a study in 2010, the average daily TFA intake for adults was around 2.5 to 3.49% of daily energy intake in the U.S. (Micha et al., 2014), which was much higher than the 1% recommended limit. About 10 years later in 2015, the U.S. Food and Drug Administration (FDA) ruled that industrial TFA are not safe in food and set a June 2018 deadline for their removal from the food system (USDA, 2020). The official ban on industrial TFA has left ruminant-derived fats as the sole dietary source of TFA in the U.S., as well as in Canada and most European countries.

The estimated dietary intake of ruminant TFA varies between 0.8% to 1.7% of total energy intake depending on the country, with the average intake of ruminant TFA in the U.S. estimated to be about 1.2 % of energy intake (Gebauer et al., 2011). In a more recent study, however, ruminant TFA intake in Europe and the U.S. was estimated to be around 0.5% of energy intake (Brouwer et al., 2013). Given recent bans on industrial TFA, there is need for new studies to determine the current intake of TFA from ruminant derived foods.

Factors affecting concentrations of TFA in ruminant-derived foods.

The TFA composition of ruminant derived foods is largely influenced by dietary, management and animal factors. Adding sources of PUFA to the diet (e.g. plant oil and oilseeds) significantly increases the contents of TFA in ruminant milk and meat including RA, TVA and TPA (Scollan et al., 2017; Guillocheau et al., 2020; Guo et al., 2023). The source of PUFA has the largest impact, with LNA-rich oils, yielding the greatest RA, TVA and TPA contents (Bessa et al., 2015; Kliem and Shingfield, 2016). Feeding ALA-rich sources also enhances CLnA content in ruminant fats (Kliem and Shingfield, 2016; Chikwanha et al., 2018). Seemingly effective novel alternative oil

sources for increasing TFA, such as insect oils, warrant further investigation (Hervás et al., 2022). Furthermore, the amount of PUFA increases TFA content, reaching a peak when feeding between 50 and 80 g/kg DM intake (Scollan et al., 2017; Chikwanha et al., 2018). Besides PUFA, feeding forage-based diets, as opposed to grain-based diets, effectively increases TFA with trans-11 double bonds (TVA and RA) in ruminant fats (Chikwanha et al., 2018; Cabiddu et al., 2022). However, the effectiveness of forage-based diets at increasing TFA is determined by several factors relating to the source of forage including species diversity, cultivars, phenological stage, maturity, conservation method, particle length, presence of bioactive compounds and seasonality (Frutos et al., 2020; Cabiddu et al., 2022). Noteworthy, feeding a combination of forages and PUFA for an extend period substantially enhances presence of TFA in ruminant fats (Kliem and Shingfield, 2016; Vahmani et al., 2020; Alves et al., 2021), particularly when forage and PUFA sources are feed separately (Vahmani et al., 2017).

High-grain diets supplemented with LNA substantially reduces increases contents trans10-18:1 and trans10,cis12-CLA (i.e., trans10-shift) in ruminant meat and milk (Mapiye et al., 2015; Kliem and Shingfield, 2016). The t10-shift is exacerbated by feeding small grains (i.e. barley and wheat versus corn), pelleting, and increasing feeding duration (Mapiye et al., 2012; Mutsvangwa et al., 2012). A multitude of strategies to avoid the trans10-shift such as the addition of forages, non-starch fibers, strong buffers, antibiotics, antioxidants, yeast, chitosan, and agro-industrial by-products to high-grain diets with varying success (Alves et al., 2021; Amin and Mao, 2021; Hervás et al., 2022). Additionally, PUFA protective treatments and adsorbents inhibit the trans10-shift to a limited extent (Guo et al., 2023).

Notably, contents of TFA are more effectively increased in milk versus meat and small versus large ruminants (Chilliard et al., 2007; Dugan et al., 2011; Chikwanha et al., 2018). The TFA

contents in milk are somewhat influenced by animal individuality, breed, stage of lactation and parity (Samková et al., 2012). In meat animals, breed, sex, slaughter age and weight, anatomical location of fat depot and muscle type have marginal effects on TFA contents (Mapiye et al., 2015).

Metabolism of ruminant TFA in the human body

Most published data on TFA metabolism comes from studies on trans 18:1, which are the predominant fatty acid type in both ruminant and industrial TFA. Trans 18:1 isomers are intestinally absorbed to the same extent as cis 18:1 isomers and the double-bond position has little or no effect on their absorption (Baer et al., 2003). After absorption, trans 18:1 isomers can be incorporated into cell membrane phospholipids, or stored in the triacylglycerols of adipose tissues. Trans 18:1 isomers can be metabolized by oxidation, elongation, and desaturation processes, which result in isomer-specific metabolites with different biochemical properties (Vahmani et al., 2020). For TVA, two main metabolic fates have been characterized which include chain shortening to TPA and delta-9 desaturation to RA (Figure 2). It has been estimated that ~19% of TVA, the main t-18:1 isomer in milk and meat from forage-fed ruminants consumed by humans, can be converted to RA by tissue-level Δ-9 desaturation which is catalyzed by the stearoyl-CoA desaturase 1 (SCD1) enzyme (Turpeinen et al., 2002; Miller et al., 2003).

In addition to Δ-9 desaturation, chain shortening of TVA by β-oxidation (peroxisomal β-oxidation of TVA) can lead to elevated levels of TPA in the plasma or tissues after consuming foods containing TVA, particularly grass-fed beef and dairy products. The conversion rate of TVA to TPA has been estimated to be 10% in cultured rat hepatocytes incubated with TVA (Jaudszus et al., 2014), however, the whole body (in vivo) conversion rate of TVA to TPA is not known. Given the very low concentration of TPA in the food supply including ruminant-derived foods (<0.05%

of total fatty acids), the major origin of circulating TPA in humans is assumed to be from TVA intake from consumption of ruminant derived foods (Jaudszus et al., 2014).

Health effects of ruminant TFA

The effects of ruminant- versus industrial- TFA on human health have been controversial and a subject of debate for many years. The recent removal of industrial TFA from the food supply in developed countries has renewed interest in understanding the human health effects of ruminant TFA. Several epidemiological studies have shown that, in contrast to industrial TFA, ruminant TFA do not appear to increase cardiovascular disease (CVD) risk and mortality (Ascherio et al., 1994; Pietinen et al., 1997; Jakobsen et al., 2008; Bendsen et al., 2011; Sacks et al., 2017). This has been attributed to the different isomeric profile of trans18:1 between ruminant and industrial TFA, as well as the low concentrations of total TFA in ruminant fats (2-6% of total fatty acids) compared to partially hydrogenated vegetable oils (60-65 % of total fatty acids) (Stender et al., 2008). Conversely, based on recent human clinical trials, both industrial- and ruminant- TFA adversely affect cholesterol homeostasis (i.e. increased blood LDL-cholesterol and reduced HDL cholesterol) when consumed at comparable levels (Motard-Bélanger et al., 2008; Brouwer et al., 2013; Gebauer et al., 2015; Stender, 2015; Verneque et al., 2022). However, there is limited information available on the effect of ruminant TFA on other cardiovascular disease risk markers and the development of other chronic diseases such as type 2 diabetes. In the following section, we summarize published data from observational studies, clinical trials and animal studies on the effects of ruminant TFA on metabolic parameters related to type 2 diabetes.

Observational studies. Since 2010, several prospective epidemiological studies have consistently shown that increased blood levels of TPA, the chain shortening product of TVA (the

most abundant TFA in ruminant fats) was associated with lower risk and incidence of type 2 diabetes (Mozaffarian et al., 2010). Similarly, two meta-analyses reported circulating TPA was inversely associated with type 2 diabetes (Imamura et al., 2018). In a more recent epidemiological study, circulating TVA but not TPA was inversely associated with diabetes risk (Prada et al., 2022). This discrepancy could be in part due to the limitations in the analytical methods used to determine TFA isomeric profile in human blood samples (Guillocheau et al., 2020). Although available data from observational studies point towards potential antidiabetic properties of ruminant TFA, a cause-and-effect relationship has not yet been proven in humans.

Clinical trials. There is very limited clinical data on the human health effects of ruminant TFA. Gebauer et al. (2015) compared the effects of isocaloric diets containing different TFA isomers in a randomized, crossover feeding trial in 106 healthy subjects who were each provided the diets for 24 days. Diets were designed to have stearic acid replaced with the following TFA isomers (percentage of energy): ~3% pure TVA, ~3% mixed isomers of industrial TFA from partially hydrogenated vegetable oil, or 1% pure RA. In this study, there was no difference among treatments in terms of metabolic parameters related to type 2 diabetes including blood glucose, insulin, or HOMA-IR. Another study using a hyperinsulinemic-euglycemic clamp in abdominally obese male subjects reported that supplementation with pure RA (~1% of energy intake for 12 weeks) reduced insulin sensitivity compared with an olive oil placebo (Risérus et al., 2004). To our knowledge, there has been no clinical study investigating the health effect of TPA supplementation in humans, likely due to the lack of pure TPA (Guillocheau et al., 2020). In fact, the above two studies are *the only two human clinical studies* examining health effects of pure ruminant TFA isomers. However, there are four published clinical studies in which ruminant TFA-enriched dairy fats were fed. In these studies, TVA+RA-enriched butter (1–1.5%

of energy intake from TVA+RA) did not alter blood *glucose and insulin, insulin resistance* index (HOMA-IR) or glucose tolerance in healthy subjects compared with standard butters (Tholstrup et al., 2006; Tricon et al., 2006; Brown et al., 2011; Penedo et al., 2013; Werner et al., 2013). Overall, available data from clinical trials do not *support* a beneficial effect of ruminant TFA on glucose homeostasis in humans. It is noteworthy, however, almost all of these clinical trials were done in healthy subject and not in people with prediabetes or type 2 diabetes.

Animal studies. Most available data regarding the promising health effects of ruminant TFA including their postulated antidiabetic properties come from animal model studies. Feeding a diet enriched with 1% pure TVA (~4.5% of energy intake) to obese insulin resistant JCR-LA:cp rats resulted in significant reductions in fasting *and postprandial insulin levels and an increase in insulin sensitivity (lower HOMA-IR) (Jacome-Sosa et al., 2014). T*he authors attributed these insulin-sensitizing effects of TVA to activation of peroxisome proliferator-activated receptor-γ. In fact, both TVA and its delta-9 desaturation product, RA, have been shown to act as ligands for PPARγ and PPARα, *which* are transcription factors for several genes involved in lipid and glucose metabolism (Moya-Camarena et al., 1999; Wang et al., 2012). Feeding beef fat enriched with TVA and RA to obese/insulin-resistant JCR:LA-*cp* rats reduced fasting insulin and HOMA-IR, and lowered insulin secretion following a meal tolerance test, which were accompanied by higher protein expression of PPARγ and PPARα in the liver (Diane et al., 2016). Similarly, feeding Wister rats a high-fat diet containing TVA+RA-enriched butter reduced fasting serum insulin and increased hepatic PPARγ protein expression compared to rats fed a control high-fat diet containing standard butter (De Almeida et al., 2014). The apparent insulin-sensitizing effects of TVA and RA in the above rodent studies were attributed their potential to bind and activate PPARγ-regulated pathways in the liver and adipose tissues. Moreover, it has been suggested that

TVA can also *restore glucose homeostasis* by promoting insulin secretion from pancreatic islets. In diabetic Sprague–Dawley rats (induced by high-fat diet/streptozotocin), 8 weeks supplementation with pure TVA (1.2% of diet mass) reduced both fed and fasting blood glucose, and increased β-cell area (Wang et al., 2016). In addition, in this study, hyperglycemic clamp showed that TVA increased glucose turnover in diabetic rats, accompanied by an elevated plasma C-peptide concentration, suggesting improved insulin secretion. Moreover, isolated islets from TVA fed diabetic rats had higher glucose-stimulated insulin secretion (GSIS) than the control diabetic rats (Wang et al., 2016). Thus, the authors concluded that TVA may improve glucose homeostasis in diabetic rats in part by promoting insulin secretion (Wang et al., 2016). Consistent with these findings, Wang et al. recently reported that feeding pure TVA to diabetic rats could promote insulin secretion through stimulating G-protein coupled receptor 40 (GPR40) expression and signaling in islets (Wang et al., 2019).

The above studies should be *weighed against other studies* that have not found ruminant TFA to improve glucose homeostasis in *animal models of insulin* resistance and type 2 diabetes. In obese/insulin resistant JCR-LA:cp rats, feeding a diet enriched with pure TVA (1.5% of diet as TVA) did not alter fasting levels of insulin or glucose, nor insulin and glucose responses to a meal tolerance test (Wang et al., 2008). Another study using Wistar rats showed that 8 weeks of feeding diets enriched (4% of energy intake) with either TVA, mixed industrial TFA mixed isomers or oleic acid to did not alter insulin and glucose responses to an intraperitoneal glucose tolerance test (Tardy et al., 2008). Similarly, dietary supplementation with pure TPA (0.7% of energy intake) did not modify glucose homeostasis in high fat diet-induced obese (DIO) mice as measured by glucose/insulin tolerance tests and insulin-mediated Akt activation (Chávaro-Ortiz et al., 2022). Another recent study using DIO mice showed that feeding a high-fat diet containing

beef fat naturally enriched with TVA and RA (beef fat from flaxseed-fed cattle) for 19 weeks worsened glucose tolerance and liver steatosis compared to mice fed a control high fat diet (Xu et al., 2022). The authors partly attributed the adverse effects on glucose tolerance and liver health to other TFA present in TVA+RA enriched beef fat. It is noteworthy that no study to date has tested the effects of pure TVA or RA in DIO mouse model which is one of the most clinically translatable animal models to test the efficacy of natural compounds and/or drugs against prediabetes and type 2 diabetes.

Conclusion

Taken together, although TVA and its metabolites (TPA and RA) have been touted as having antidiabetic properties based on data from observational studies and a small number of animal studies, while the same effects have not been observed in other animal studies or in human clinical trials. On the other hand, a limited number of human clinical trials in which pure TVA or TVA+RA enriched dairy fats were fed, have shown that ruminant TFA, like industrial TFA, can result in reduced levels of HDL-cholesterol, a known marker of cardiovascular disease risk (Melin et al., 2019). Additional *clinical and mechanistic studies* are needed to better understand the isomer-specific effects of ruminant TFA. Nevertheless, based on the current knowledge regarding potential detrimental effects of ruminant TFA on HDL-cholesterol, production practices resulting in increased levels of this group of fatty acids in ruminant milk and meat should be carefully reconsidered.

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Chapter Two: Beef Fat Enriched with Vaccenic acid and cis9, trans11-Conjugated Linoleic Acid Promotes Hepatic Steatosis and Glucose Intolerance in Diet-Induced Obese Mice

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Abstract

Vaccenic acid (TVA, trans11-18:1) and cis-9, trans11-conjugated linoleic acid (also known as rumenic acid; RA) have received widespread attention as “beneficial” trans fatty acids (TFA) due to their putative health benefits, including anti-diabetic properties. Feeding oilseeds (e.g., sunflower seeds or flaxseeds) to cattle given forage-based diets has been shown to enrich their meat and milk fat with TVA and RA. However, there is a shortage of studies evaluating the health effects of the resulting enriched fat. The objective of this study was to determine the effects of beef fat enriched with TVA and RA on parameters related to glucose homeostasis and associated metabolic markers in diet-induced obese (DIO) mice. We hypothesized that beef fat enriched with TVA and RA would improve glucose tolerance and insulin sensitivity in DIO mice. Thirty-six male C57BL/6J mice (8 weeks old) were fed for 19 weeks with either a control low-fat diet (CLF; 10% total calories from fat), control high-fat diet containing lard (CHF), or an enriched-high fat diet containing beef fat enriched with TVA and RA (EHF). Compared with CLF, feeding either CHF or EHF resulted in adverse metabolic outcomes associated with high-fat diets, including adiposity, impaired insulin and glucose tolerances, and hepatic steatosis. However, the EHF diet induced a higher (P < 0.05) liver weight and liver triglyceride content compared with the CHF diet. In parallel with steatosis, plasma levels of ALT and hepatic expression of inflammation markers (MCP-1 and F4/80) were highest in the EHF group. In addition, the area under the curve (AUC) for the glucose tolerance test (GTT) in EHF mice was higher (P < 0.05) than in the CHF group, indicating worse glucose intolerance. Collectively, the findings from this study, suggest that feeding beef fat enriched with TVA and RA (i.e. EHF) worsens glucose tolerance and liver steatosis in DIO mice. Additional studies are needed to determine if the adverse effects of EHF on glucose tolerance and liver health are related to TVA and RA or due to other TFA present in EHF.

Key words: Beef, biohydrogenation, trans fatty acids, type2 diabetes

Introduction:

Milk and meat fats from ruminant animals (e.g., cattle, sheep, and goats) have the most complex fatty acid composition (> 100 different fatty acids) among all edible fats, in part due to the biohydrogenation process (Jenkins et al., 2008). In ruminants, dietary unsaturated fatty acids are toxic to rumen bacteria. To cope, rumen bacteria convert them to less toxic saturated fatty acids through biohydrogenation. During this process, numerous biohydrogenation intermediates are produced, and a portion of them pass from the rumen and subsequently into tissues and milk after post-ruminal absorption. Given that the majority of these intermediates contain at least one *trans* double bond, they are generally referred to as "ruminant *trans* fatty acids (TFA)" or "natural TFA*"*. There are at least 40 different TFA isomers found in ruminant-derived fats, with *trans*(*t*) vaccenic acid (TVA; *t*11-18:1) and *cis*(*c*)-9, *t*-11 conjugated linoleic acid (c9,t11-CLA; also known as rumenic acid, RA) being the most predominant ones, accounting for 50- 70% of total TFA in ruminant-derived fats (Lock and Bauman, 2004). Trans vaccenic acid can also be converted to RA in the body via Δ-9 desaturation, with the conversion rate estimated to be approximately 19% in humans (Lock and Bauman, 2004).

In contrast to partially hydrogenated vegetable oils (also known as industrial TFA), which have undisputable adverse health effects, particularly increased cardiovascular disease (CVD) risk and mortality, TVA and RA have been associated with reduced risk of some disease conditions including type 2 diabetes (Field et al., 2009; Gebauer et al., 2011; Yu et al., 2012; Prada et al., 2022). In rodent studies, supplementation with pure TVA or RA reduces fasting and postprandial insulin levels and HOMA-IR (Moloney et al., 2007; De Almeida et al., 2014; Jacome-Sosa et al., 2014; Diane et al., 2016). The apparent insulin-sensitizing effects of TVA and RA in these studies were mainly attributed to their potential to bind and activate PPARγ-regulated pathways in the

liver and adipose tissues (Jacome-Sosa et al., 2014; Diane et al., 2016; De Brito Medeiros et al., 2021). Moreover, TVA has been shown to restore glucose homeostasis in diabetic rats by promoting insulin secretion from pancreatic islets (Wang et al., 2019).

Given the postulated health benefits of TVA and RA, ruminant nutritionists have sought to develop feeding strategies to enhance the content of these fatty acids in beef and dairy products (Vahmani et al., 2020). The findings from these studies have shown that significant enrichment with TVA and RA can be achieved by feeding cattle forage-based diets that are supplemented with polyunsaturated fatty acids (PUFA) sources, such as oilseeds (flaxseed or sunflower seeds).

A limited number of rodent model studies have shown that feeding butter from oilseed-feed dairy cattle (i.e., TVA+RA enriched butter) improved plasma lipoprotein profiles compared with those fed regular butter (Lock et al., 2005; Valeille et al., 2006; Bassett et al., 2010). Furthermore, short-term (3 weeks) feeding of beef fat from flaxseed-fed cattle (i.e. TVA+RA enriched beef fat) alleviated insulin resistance in JCR:LA-cp rats compared to those fed regular beef fat (Diane et al., 2016). However, the long-term effects of feeding TVA+RA ruminant fats on glucose homeostasis has not been studied. Thus, we sought to determine whether long-term supplementation with TVA+RA-enriched beef fat would improve glucose homeostasis and associated metabolic markers in diet-induced obese (DIO) mice. We hypothesized that dietary supplementation with TVA+RA enriched beef would attenuate glucose intolerance and insulin resistance, and other obesity-associated metabolic impairments in DIO mice.

Materials and Methods

Animals and diets

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of California- Davis and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

A total of 36 eight-week-old C57BL/6J male mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were group-housed (4 mice/cage) and kept under a 12 h light cycle (7:00. A.M. lights on, 7:00 P.M. lights off) in a temperature (22°C) and humidity-controlled vivarium with *ad libitum* access to food and water. After 1 week of acclimation to a standard rodent-chow diet, each cage was randomly assigned to either a control low-fat diet (CLF; 10% energy from fat), a control high-fat diet (CHF; 45% energy from fat) with no TFA, or an enriched high-fat diet (45% energy from fat) containing beef tallow enriched with TVA and RA (EHF) (Table 2). The detailed fatty acids profiles are presented in Table 4. The CHF and EHF diets were designed to have a similar content of total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and a similar n-3/n-6 PUFA ratio (Table 3). The TVA+RA enriched beef tallow was sourced from subcutaneous fat of steers fed a diet containing 75% hay and 25% flaxseed-based concentrate (Vahmani et al., 2017). Briefly, the subcutaneous fat (i.e., backfat) was ground through a 6 mm plate (Butcher Boy meat grinder Model TCA22, Lasar Manufacturing Co, Los Angeles, CA, USA), vacuum packaged, frozen, and held at -40 °C until rendering. Prior to rendering, vacuum-packaged ground fat was melted and heated to 60 °C in a water bath. The melted fat was strained through cheesecloth and added to an equal volume of 60 °C water. The fat-water mixture was then left to cool overnight at 2 °C, and rendered beef fat was

collected from the surface. The resulting fat was analyzed for fatty acids and then sent to Research Diets, Inc. (Brunswick, NJ, USA) to be incorporated into a high-fat diet (Table 2).

Body weight and food intake were measured weekly throughout the study. Energy intake was calculated from food intake and energy density (kcal/g) of diets. At week 15, blood sampling (tail vein) was performed in fed and 12-h fasted animals for measurement of blood glucose and insulin levels. After 19 weeks of dietary treatments, mice were euthanized using cervical dislocation. Blood was collected from the abdominal aorta into EDTA anticoagulant tubes, and plasma was obtained after centrifugation at 1000*g* for 15 min at 4 °C. Plasma samples were stored at −80°C until used for analyses. Epididymal adipose tissue and liver were collected and weighed. Tissues were flash-frozen in liquid nitrogen and then stored at −80 °C until further analysis.

Measurement of plasma metabolites

Glucose levels were measured with a glucometer (Easy Plus II, Home Aid Diagnostics Inc, Deerfield Beach, FL, USA) via tail vein blood. Insulin levels were determined by an ELISA (Ultra Sensitive Mouse Insulin ELISA kit-Crystal Chem, Downers Grove, IL, USA) according to the manufacturer's instructions. Plasma triglycerides and total cholesterol esters were measured using Infinity^{TM} reagents (TR22421 and TR13421; Thermo Fisher Scientific, Altham, MA, USA). Plasma aspartate aminotransferase (AST) and alanine transaminase (ALT) were analyzed by the University of California, Davis (UC Davis) Comparative Pathology Laboratory.

Insulin and glucose tolerance tests

A glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed during week 16 and week 17, respectively. For ITT, mice were fasted for 4 h and injected intraperitoneally with 1 U/Kg body weight human insulin (Novolin-R, Novo Nordisk, Bagsværd, Denmark). Values

were measured before injection and at 15, 30, 45, 60, 90, and 120 min post-injection. For GTT, overnight fasted mice were injected with D-glucose (2 g/kg body weight), and blood glucose was measured before injection and at 15, 30, 60, and 120 min post-injection. Glucose levels at indicated time points for ITT and GTT were measured from tail vein blood as described above.

Liver triglyceride content and staining

Liver samples were homogenized and put into a 2:1 chloroform and methanol mix and stored at 4 ºC overnight. Next, 0.7% sodium chloride was added to the mix and stored for another 24 hours at 4 ºC. The aqueous upper phase was aspirated and discarded, and the bottom phase was removed and dried down with nitrogen gas. The sample was reconstituted with 2-propanol and triglyceride levels were quantified using Infinity™ reagents (TR22421; Thermo Fisher Scientific, Altham, MA, USA). For liver histological analyses, 4% paraformaldehyde-fixed liver samples were paraffin-embedded, sectioned, and hematoxylin/eosin (H&E)-stained by the UC Davis Comparative Pathology Laboratory.

Quantitative real-time PCR

Frozen livers were homogenized, and extracted using reagent Trizol (Ambion, Austin, TX, USA) and RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), with the quantity and quality determined using a NanoDrop ND-2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). After that, cDNA was generated using a Maxima First Strand cDNA synthesis kit with same total RNA amount for every sample (Thermo Scientific Inc., Waltham, MA, USA). Samples were mixed with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and relevant primer pairs to determine the threshold cycle (Ct) by an Applied Biosystems MiniAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences for

the internal control gene (TATA box-binding protein; Tbp) and target genes involved in inflammation including adhesion G protein-coupled receptor E1 (EMR1, also known as F4/80), monocyte chemoattractant protein 1 (MCP-1), interleukin-1 (IL-1) and cluster of differentiation 36 (CD36) are listed in Table 5. The amplification efficiency for each primer pair was calculated from the slope of the standard curve generated with serial dilutions of a pooled cDNA sample using the formula ($E = 10^{(-1/slope)}$). The amplification efficiencies were between 90 and 105% for all primer pairs used in this study. Relative mRNA expression of target genes was calculated using the ΔCt method with Tbp as the internal control gene. Each 96-well qPCR plate was set up to include reactions for both the target gene and the internal control gene for each cDNA sample. Target gene cycle threshold (Ct) values were normalized to that of Tbp using $2^{-\Delta Ct}$ where ΔCt = Ct target gene Ct Tbp (Schmittgen and Livak, 2008). Statistical analysis was performed with SAS on $2^{-\Delta Ct}$ data, and the results were expressed as fold change relative to control.

Fatty Acid Analysis

The fatty acid composition of the liver was determined using gas chromatography (GC). Briefly, tissue samples were freeze-dried and direct methylated using dual acid-base methylation with sodium methoxide followed by methanolic HCl (Jenkins, 2010). *Cis*-10–17:1 methyl ester (Nu-Check Prep Inc., Elysian, Mn, USA) was added as an internal standard prior to the methylating reagent. Fatty acid methyl esters (FAME) were analyzed by GC using a CP-Sil88 column (100 m, 25 μm ID, 0.2 μm film thickness) in a TRACE 1310 gas chromatograph (Thermo Fisher Scientific) equipped with a flame-ionization detector (GC-FID, Thermo Fisher Scientific). Each sample was analyzed twice by GC using a 175°C plateau temperature program (Dugan et al., 2007). The

FAME were quantified using chromatographic peak area and internal standard-based calculations (Vahmani et al., 2017).

Data Analysis

Data were analyzed using the mixed models procedure of SAS (v 9.3; SAS Institute, Cary, IN) with cage as a random effect and treatment as a fixed effect, and time as a repeated measure when applicable (i.e., for ITT and GTT data). Prior to analysis, data were checked for normality using the Anderson−Darling test, and all data were normally distributed. Differences between means were considered significant at $P < 0.05$ using the Tukey−Kramer multiple comparison test. Data are expressed as means ± standard deviation.

Results

Both high-fat diets, regardless of their fatty acid composition, induced obesity, hyperglycemia and hyperinsulinemia in mice

The average food intakes during the experimental period was similar among the treatments (Fig 3A). As expected, the energy intake was higher ($P < 0.01$) in both high-fat fed groups (CHF and EHF) compared to the CLF group (Fig 3B). Although the cumulative energy intake over the 19-week experiment was higher in EHF than CHF (1912.61 vs. 1710.56 kcal; $P = 0.0019$), the final body weight was comparable between the two groups (Fig 4). Consistent with the increased body weight in high-fat fed mice, adiposity was comparably increased, which was reflected in epididymal fat pad mass (Fig 4C).

Consumption of high-fat diets for 19 weeks resulted in hyperglycemia and hyperinsulinemia in C57BL/6J mice (Table 6). Fasting plasma levels of cholesterol were increased by both high-fat treatments compared with the CLF, whereas plasma triglyceride levels were not different among treatment groups (Table 6).

EHF consumption was associated with high-fat diet induced glucose intolerance

Both high-fat diets induced glucose intolerance and decreased insulin sensitivity as evidenced by glucose and insulin tolerance tests, respectively (Fig 5). Notably, the area under the curve for GTT in EHF mice was higher ($P < 0.05$) than in CHF group (Fig 5), suggesting that EHF worsened high-fat diet induced glucose intolerance in mice. Although our ITT data did not achieve statistical significance, the area under the curve for ITT, as a measure of whole-body insulin sensitivity, tended ($P = 0.09$) to be higher in the EHF group compared to CHF fed mice. Similarly, there was a trend toward higher fasting glucose levels in the EHF group compared with the CHF group ($P = 0.07$).

EHF consumption was associated with elevated hepatic steatosis and inflammation.

The effects of TVA+RA enriched beef fat on hepatic steatosis and inflammation were assessed by measuring liver triglyceride content using a biochemical assay, inflammation markers by qPCR, and serum markers of hepatic inflammation/damage, including AST and ALT (liver damage markers), and liver histology. Both high-fat diets induced steatosis and inflammation as evidenced by liver triglyceride content (Fig 6D) and histology (Fig 6H), and mRNA expression of inflammation markers (Fig 6G). Furthermore, both the CHF and EHF groups had higher ($P < 0.01$) plasma ALT levels compared to the control group (Fig 6E). When comparing between the two high fat fed groups, EHF group had a higher ($P < 0.01$) liver weight (Fig 6A) and hepatic triglyceride content (Fig 6D), which were accompanied by increased ($P = 0.05$) hepatic expression of inflammation markers including MCP1 and F4/80 (Fig 6G) and elevated ($P < 0.01$) plasma ALT levels (Fig 6E).

EHF increased TFA content of liver while reduced the contents of n-6 and n-3 PUFA

Chronic consumption of high-fat diets (CHF and EHF) increased the hepatic concentrations of cis-MUFA at the expense of n-3 and n-6 PUFA (Fig 8). Compared to CHF group, EHF group had a higher content of TFA including trans 18:1 isomers, atypical dienes; CLA and conjugated linolenic acids (Table 7), while had a lower content of n-3 and n-6 PUFA including α-linolenic acid (18:3n-3), docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6; Table 7). This suggests that consuming EHF further reduced liver content of n-3 and n-6 PUFA in high-fat fed mice.

Discussion

Previously, it has been demonstrated that short-term feeding (3-4 weeks) of pure TVA or TVA+RA enriched beef fat can improve glucose homeostasis and insulin sensitivity in JCR:LA-*cp* rats, a rodent model of dyslipidemia and insulin resistance (Jacome-Sosa et al., 2014; Diane et al., 2016). However, whether long-term consumption of these fatty acids can attenuate diet-induced glucose intolerance and insulin resistance is not clear. Thus, we conducted this study to evaluate whether long-term supplementation with a TVA+RA enriched fat can attenuate high-fat diet induced glucose intolerance and insulin resistance. We used high-fat diet (45 kcal% fat) fed male C57Bl6J mice, which is a reliable model for visceral obesity, glucose intolerance and insulin resistance. For the EHF diet, we used a tallow made from the subcutaneous fat of flaxseed-fed beef cattle, which is naturally enriched with TVA and RA (Vahmani et al., 2017). We designed the CHF diet in a way that matched the trans fatty acid contents (e.g. trans 18:1 and CLA isomers) in EHD diet with their cis equivalents (e.g. oleic acid and LNA) to keep the total SFA, MUFA, and PUFA similar between the two high-fat diets (Table 3). We used an 18-week feeding period with the diets provided at the start of the intervention to examine the ability of EHF to prevent (as opposed to treating) glucose intolerance and insulin resistance in DIO mice.

Feeding EHF did not affect body weight gain and epididymal fat pad weight compared to CHF, implying that both high-fat diets similarly affected body weight gain and adiposity. Consistent with our findings, feeding a high fat diet containing butter enriched with VA and RA to Wistar rats did not affect body weight or body composition compared to those fed a control high fat diet (De Almeida et al., 2014). Similarly, feeding pure TVA or RA had no effect on body weight and body fat accumulation in rodent models when compared to diets containing similar amounts of pure oleic acid or linoleic acid, respectively (Clément et al., 2002; Wang et al., 2008).

Beef fat enriched with VA and RA did not alleviate the worsened glucose control caused by high fat feeding in mice

We conducted this study mainly to resolve the contradictions in the literature concerning the effect of ruminant TFA on glucose homeostasis. To the best of our knowledge, this is the first study to test the effects of a ruminant fat naturally enriched with TVA and RA in high-fat diet induced obese mice. In contrast to previous rodent studies in which pure TVA or RA or TVA+RA enriched ruminant fats were fed (De Almeida et al., 2014; Jacome-Sosa et al., 2014; Diane et al., 2016), we found that long-term feeding of EHF did not improve glucose homeostasis in DIO mice, as evidenced by fasting blood glucose and GTT area under the curve. Notably, our findings are similar to those reported in previous mouse studies and human clinical trials, in which industrial TFA were fed (Clément et al., 2002; Risérus et al., 2002; Koppe et al., 2009). Given the TVA+RA enriched beef fat used in our study also contained a relatively high content of several other trans 18:1, 18:2 and 18:3 isomers (Table 4), it is possible that EHF-induced detrimental effects on glucose homeostasis were caused by TFA other than TVA and RA. While the underlying mechanisms are not fully understood, it has been suggested that industrial trans fatty acids may in part cause insulin resistance by decreasing the PUFA levels, in particular arachidonic acid (20:4n-6), in liver and adipose tissue, which in turn could impair cell membrane lipid fluidity, therefore

reducing responsiveness to insulin (Ibrahim et al., 2005; Koppe et al., 2009; Obara et al., 2010). The fatty acid analysis of liver in the present study also revealed a decrease in long-chain PUFA levels such as arachidonic acid and docosahexaenoic acid in the liver of EHF-fed mice compared to that of CHF-fed mice (Table 4). Nevertheless, the potential role of decreased long-chain PUFA levels in inducing insulin resistance and progression to steatosis needs further investigations.

Beef fat enriched with TVA and RA resulted in increased steatosis, markers of inflammation and liver injury in DIO mice

In addition to insulin resistance, steatosis is as one of the major consequences of obesity and a hallmark of high-fat fed mice. Compared to natural saturated and cis-unsaturated, consumption of industrial TFA (partially hydrogenated vegetable oils or mixture of trans 18:1 isomers) have been consistently shown to cause steatosis in mouse studies (Collison et al., 2009; Koppe et al., 2009; Obara et al., 2010; Oteng et al., 2019). Several studies have shown that industrial TFA can cause preferential fat accumulation in the liver at the expense of adipose tissues when compared with saturated or *cis*-unsaturated fatty acids (Oteng and Kersten, 2020). In addition, pure elaidic acid (trans9-18:1; a major trans 18:1 isomer found in industrial TFA) have been shown to promote fat accumulation in the liver by stimulating SREBP1c-dependent lipogenesis pathways (Oteng et al., 2019). In contrast, short-term rodent studies with pure TVA and RA found either no effect or reduced liver fat content when compared to oleic acid and linoleic acid, respectively (Clément et al., 2002; Jacome-Sosa et al., 2014). With regards to liver health, the EHF effects on the liver in the present study (increased liver weight and triglyceride content and markers of inflammation and liver injury) resemble that of previously reported for industrial TFA (Clément et al., 2002; Collison et al., 2009; Koppe et al., 2009; Obara et al., 2010; Oteng et al., 2019). Thus, it is possible that TFA other than TVA and RA, contributed to the adverse effects of EHF on liver health.

Conclusion

Contrary to our hypothesis, long-term supplementation TVA+RA enriched beef fat was associated with worse glucose hemostasis and liver steatosis in DIO mice, which is similar to that previously reported for industrial TFA. However, we cannot rule out whether the effects seen were caused by TVA and RA or other TFA isomers present in TVA+RA enriched beef fat. Thus, additional studies are needed to determine isomer specific effects of ruminant TFA. Until then, production practices resulting in increased levels of this group of fatty acids in ruminant milk and meat should be carefully reconsidered.

CHAPTER 3 – Conclusion and Future directions

Recent government bans on industrial TFA in developed countries has left naturally occurring TFA from ruminant products (e.g., dairy, beef, and lamb) as the sole source of TFA in the food supply. In contrast to industrial TFA, which have undisputed adverse health effects, ruminant TFA such as TVA, RA and TPA have been associated with reduced risk for some diseases such as type 2 diabetes. However, whether long-term consumption of these fatty acids can attenuate diet-induced glucose intolerance and insulin resistance is unclear. Thus, we conducted this research to evaluate whether long-term supplementation with a TVA+RA enriched fat (i.e. tallow from flaxseed-fed cattle) can attenuate high-fat diet induced glucose intolerance and insulin resistance. We used high-fat diet (45 kcal% fat) fed male C57Bl6J mice, which is known to be a reliable model for visceral obesity, glucose intolerance and insulin resistance. Our findings showed that long-term supplementation TVA+RA enriched beef fat was associated with worse glucose hemostasis and liver steatosis in DIO mice, which is similar to that previously reported for industrial TFA. However, we cannot rule out whether the effects seen were caused by TVA and RA or other TFA isomers present in TVA+RA enriched beef fat. Thus, additional feeding trials with pure isomers are needed to better understand how ruminant TFA impact whole-body glucose homeostasis and insulin signaling pathways. This would include supplementation of DIO diets with purified trans fatty acids including VA, RA and trans9-18:1 (the major isomer in industrial TFA) to assess their effects on liver health (e.g. fat content, inflammation markers and lipogenic gene expression) and glucose homeostasis. The latter can achieved by measuring fasted and fed blood glucose and insulin concentrations, as well as GTT and ITT assays. It is also important to assess the effects of pure trans fatty acids (VA, RA and trans9-18:1) on insulin signaling pathways in DIO mice to better understand their effects on insulin sensitivity at the molecular level. To

achieve this, mice can be intraperitoneally injected with either saline or insulin 10 min before euthanasia. Then, the insulin sensitive tissues including liver, muscle and adipose can be collected to assess the basal and insulin-stimulated phosphorylation of major components of the insulin signaling cascade such as protein kinase B (Akt) using Western blot. Data generated from these studies will be key to determining the bioactivity and health effects of ruminant TFA.

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FIGURES

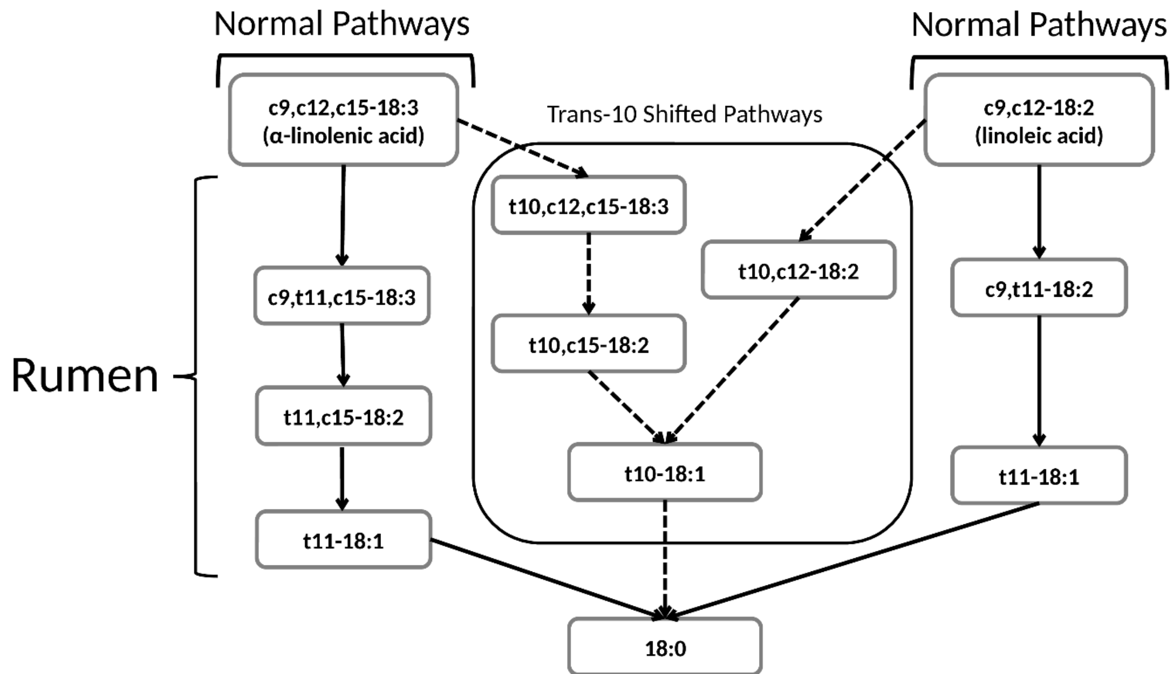


Figure 1. Normal and trans-10 shifted biohydrogenation pathways of linoleic acid (cis9,cis12-18:2) and alpha-linolenic acid (cis9,cis12,cis15-18:3). Arrow with solid lines show the main pathways in ruminants fed forage-based diets, and arrows with dashed lines show pathways in ruminants fed grain-based diets (Adopted from Alves et al., 2021)

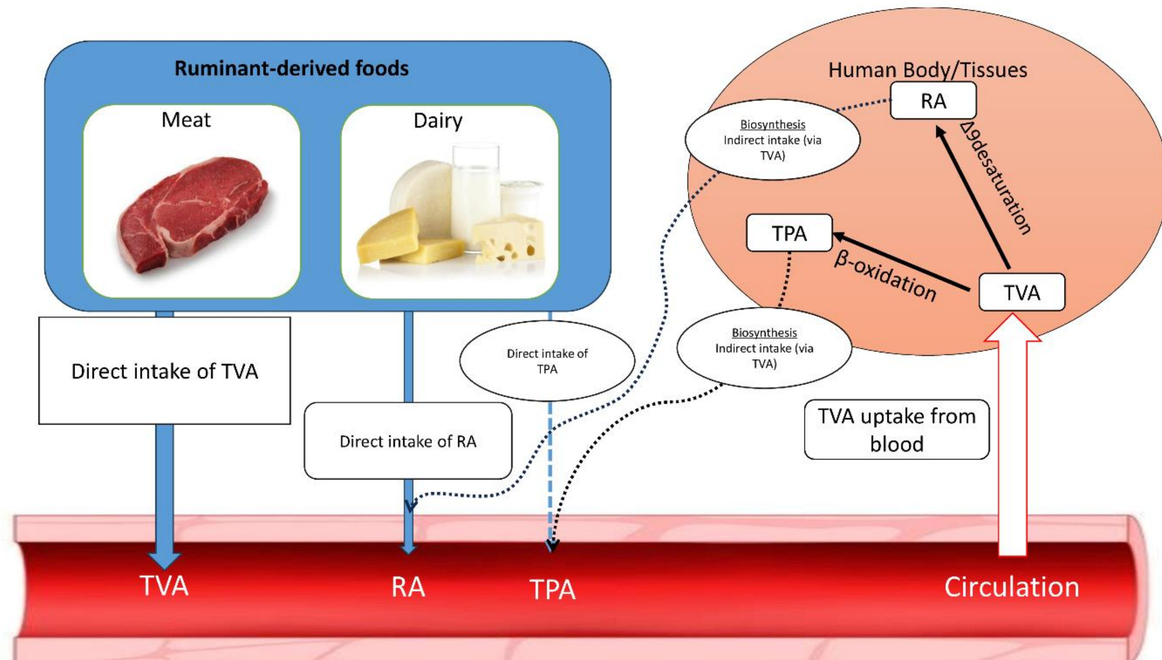


Figure 2. Origin of ruminant trans fatty acids in human blood. TPA, trans-palmitoleic acid (trans9-16:1); TVA, trans vaccenic acid (trans11-18:1); RA, rumenic acid (cis9,trans11-18:2). Arrows with thick solid lines describes the major origins, arrow with narrow lines indicates minor origins, arrows with dashed lines shows the very minor origins (Turpeinen et al., 2002; Miller et al., 2003; Jaudszus et al., 2014).

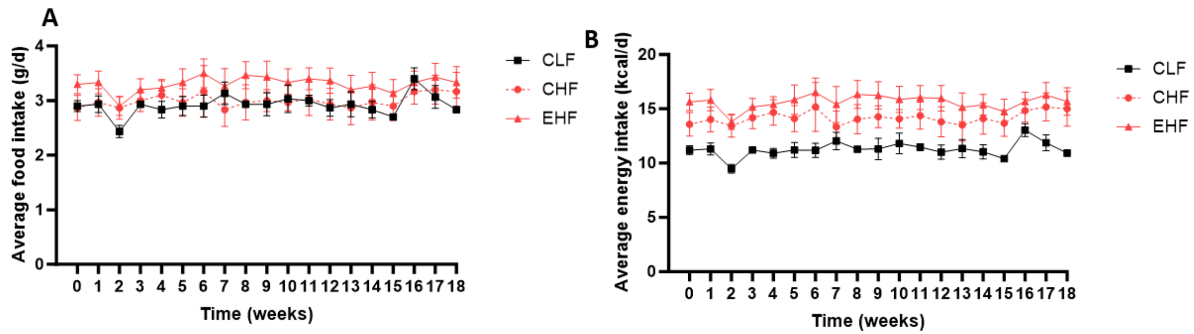


Figure 3. **Food intake (A) and energy intake (B) of male C57BL/6J mice fed experimental diets: control low-fat diet (CLF, 10% kcal from fat), high-fat diet with lard (CHF; 45% kcal from fat), and high-fat diet with TVA and RA-enriched tallow (EHF, 45% kcal from fat) for 19 weeks.** Body weight (n=12/group) and energy intake (n=12/group) were measured weekly during the feeding period. Data were analyzed using the mixed models procedure of SAS. Differences between means were considered significant at $P < 0.05$ using the Tukey−Kramer multiple comparison test. Values are expressed as mean ± standard deviation.

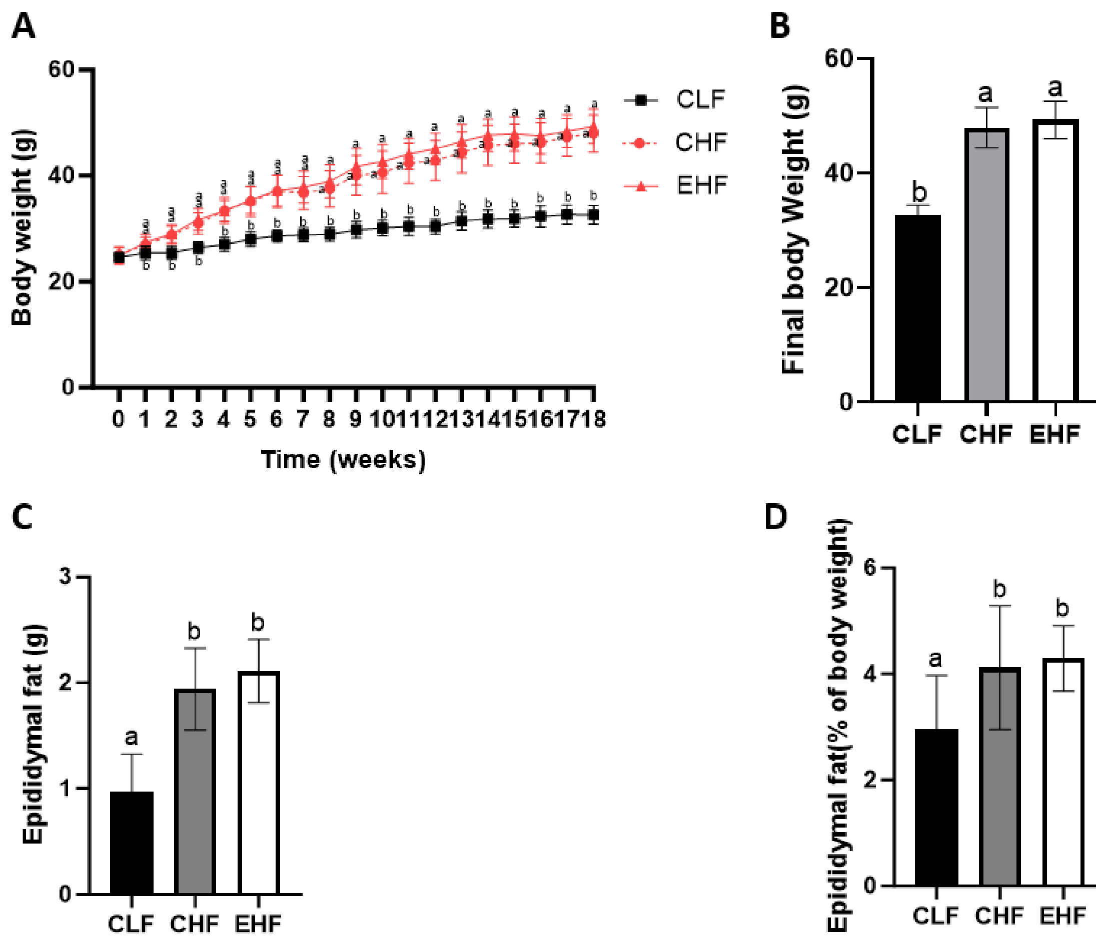
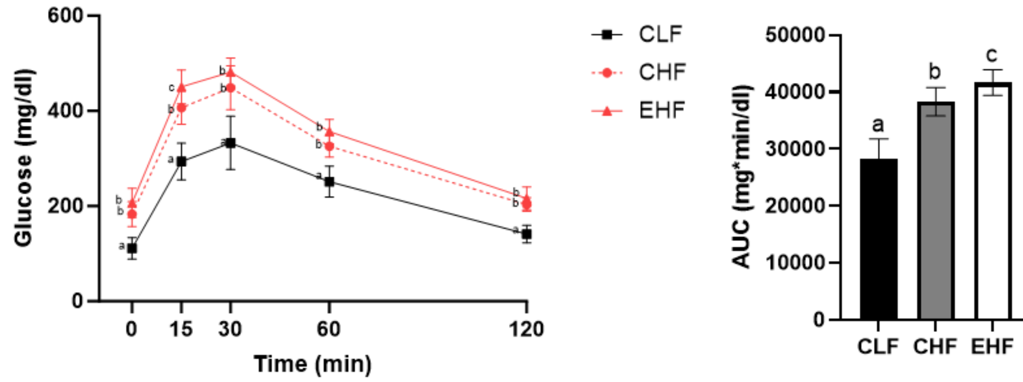


Figure 4. Body weight change over time (A), final body weight (B), epididymal fat pad weight in grams (C), epididymal fat pad weight as percentage of body weight (D) of male C57BL/6J mice fed experimental diets: control low-fat diet (CLF, 10% kcal from fat), high-fat diet with lard (CHF; 45% kcal from fat), and high-fat diet with TVA and RA-enriched tallow (EHF, 45% kcal from fat) for 19 weeks. Body weight (n=12/group) and energy intake (n=12/group) were measured weekly during the feeding period. Data were analyzed using the mixed models procedure of SAS. Differences between means were considered to be significant at $P < 0.05$ using the Tukey−Kramer multiple comparison test. Values are expressed as mean ± standard deviation. Time points and bars not sharing common letters (a-b) are significantly different ($P < 0.05$).

A) GTT



B) ITT

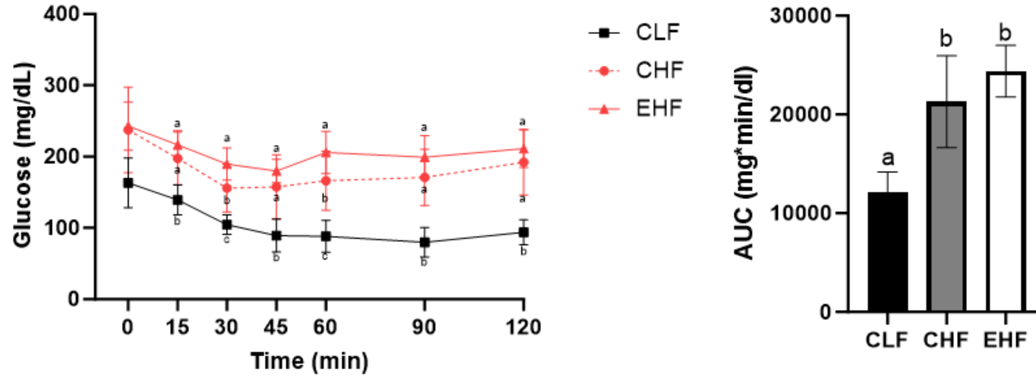


Figure 5. Glucose and insulin tolerance test in male C57BL/6J mice fed experimental diets Glucose tolerance test (GTT) and its area under curve (AUC) at week 16 (A), and insulin tolerance test (ITT) and its AUC at week 17 (B) in male C57BL/6J mice fed experimental diets: control low-fat diet (CLF, 10% kcal from fat), high-fat diet with lard (CHF; 45% kcal from fat), and high-fat diet with TVA and RA-enriched tallow (EHF, 45% kcal from fat) for 19 weeks. Values are expressed as mean ± standard deviation and are the average of 12 animals/group. Values not sharing common letters (a-c) are significantly different ($P < 0.05$).

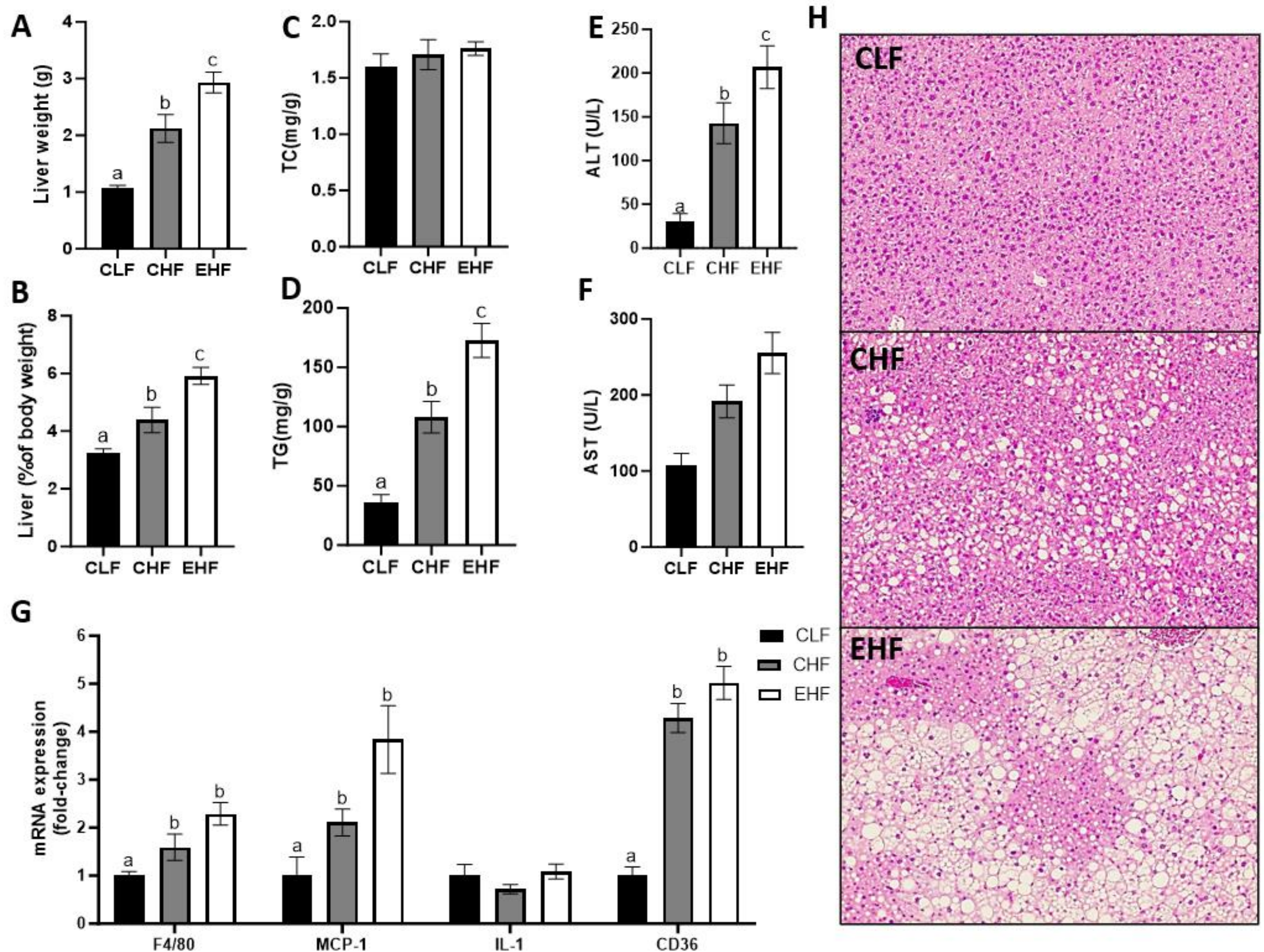


Figure 6. Liver weight in grams (A), liver weight as percentage of body weight (B), liver total cholesterol (TC) content (C), liver triglyceride (TG) content (D), alanine aminotransferase (ALT) concentration in plasma (E), aspartate aminotransferase (AST) concentration in plasma (F), mRNA expression inflammation markers in liver (G), Hematoxylin and eosin (H&E)-stained liver sections (H) from mice fed different experimental diets. Male C57BL/6J mice fed experimental diets: control low-fat diet (CLF, 10% kcal from fat), high-fat diet with lard (CHF; 45% kcal from fat), and high-fat diet with TVA and RA-enriched tallow (EHF, 45% kcal from fat) for 19 weeks. Data were analyzed using the mixed models procedure of SAS. Differences between means were considered significant at $P < 0.05$ using the Tukey−Kramer multiple comparison test. Values are expressed as mean ± standard error of the mean and are the average of 12 animals/group. Values not sharing common letters (a-c) are significantly different ($P < 0.05$).

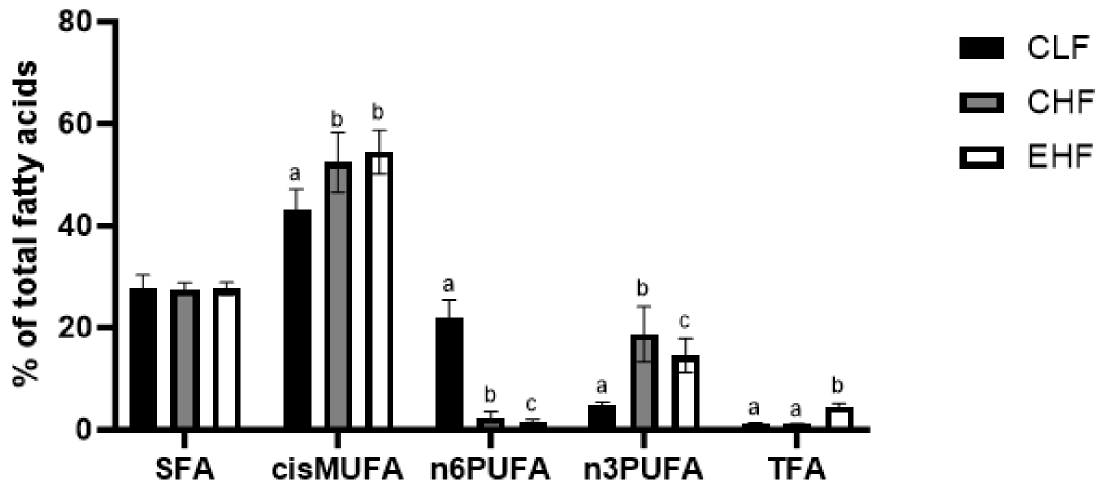


Figure 7 Hepatic content of different fatty acid groups in male C57BL/6J mice fed experimental diets: control low-fat diet (CLF, 10% kcal from fat), high-fat diet with lard (CHF; 45% kcal from fat), and high-fat diet with TVA and RA-enriched tallow (EHF, 45% kcal from fat) for 19 weeks. Total saturated fatty acids (SFA), total cis monounsaturated fatty acids (cis-MUFA), total omega-6 polyunsaturated fatty acids (n6PUFA), Total omega3 polyunsaturated fatty acids (n-3 PUFA), total trans fatty acids (TFA). Data were analyzed using the mixed models procedure of SAS. Differences between means were considered to be significant at $P < 0.05$ using the Tukey−Kramer multiple comparison test. Values are expressed as mean ± standard deviation and are the average of 12 animals/group. Within each fatty acid type, bars not sharing common letters (a-c) are significantly different ($P < 0.05$).

TABLES

Table 1. The TFA composition of common ruminant fats

	Bovine milk fat [1]		Grass-fed beef fat [2]		Grain-fed beef fat [2]	
Fatty acid	% of total FA	% of TFA	% of total FA	% of TFA	% of total FA	% of TFA
t6-8-16:1	0.24	3.41	0.51	6.35	0.28	5.78
t9-16:1	0.05	0.77	0.09	1.10	0.02	0.37
t10-16:1	0.01	0.19	0.01	0.15	0.01	0.11
t11-12-16:1	0.04	0.59	0.05	0.68	0.03	0.70
t14-16:1	0.02	0.33	0.04	0.47	NR	NR
∑trans16:1	0.37	5.29	0.70	8.74	0.34	6.96
t4-18:1	0.04	0.59	0.02	0.27	0.02	0.39
t5-18:1	0.04	0.62	0.02	0.23	0.02	0.44
t6-8-18:1	0.43	6.14	0.17	2.09	0.40	8.22
t9-18:1	0.39	5.52	0.21	2.65	0.37	7.62
t10-18:1	0.73	10.32	0.19	2.36	2.05	42.30
t11-18:1	1.18	16.70	3.37	42.25	0.52	10.81
t12-18:1	0.58	8.17	0.12	1.57	0.10	2.14
t13-14-18:1	1.09	15.48	0.32	4.07	0.15	3.03
t15-18:1	0.75	10.65	0.15	1.88	NR	NR
t16-18:1	0.41	5.74	0.34	4.32	0.09	1.87
∑trans18:1	5.64	79.93	4.92	61.67	3.57	73.79
t11,t15-18:2	0.01	0.08	0.09	1.18	0.01	0.16

t9,t12-18:2	0.01	0.18	0.02	0.25	0.01	0.26
c9,t13-/t8,c12-18:2	0.23	3.30	0.25	3.14	0.15	3.18
t8,c13-18:2	0.09	1.32	0.12	1.52	0.07	1.35
c9,t12-18:2	NR	NR	NR	NR	0.06	1.21
t9,c12-18:2	0.03	0.43	NR	NR	0.01	0.29
t11,c15-18:2	0.04	0.53	0.57	7.18	0.04	0.92
∑AD	0.41	5.83	1.06	13.27	0.36	7.37
t7,c9-18:2	0.06	0.79	NR	NR	NR	NR
c9,t11-18:2/t8,c10-18:2	0.44	6.18	0.90	11.34	0.39	8.07
t10,c12-18:2	0.03	0.41	0.01	0.12	0.04	0.87
t11,c13-18:2	0.01	0.15	0.12	0.22	0.02	0.34
t12,t14-/t13,t15-18:2	0.01	0.15	0.03	0.34	0.02	0.37
t11,t13-18:2	0.03	0.40	0.05	0.67	0.02	0.44
t7,t9-t10,t12-18:2	0.04	0.50	0.02	0.31	0.02	0.49
∑CLA	0.61	8.57	1.15	14.46	0.55	11.31
c9,t11,t15-18:3	0.01	0.10	0.06	0.77	0.01	0.29
c9,t11,c15-18:3	0.02	0.27	0.09	1.09	0.01	0.29
ΣCLnA	0.03	0.37	0.15	1.86	0.03	0.57

NR , not reported; TFA, total trans fatty acids;

***c*, *cis*; *t*, *trans*; ΣCLA, sum of conjugated linoleic acid isomers; ΣAD, sum of atypical dienes (non-conjugated non-methylene interrupted 18:2; ΣCLnA, sum of conjugated linolenic acid (18:3) isomers.**

[1] (Rosemond, 2021)

[2](Klopatek et al., 2022)

Table 2. Diet formulations

	Control low-fat (CLF)		Control high-fat (CHF)		Enriched high-fat (EHF)	
Ingredients (g/kg diet)	g	kcal%	g	kcal%	g	kcal%
Casein, 30 Mesh	189.56	19.72	233.06	19.72	233.06	19.72
L-Cystine	2.84	0.30	3.50	0.30	3.50	0.30
Corn Starch	428.61	44.58	84.83	7.17	84.83	7.17
Maltodextrin 10	71.09	7.39	116.53	9.86	116.53	9.86
Sucrose	163.78	17.04	201.36	17.03	201.36	17.03
Cellulose, BW200	47.39	0.00	58.26	0.00	58.26	0.00
Soybean Oil	23.70	5.55	28.31	5.39	0.00	0.00
Lard	18.96	4.44	179.33	34.15	0.00	0.00
Flax fed Beef tallow	0.00	0.00	0.00	0.00	169.89	32.35
Safflower oil	0.00	0.00	11.80	2.25	30.68	5.84
Cocoa butter	0.00	0.00	0.00	0.00	18.88	3.59
Palm oil	0.00	0.00	16.52	3.15	16.52	3.15
Mineral Mix S10026	9.48	0.00	11.65	0.00	11.65	0.00
DiCalcium Phosphate	12.32	0.00	15.15	0.00	15.15	0.00
Calcium Carbonate	5.21	0.00	6.41	0.00	6.41	0.00
Potassium Citrate, 1 H2O	15.64	0.00	19.23	0.00	19.23	0.00
Vitamin Mix V10001	9.48	0.99	11.65	0.99	11.65	0.99
Choline Bitartrate	1.90	0.00	2.33	0.00	2.33	0.00
FD&C Red Dye #40	0.01	0.00	0.00	0.00	0.06	0.00
FD&C Blue Dye #1	0.00	0.00	0.06	0.00	0.00	0.00
FD&C Yelloe Dye #5	0.04	0.00	0.00	0.00	0.00	0.00
Total	1000	100	1000	100	1000	100

Table 3. Diet composition

	Control low-fat (CLF)		Control high-fat (CHF)		Enriched high-fat (EHF)	
Nutrients and Energy	Diet%	kcal%	Diet%	kcal%	Diet%	kcal%
Protein	19.2	20.0	24.0	20.0	24.0	20.0
Carbohydrate	67.3	70.0	41.0	35.0	41.0	35.0
Fat	4.3	10.0	24.0	45.0	24.0	45.0
kcal/g	3.9		4.7		4.7	
Fatty acid (FA)*	FA%	kcal%	FA%	kcal%	FA%	kcal%
C16:0 (palmitic acid)	16.0	1.6	21.0	9.4	21.6	9.7
C18:0 (stearic acid)	7.7	0.8	11.6	5.2	10.1	4.6
Cis9-18:1 (oleic acid)	24.9	2.5	37.4	16.8	27.3	12.3
Trans11-18:1 (trans vaccenic acid)	0.2	0.0	0.1	0.1	5.3	2.4
18:2n-6 (linoleic acid)	38.4	3.8	18.6	8.4	12.5	5.6
18:3n-3 (α-linolenic acid)	5.3	0.5	1.3	0.6	0.8	0.4
Cis9,trans11-18:2 (Rumenic acid)	0.1	0.0	0.1	0.0	2.0	0.9
∑TFA	0.9	0.1	0.9	0.4	14.3	6.4
∑SFA	25.6	2.6	34.7	15.8	36.2	16.3
∑MUFA	3.9	0.4	43.1	19.4	41.9	18.9
∑PUFA	44.7	4.5	21.3	9.6	20.3	9.1
n6-/n3-PUFA	7.1	-	12.3	-	13.4	-

∑TFA: Sum of fatty acids that contain at least one trans double bond (trans-18:1+conjugated 18:2+non conjugated non-methylene interrupted 18:2+ conjugated 18:3)
∑SFA: Sum of saturated fatty acids
∑MUFA: Sum of monounsaturated fatty acids (trans-16:1+trans-18:1+cis16:1+cis-18:1)
∑PUFA: Sum of polyunsaturated fatty acids (n-6PUFA+n-3-PUFA+ conjugated 18:2+non conjugated non-methylene interrupted 18:2+ conjugated 18:3)
*Fatty acids are presented as % of total fatty acids (FA%) and as % of dietary energy (kcal%)

Table 4. Detailed fatty acid composition of diets (% of total fatty acids)

Fatty acid	Control low-fat (CLF)	Control high-fat (CHF)	Enriched high-fat (EHF)
14:0	1.01	1.21	2.99
16:0	16.01	20.98	21.64
18:0	7.71	11.62	10.13
20:0	0.21	0.22	0.18
22:0	0.14	0.08	0.07
24:0	0.04	0.04	0.04
∑SFA	25.58	34.74	36.15
c7-16:1	0.15	0.18	0.12
c9-16:1	0.80	1.52	2.98
c11-16:1	0.03	0.02	0.21
c9-18:1	24.94	37.43	27.29
c11-18:1	1.54	2.23	1.03
c12-18:1	0.01	0.02	0.17
c13-18:1	0.06	0.09	0.31
∑cisMUFA	28.17	42.51	33.87
t6-t8-16:1	0.03	0.02	0.22
t9-16:1	0.03	0.01	0.10
t10-16:1	0.02	0.00	0.01
t11-t12-16:1	0.03	0.00	0.06
t14-16:1	0.01	0.01	0.02
t6-t8-18:1	0.03	0.02	0.32
t9-18:1	0.06	0.11	0.35
t10-18:1	0.06	0.09	0.33
t11-18:1	0.21	0.14	5.27
t12-18:1	0.02	0.02	0.32
t13-t14-18:1	0.10	0.06	0.69
c14-t16-18:1	0.03	0.02	0.20
∑transMUFA	0.68	0.59	7.98
18:2n-6	38.41	18.61	12.48
18:3n-6	0.04	0.02	0.01
20:2n-6	0.24	0.31	0.02
20:3n-6	0.04	0.05	0.03
20:4n-6	0.10	0.14	0.03
∑n-6PUFA	38.85	19.22	12.57
18:3n-3	5.34	1.34	0.78
20:3n-3	0.05	0.08	0.03
20:4n-3	0.01	0.02	0.03

20:5n-3	0.02	0.02	0.03
22:3n-3	0.01	0.01	0.00
22:5n-3	0.04	0.06	0.06
22:6n-3	0.01	0.02	0.01
∑n3PUFA	5.47	1.56	0.94
t11,t15-18:2	0.01	0.02	0.33
c9,t13-/t8,c12-18:2	0.03	0.03	0.46
t8.c13-18:2	0.01	0.01	0.13
t9,c12-18:2	0.06	0.05	0.10
t11,c15-18:2	0.03	0.03	2.03
c9,c15-18:2	0.06	0.10	0.11
c12,c15-18:2	0.01	0.01	0.04
∑AD	0.23	0.28	3.22
c9,t11-18:2	0.08	0.08	2.02
t11,c13-18:2/c9,c11-18:2	0.01	0.02	0.65
t11,t13-18:2	0.01	0.00	0.08
other t,t-CLA	0.04	0.05	0.10
∑CLA	0.16	0.17	2.85
c9,t11,t15-18:3	0.01	0.00	0.26
c9,t11,c15-18:3	0.02	0.02	0.43
∑CLnA	0.02	0.02	0.69
∑transFA	0.93	0.97	14.25

c = *cis*; *t* = *trans*; SFA: Saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: Polyunsaturated fatty acid; AD: Atypical dienes; CLA: conjugated linoleic acids; CLnA: conjugated linolenic acids; TFA: fatty acids with at least one trans double bond .

Table 5 Gene-specific forward and reverse primer sequences used for qPCR

Gene	Forward primer (5'-3')	Reverse primer (3'-5')	source
F4/80	TGTGTCGTGCTGTTCAGA ACC	AGGAATCCCGCAATGATGG	(van der Heijden et al., 2015)
MCP-1	GCTGGAGAGCTACAAGA GGATCA	ACAGACCTCTCTCTTGAGCT TGGT	(van der Heijden et al., 2015)
IL-1	TGCAGCTGGAGAGTGTG G	TGCTTGTGAGGTGCTGATG	(van der Heijden et al., 2015)
CD36	GATGACGTGGCAAAGAA CAG	TCCTCGGGGTCCTGAGTTAT	(van der Heijden et al., 2015)

F4/80, adhesion G protein-coupled receptor E1; MCP-1, monocyte chemoattractant protein 1; IL-1 interleukin-1; CD36, cluster of differentiation 36.

Table 6. Metabolic parameters

Parameter	Control low-fat (CLF)	Control high-fat (CHF)	Enriched high-fat (EHF)	P-value
Total body weight gain, g	8.0±1.6[a]	23.0±3[b]	24.5±3.2[b]	<0.0001
Total food intake, g	358.8±15.35	361.64±27.85	404.36±20.79	0.0766
Glucose, mg/dl	96.3±13.6[a]	152.5±40.5[b]	178±27.9[b]	0.0001
Insulin, pg/ml	381±368.9[a]	2428±1025[b]	2363±770.1[b]	<0.0001
TG, mg/ml	0.8±0.2	0.7±0.1	0.7±0.1	0.1299
TC, mg/dl	73.4±15.0[a]	122.0±28.1[b]	119.6±21.2[b]	<0.0001

CLF: control low fat (10% kcal from fat), CHF: high-fat diet with lard (45% kcal from fat), EHF: high-fat diet with TVA+RA enriched tallow (45% kcal from fat); Total body weight gain and food intake during 18 weeks on experimental diets; Fasting plasma glucose, insulin, total cholesterol (TC) and triglyceride (TG) concentrations on week 15. Values are expressed as mean ± standard deviation and are the average of 12 animals/group. Means not sharing common letters (a-c) are significantly different ($P < 0.05$).

Table 7 Fatty acid composition of liver (% of total fatty acids)

Fatty acid	Control low-fat (CLF)	Control high-fat (CHF)	Enriched high-fat (EHF)
14:0	0.50±0.20[a]	0.39±0.05[b]	0.48±0.04[a]
16:0	22.00±1.40[a]	23.72±1.37[b]	24.15±0.77[b]
18:0	5.01±1.46[a]	3.09±0.86[b]	2.36±0.59[b]
20:0	0.15±0.08	0.13±0.04	0.14±0.06
22:0	0.07±0.03[a]	0.03±0.01[b]	0.04±0.01[ab]
∑SFA	27.98±2.38	27.59±1.2	27.63±1.25
c7-16:1	0.91±0.14[a]	1.9±0.36[b]	2±0.34[b]
c9-16:1	5.80±1.17[a]	3.11±0.24[b]	3.97±0.46[b]
c11-16:1	0.04±0.01[a]	0.03±0.00[a]	0.15±0.02[b]
c9-18:1	31.10±3.14[a]	42.24±4.33[b]	41.60±3.34[b]
c11-18:1	4.23±1.50[a]	3.87±0.85[a]	4.81±0.79[a]
c12-18:1	0.00±0.00[a]	0.00±0.00[a]	0.06±0.01[b]
c13-18:1	0.06±0.03[a]	0.04±0.01[b]	0.35±0.03[c]
∑cisMUFA	43.07±4.13[a]	52.44±5.84[b]	54.43±4.27[b]
t6-t8-16:1	0.00±0.00[a]	0.00±0.00[a]	0.04±0.01[b]
t9-16:1	0.07±0.01[a]	0.05±0.01[a]	0.17±0.05[b]
t10-16:1	0.00±0.00[a]	0.00±0.00[a]	0.02±0.00[b]
t11-t12-16:1	0.00±0.00[a]	0.00±0.00[a]	0.08±0.01[b]
t14-16:1	0.02±0.01[a]	0.01±0.00[b]	0.04±0.00[c]
t6-t8-18:1	0.00±0.00[a]	0.00±0.00[a]	0.04±0.01[b]
t9-18:1	0.06±0.01[a]	0.05±0.01[b]	0.08±0.01[c]
t10-18:1	0.00±0.00[a]	0.00±0.00[a]	0.09±0.01[b]
t11-18:1	0.08±0.03[a]	0.02±0.01[b]	0.55±0.16[c]
t12-18:1	0.00±0.00[a]	0.00±0.00[a]	0.05±0.01[b]
t13-/t14-18:1	0.12±0.04[a]	0.16±0.03[ab]	0.18±0.04[b]
(c14-/)t16-18:1	0.00±0.00[a]	0.00±0.00[a]	0.02±0.01[b]
∑transMUFA	0.35±0.06[a]	0.28±0.04[a]	1.41±0.27[b]
18:2n-6	13.63±4.04[a]	10.41±2.76[ab]	6.92±1.42[b]
18:3n-6	0.47±0.37	0.26±0.14	0.1±0.03
20:2n-6	0.2±0.06	0.2±0.03	0.15±0.02
20:3n-6	0.82±0.44	0.69±0.14	0.46±0.14
C20:4n-6	6.45±1.41[a]	3.62±1.61[b]	1.94±1.07[c]
∑n-6PUFA	21.82±3.68[a]	15.53±4.43[b]	9.75±2.58[c]
18:3n-3	0.50±0.23[a]	0.20±0.08[b]	0.12±0.04[b]
20:3n-3	0.07±0.04	0.06±0.02	0.07±0.02
20:4n-3	0.00±0.00[a]	0.00±0.00[a]	0.03±0.01[b]
20:5n-3	0.19±0.06[a]	0.08±0.07[b]	0.04±0.02[b]
22:5n-3	0.27±0.07[a]	0.20±0.08[ab]	0.13±0.06[b]
22:6n-3	3.68±0.60[a]	1.87±0.91[b]	1.12±0.44[c]
∑n3PUFA	4.70±0.72[a]	2.42±1.13[b]	1.52±0.54[c]
t11,t15-18:2	0.00±0.00[a]	0.00±0.00[a]	0.04±0.01[b]
c9,t13-/t8,c12-18:2	0.00±0.00[a]	0.00±0.00[a]	0.23±0.02[b]

t9,c12-18:2	0.00±0.00[a]	0.00±0.00[a]	0.02±0.01[b]
t11,c15-18:2	0.18±0.05[a]	0.18±.04[a]	0.62±0.06[b]
c9.c15-18:2	0.03±0.01[a]	0.07±0.01[b]	0.11±0.02[c]
c12,c15-18:2	0.00±0.00[a]	0.00±0.00[a]	0.01±0.01[b]
∑AD	0.21±0.06[a]	0.25±0.04[a]	1.04±0.09[b]
c7,t9-/c9,t11-CLA	0.06±0.01[a]	0.03±0.01[a]	1.44±0.19[b]
t11,c13-18:2/c9,c11-18:2	0.07±0.02[a]	0.07±0.02[a]	0.21±0.02[b]
t11,t13-18:2	0.10±0.07	0.18±0.06	0.17±0.02
other t,t-CLA	0.10±0.10	0.02±0.02	0.04±0.01
∑CLA	0.32±0.06[a]	0.31±0.06[a]	1.86±0.18[b]
c9,t11,t15-18:3	0.02±0.01[a]	0.02±0.01[a]	0.09±0.02[b]
c9,t11,c15-18:3	0.27±0.14	0.27±0.07	0.32±0.03
∑CLnA	0.29±0.15	0.29±0.07	0.41±0.05
∑TFA	1.13±0.23[a]	1.06±0.19[a]	4.6±0.56[b]

t, trans; c, cis; SFA, Saturated fatty acid; MUFA, monounsaturated fatty acid; n-6 PUFA, omega6 polyunsaturated fatty acids; n-3 PUFA, omega3 polyunsaturated fatty acids; PUFA, polyunsaturated fatty acids, AD, atypical dienes; CLA, conjugated linoleic acids, CLNA, conjugated linolenic acids; TFA: total fatty acids with at least one trans double bond. Values are expressed as mean ± standard deviation and are the average of 12 animals/group. Means not sharing common letters (a-c) are significantly different ($P < 0.05$).