IMPACT OF FINISHING DIET AND BREED ON THE FATTY ACID AND PHYTOCHEMICAL PROFILE OF GRASS-FINISHED BEEF

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Food Science – Doctor of Philosophy

2023

ABSTRACT

While there are concerns regarding the effects of red meat consumption on non-communicable diseases and climate change, this view does not consider the impact of different production practices. Grass-finished beef (GFB) generally aligns with the demands of consumers who are concerned about nutrition and the environment. GFB usually contains higher levels of omega-3 polyunsaturated fatty acids (*n*-3 PUFAs), conjugated linoleic acid (CLA), vitamin E, and phytochemicals desired by health-conscious consumers. However, a recent survey of commercially available GFB found large variations in the nutritional profile of beef coming from different producers. In some cases, GFB had a higher omega-6:omega-3 (*n*-6:*n*-3) ratio than grain-finished beef. This dissertation aims to explain nutritional variations in GFB and investigate the influence of breed and feeding practices on its composition including fatty acids (FAs), micronutrients, and phytochemicals.

In the first study, Red Angus (RA) and Red Angus x Akaushi (AK) steers (n = 104) were randomly allocated to either a pasture or a grain diet. Feed samples were collected to determine variations in the FA and antioxidant profiles of the biodiverse pasture, and different plant species were correlated with specific weather data and bioactive compounds (chapter 2). Beef samples were profiled for FAs and micronutrients (chapter 3). In the second study (n = 117), three groups of steers were kept on pasture and supplemented with either hay, baleage, or soybean hulls (SH). A fourth group was fed baleage and SH in feedlot. Beef FAs, micronutrients, and lipid peroxidation values were analyzed (chapter 4). Chapter 5 involved 54 RA steers fed either a grass diet, a total mixed ration, or a total mixed ration supplemented with grapeseed extracts (GSE). Beef FAs, micronutrients, and phytochemicals were analyzed. FAs were analyzed by gas chromatography-

mass spectrometry (GC-MS), and phytochemicals were analyzed by ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

In chapter 2, the diverse pasture yielded higher levels of beneficial compounds (phenols, chlorophyll, carotenoids, *n*-3 PUFAs) compared to the total mixed ration, and early and late grazing season displayed the most beneficial nutritional profiles. In chapter 3, GFB exhibited higher levels of *n*-3 PUFAs, CLA, iron, and vitamin E compared to grain-finished beef, indicating a strong diet effect. Breed effects were only observed for a few specific FAs and micronutrients, and the interaction between diet and breed was not significant. In chapter 4, SH increased the *n*-6:*n*-3 ratio of beef, while supplementing GFB with hay resulted in the most favorable nutritional profile, with higher levels of long-chain *n*-3 PUFAs and a lower *n*-6:*n*-3 ratio compared to the other groups. However, the ratio remained under 2:1 for all treatments tested. In chapter 5, GFB exhibited a richer phytochemical profile compared to grain-finished beef and beef supplemented with GSE. Specific phytochemicals were also identified to discriminate between finishing diets.

Altogether, GFB offers potential health benefits due to its beneficial FA profile and increased levels of phytochemicals compared to grain-finished beef. It was established that the diet had the strongest potential to modify the nutritional profile of beef. The supplemental feeds tested in this dissertation could not explain the large nutritional variations observed previously. Not all beef is equal in nutrient density, and distinctions should be made when considering the nutrient profile of beef. Future research should focus on the standardization of labeling practices for GFB and the effects of beef consumption from varying productions systems on human health.

Dedicated to all the international students who couldn't go home during the COVID-19 pandemic.

ACKNOWLEDGMENTS

I would like to start by thanking my doctoral advisor Dr. Jenifer Fenton for her invaluable help and support throughout my graduate journey. You have been a continuous source of encouragement, always reminding me that "things always work out." You taught me how to become a scientist, and I hope that we can keep collaborating for a long time. I truly appreciate your mentorship and friendship.

I would also like to thank my committee members Dr. Ilce Medina-Meza, Dr. Jason Rowntree, and Dr. Gale Strasburg for their guidance and for the difficult questions they asked during my comprehensive exam. You helped me think like a scientist and significantly improved my research projects. Dr. Medina-Meza, thank you for introducing me to the world of metabolomics and for always being available when I needed your advice. Dr. Rowntree, thank you for your mentorship and for your encouraging words. I am extremely grateful for your trust even when I did not have any agricultural/animal science experience! Dr. Strasburg, thank you for always challenging me to think about the real-life applications of my research and for showing me how to become a better teacher.

I also want to thank our collaborators and colleagues involved with the projects, without whom this research would have not been possible. Dr. Isabella Maciel, thank you for driving to KBS and for helping me collect samples at the farm! Dr. Joao Sacramento, thank you for reviewing the last chapter of this dissertation on such short notice. Thank you to the Greenacres Foundation for their invaluable help and trust with our research projects, with a special thanks to Chad Bitler. To KBS, thank you for taking care of our animals and for making sure that the trial was successful. To the MSU Meat Lab staff, thank you for taking the time to help with sample preparation. Dr. Andrea Garmyn, thank you for helping with carcass grading. To the CANR Statistical Consulting

Center, thank you for always being available to help with data analysis. Dr. Todd Lydic, thank you for helping us troubleshoot our beef phenol method. Dr. Stephan van Vliet, thank you for mentoring me and teaching me about metabolomics. I would also like to thank the members of the Fenton Lab, without whom none of this would have been possible. I was lucky enough to be surrounded by caring and talented scientists, and I will always be thankful for your help. A special thanks to Selin who taught me most of what I know about GC-MS.

Finally, I would like to thank my friends and family for their support. Thank you, Rick, for helping with grad classes and for always being available for lunch. To the Morgan State Tennis Team, thanks for an amazing undergraduate journey and for making the U.S. feel like home. I will always remember our moments (good and bad) and I consider all of you lifelong friends. To June, Jeremy, and Felix, thank you for always checking on me and for always being there when I need it. Your words of encouragement helped me tremendously. To my family, who always believed in me and supported me, even when I couldn't go home for extended periods of time. I always kept you close to my heart. To my mom Ursula and dad Raphaël, who always believed that I could become a doctor one day and who supported my dreams even if it meant moving across the Atlantic. I also have a big thought for my maternal grandmother who unfortunately was not able to see me graduate. I know that she would be proud. Thank you Giorgia and Sergio for being so welcoming and for making Michigan feel like home. And to Maria, thank you for being the most amazing girlfriend and for always believing in me. You always asked about my research and made it seem like it was the coolest topic ever. I will always be grateful for your support and for your unconditional love.

Thank you all.

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CHAPTER 1: LITERATURE REVIEW – ATTENTION TO THE DETAILS: HOW VARIATIONS IN THE U.S. GRASS-FED AND GRASS-FINISHED CATTLE-FEED SUPPLEMENTATION AND FINISHING DATE INFLUENCE HUMAN HEALTH

This chapter has been published in:

Krusinski, L., Sergin, S., Jambunathan, V., Rowntree, J.E., and Fenton, J.I. (2022). Attention to the details: How variations in the U.S. grass-fed cattle-feed supplementation and finishing date influence human health. *Front. Sust. Food Syst.* 6, 851494. doi: 10.3389/fsufs.2022.851494

1.1 Abstract

As the global population increases, so does meat consumption. This trend is accompanied by legitimate concerns regarding the meat industry, and consumers are demanding transparency on the environmental and health effects of the products they are purchasing. Multiple epidemiological studies have pushed leading health organizations to make recommendations to reduce red meat consumption. Nevertheless, no differentiation is made among red meats and beef. The beef production system is generally ignored despite nutritional differences between grain- and grass-fed beef. Compared to grain-fed beef, grass-fed beef contains a healthier fatty acid profile, including more omega-3 polyunsaturated fatty acids and conjugated linoleic acid, and increased concentrations of phytochemicals desired by health-conscious customers. However, there is a lack of consistency among grass-fed beef in the United States regarding clear product labeling and cattle dietary components. Grass-fed beef labeling confusion has emerged, including misunderstandings between grass-fed and grass-finished beef. Along with this, previous studies observed significant nutritional variation among grass-finished beef from different producers across the country. Cattle diet has the strongest influence on the nutritional composition of beef. Therefore, understanding differences in feeding practices is key to understanding differing nutritional quality of grass-fed beef. Feeding cattle diverse pastures composed of multiple plant species including grasses and legumes managed in a rotational grazing fashion results in higher

omega-3 polyunsaturated fatty acids and phytochemical levels in beef compared to feedlots and monocultures. Seasonal differences including changes in temperature, rainfall, grazing practices, and plant growth cycles affect the nutritional composition of feeds and ultimately meat. Additional feeds utilized in grass-fed beef production systems such as conserved forages may reduce or increase health-promoting nutrients in grass-fed beef, while supplements such as grape byproducts and flaxseed may improve its nutritional profile. Further research should measure the effects of individual feedstuff and the finishing period on the nutritional profile on grass-fed beef. A better understanding of these details will be a step towards the standardization of pasture-raised ruminant products, strengthening the relationship between grass-fed beef consumption and human health.

1.2 Introduction

Globally, meat consumption continues to increase, along with population and per capita income (Godfray et al., 2018). However, there are legitimate concerns regarding the sustainability of meat; greater consumption requires increased production and consequently, greater global warming, pollution, and water waste (Ritchie et al., 2018). Meat production is a source of methane emissions, accounting for approximately 15% of all anthropogenic emissions (Gerber et al., 2013). Further, it accounts for a third of all agricultural water use (Godfray et al., 2018). Consumers are becoming more cognizant of what they are purchasing and how it was produced, and are willing to pay a premium price for local, healthy, and environmentally friendly products (Asioli et al., 2017). Some consumers are moving towards alternatives like plant-based products and are reducing their meat consumption (Hodson and Earle, 2018;Delon, 2019).

Meat is important in many cultures and humans have consumed meat for centuries because of its nutritional qualities as well as its taste (Pighin et al., 2016;Melendrez-Ruiz et al., 2019). Beef is highly nutrient-dense, providing energy, protein, fat, and other micronutrients like zinc, iron,

selenium, and B vitamins (Omaye and Omaye, 2019). Beef is a significant source of desirable omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs), including α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) as well as ruminal *trans* fatty acids (FAs) such as conjugated linoleic acid (CLA) and *trans* vaccenic acid (TVA). These ruminal *trans* FAs are purported to have health-promoting benefits, including protection against the development of coronary heart disease (Scollan et al., 2014). However, the health benefits of ruminal *trans* FAs still require investigation. Some studies exploring the health effects of ruminal versus industrial *trans* FAs found possible negative impacts (Gebauer et al., 2015;Verneque et al., 2020).

Grass-finished beef (GFB) meets the demands of consumers who are concerned about nutrition and the environment (Xue et al., 2010). Compared to grain-fed beef, grass-fed and GFB contain less total fat, less cholesterol, and less myristic and palmitic acid— saturated fatty acids (SFAs) found to be more deleterious for cholesterol levels (Ponnampalam et al., 2006;Alfaia et al., 2009). GFB contains twice as much CLA and up to 25% more PUFAs compared to conventional beef (Van Elswyk and McNeill, 2014;Berthelot and Gruffat, 2018;Lenighan et al., 2019;Prache et al., 2020;Butler et al., 2021). Grass-fed production increases *n*-3 PUFAs without increasing omega-6 (*n*-6) PUFAs, reducing the *n*-6:*n*-3 ratio (Daley et al., 2010). Health-promoting phytochemicals including phenolics, terpenoids, and carotenoids are observed to be higher in GFB (van Vliet et al., 2021b). Further, properly managed grass-fed systems encourage plants to sequester more carbon, promote plant diversity, and improve the quality of fresh-water systems (Godfray et al., 2018;van Vliet et al., 2021b). Grass-fed systems utilize forage as a sustainable and available source of long-chain *n*-3 PUFAs as an alternative to marine sources (Scollan et al., 2014).

Grass-fed products usually command premiums in retail markets, but the definitions of what consumers are buying are not always clear (Bronkema et al., 2019). "Grass-fed" and "grass-finished" are often used interchangeably, but they do not necessarily refer to the same type of production. According to the USDA, beef can be labeled as "grass-fed" if cattle have been fed exclusively forages throughout their lifetime excluding milk from their mother and have continuous access to pasture throughout the growing season (Food Safety and Inspection Service, 2019). While no grain or grain byproducts are permissible, additional forage sources such as hay, silage, or baleage may be provided to "grass-fed" cattle. Mathews and Johnson (2013) suggested some silages may consist of large amounts of grain. Definitions for "grass-finished" are less clear, though this typically refers to cattle that were fattened only on forages prior to slaughter (Mathews and Johnson, 2013).

Variations in "grass-fed" and "grass-finished" cattle diets, finishing date, and the addition of supplemental feeds can result in significant nutritional variation among beef products (Dewhurst et al., 2001;Revello-Chion et al., 2011;Bronkema et al., 2019;Jain et al., 2020). For instance, a nutritional survey of GFB found that the *n*-6:*n*-3 ratio varied from as low as 1.8:1 to as high as 28.3:1. Mineral and antioxidant content of GFB also varied significantly by producer (Bronkema et al., 2019). Further, cattle finished in the spring had greater *n*-3 and *n*-6 PUFAs compared to cattle finished in the fall (Jain et al., 2020). The surprising variations highlight the need to determine how various factors can influence the nutritional composition of GFB. The goal of this review is to analyze the influence of cattle diet, seasonal variations, and supplementation on the nutritional quality of grass-fed and GFB and discuss how these differences can impact human health.

1.3 How grass-fed and grass-finished beef align with human health

1.3.1 Health rationale

Noncommunicable diseases account for 41 million deaths globally each year. The two leading noncommunicable diseases are cardiovascular diseases (CVDs) and cancer. Chronic disease and inflammation are influenced by environmental factors, with diet playing a significant role (Fritsche, 2015;World Health Organization, 2018;Sanchez-Rodriguez et al., 2019). Based on epidemiological studies, red meat consumption is often associated with increased risks of diabetes, CVDs, and cancer (Wolk, 2017). These claims led health organizations, such as the American Heart Association (AHA), to make public health recommendations to reduce red meat consumption (Arnett et al., 2019). However, epidemiological studies do not differentiate between production systems and types of red meat which are important factors affecting nutritional profile (Provenza et al., 2019). Beef from grass-fed production systems is more consistent with nutritional recommendations, especially regarding *n*-3 PUFAs and phytochemicals (Vannice and Rasmussen, 2014;Omaye and Omaye, 2019;van Vliet et al., 2021b). Omega-3 FAs are important compounds in foods that are linked to health benefits regarding reducing inflammation, blood triacylglycerols, and the risk of CVDs, depression, and arthritis (Calder, 2015;Saini and Keum, 2018). Further, phytochemicals including phenolic compounds also have multiple cardiovascular health benefits including protection against oxidative stress and modulation of blood pressure (Medina-Remón et al., 2015;Omaye and Omaye, 2019). Though public health recommendations suggest a decrease in red meat consumption to prevent chronic diseases, GFB addresses some of the nutritional concerns.

1.3.2 Fatty acids, phytochemicals, and human health

The typical Western diet is usually high in SFAs and *n*-6 PUFAs and deficient in *n*-3 PUFAs, related to an increased risk of developing CVDs, diabetes, obesity, and cancer (Simopoulos, 2002). However, FAs need to be considered individually to assess their effects on human health (Calder, 2015;Bloomfield et al., 2016). Saturated FAs as a whole are thought to promote inflammation and increase total low-density lipoprotein (LDL) cholesterol and insulin resistance. This is significant because LDL cholesterol is linked with incidence of coronary heart diseases (Billingsley et al., 2018). Therefore, SFAs increase the risk of CVDs, type 2 diabetes, and inflammation (Fritsche, 2015;Billingsley et al., 2018). However, not all SFAs have the same effects. Stearic acid, for example, has a neutral effect on LDL cholesterol, while myristic acid and palmitic acid have a total cholesterol-raising effect (FAO, 2010). Reduction of SFA consumption is usually linked to a replacement with other nutrients. When SFAs are replaced with refined carbohydrates, total serum cholesterol increases, along with the risk of developing CVDs (DiNicolantonio et al., 2016). Dietary intake of monounsaturated fatty acids (MUFAs) is thought to be beneficial for human health, especially when the increase of MUFAs is coupled with a decreased intake of SFAs. Oleic acid intake is associated with a lower risk of CVD and CVD mortality, while palmitoleic acid may increase insulin sensitivity and improve the blood lipid profile (Calder, 2015). Two important PUFAs are linoleic acid (LA) and ALA. The human body cannot synthesize these essential FAs, but they are important to human health as they are precursors for other long-chain PUFAs of interest including arachidonic acid, EPA, DPA, and DHA (Saini & Keum, 2018). Omega-3 PUFAs have anti-inflammatory effects while *n*-6 PUFAs do not (Simopoulos, 2006). The *n*-3 PUFAs DHA and EPA are linked to healthier cardiovascular functions and can be synthesized from the precursor ALA (Parolini, 2019;Mendivil, 2021).

However, the conversion from ALA to long-chain *n*-3 PUFAs remains low and is influenced by sex and LA concentrations (Harnack et al., 2009;Welch et al., 2010;Zhou et al., 2019). The *n*-6:*n*-3 ratio in the Western diet is estimated to be between 15:1 and 20:1 compared to 1:1 in wild animals or traditional human diets (Simopoulos, 2002;2006). A lower *n*-6:*n*:3 ratio is considered important to prevent chronic diseases (Simopoulos, 2006;Husted and Bouzinova, 2016). Overall, because each FA has a different effect, and the relative proportions of each FA can change health outcomes, it is important to analyze the FA profile of beef to understand its effects on human health.

Unsaturated *trans* FAs are an important topic in the connection between FAs and human health. Unsaturated *trans* FAs have their double bonds in the *trans* configuration. With the usual configuration of unsaturated FAs being *cis*; *trans*-FAs are formed either naturally via metabolic processes like microbial activity in ruminant animals or industrially by hydrogenation (Markiewicz-Keszycka et al., 2013;Calder, 2015). Each *cis* unsaturated FA can give multiple *trans* isomers, but the major ones include elaidic acid (*trans* C18:1 *n*-9), TVA (*trans* C18:1 *n*-11), and CLA (*c*9 *t*11 C18:2 and *c*12 *t*10 C18:2) (Calder, 2015). The *c*9 *t*11 CLA isomer is mainly found in bovine milk and meat, while the *t*10 *c*12 form is mainly found in processed oils (Lindmark Månsson, 2008;Calder, 2015;Alothman et al., 2019). Unsaturated *trans* FAs have different biological properties compared to the *cis* configuration, and their functions differ based on how they were produced. *Trans* FAs produced by industrial hydrogenation of plant oils are related to higher risks of CVDs compared to other FA classes (Calder, 2015;Del Razo Olvera et al., 2017;Qiu et al., 2018). On the other hand, *trans* FAs created by biohydrogenation in ruminants (TVA and CLA) are not associated with heart disease (Kalač, 2011). CLA, especially the *c*9 *t*11 isomer, and its precursor TVA, are purported to have health benefits, including managing insulin resistance and blood pressure as well as improving lipid metabolism, in moderate doses (Field et al.,

2009;Menaa et al., 2013;Da Silva et al., 2015). It is important to note the differences between industrial *trans* FAs, which should be avoided, and ruminant *trans* FAs, which confer some health benefits, since the two are structurally similar but have different effects. Despite this distinction, the health benefits of ruminal *trans* FAs still require investigation. Recent studies exploring ruminal *trans* FAs found potential negative health effects including increasing cardiometabolic risk factors such as the lipid profile similarly to that of industrial *trans* FAs (Gebauer et al., 2015;Verneque et al., 2020).

Other than FAs, phytochemicals such as phenolic compounds in foods are known to have numerous beneficial health effects (Serra et al., 2021). Phenolic compounds are secondary metabolites derived from plants, and their chemical structure is characterized by having at least one phenolic group. They can be divided into two categories: non-flavonoids, also called phenolic acids, and flavonoids which include flavonols, flavanones, flavones, flavanols, isoflavones, anthocyanidins, and chalcones. Although they are not essential for major biological mechanisms, they do have important ecological functions and possess antioxidant properties (Cianciosi et al., 2018;Pogorzelska-Nowicka et al., 2018). Phenolic compounds stabilize free radicals by giving up one hydrogen from their hydroxyl group; thus, the degree of antioxidant activity of each compound depends on the number of hydroxyl groups (Kumar et al., 2015;Cianciosi et al., 2018). Carotenoids, including β-carotene and lutein, are another class of phytochemicals found in plentiful amounts in plants. These compounds can act as precursors to vitamin A in humans, have antioxidative effects, and reduce the risk of metabolic diseases (van Vliet et al., 2021b). Because of the potential of phytochemicals to reduce oxidative stress and inflammation, consumers are looking for foods containing these compounds (Provenza et al., 2019).

1.3.3 Fatty acids and phytochemicals in grass-fed and grass-finished beef

Fatty acid profiles in meat vary from species to species and from animal to animal. Poultry is usually leaner and therefore contains less fat, while red meat usually contains more fat (Biesalski, 2005). Because cattle diet has the biggest impact on the nutritional profile of beef, the FA profile differs based on the production system (Berthelot and Gruffat, 2018;Lenighan et al., 2019;Prache et al., 2020). Regardless of feeding regime, SFAs are abundant in beef, with stearic acid accounting for approximately one-third of total SFAs. Previous studies mainly agree that grass-feeding or finishing results in higher levels of SFAs (around 45% total FA) compared to grain-finishing (43%) (Daley et al., 2010; Duckett et al., 2009; Van Elswyk & McNeill, 2014). Nevertheless, it is important to note that GFB products are leaner than grain-finished products (Alfaia et al., 2009). GFB has 1.4 g less SFAs than grain-finished beef per 100 g (Van Elswyk and McNeill, 2014). Furthermore, GFB contains around 3% more stearic acid (C18:0) compared to grain-finished beef, which is considered neutral in regard to effects on plasma LDL cholesterol (Leheska et al., 2008;Alfaia et al., 2009;Daley et al., 2010;Van Elswyk and McNeill, 2014). Concentrations of individual SFA were reported in the literature; unfortunately, not all articles report values using the same units, so it is difficult to compare them directly. Many sources report higher concentrations of myristic acid (C14:0) and palmitic acid (C16:0), considered to be detrimental to serum cholesterol levels, in grain-finished beef (Duckett et al., 2009a;Daley et al., 2010;Duckett et al., 2013;Van Elswyk and McNeill, 2014). Overall, GFB has a more favorable SFA profile (Daley et al., 2010).

Monounsaturated fatty acids make up nearly half of beef fat, with oleic acid (C18:1 *c*9) being the most abundant (Leheska et al., 2008). Oleic acid is the most prevalent *cis*-MUFA in the human diet, and it is widely available in plant and animal products. Its effects on human health

include lower LDL cholesterol levels and blood pressure, as well as improved insulin sensitivity. These effects are improved when oleic acid is used as a replacement of SFAs (Calder, 2015). It has been reported that GFB has between 30 and 70% less MUFAs compared to grain-finished beef. More specifically, grain-finished beef has up to 1.8 g more MUFAs per 100 g tissue (2.61 vs 0.79 g per 100 g meat) (Duckett et al., 2013;Van Elswyk and McNeill, 2014). These findings are interesting from a human-health standpoint since consumption of high-oleic acid beef was linked to increased plasma high-density lipoprotein (HDL) cholesterol (Gilmore et al., 2011;Van Elswyk and McNeill, 2014).

The key FAs of interest in GFB are the PUFAs, especially *n*-3 and *n*-6 PUFAs. Significant differences in *n*-6 concentrations have been reported in the literature with GFB containing less *n*-6 PUFAs compared to grain-finished beef (Davis et al., 2022;Klopatek et al., 2022). Typically, grass-raised products have higher levels of *n*-3 PUFAs, leading to a more favorable *n*-6:*n*-3 ratio. The *n*-6:*n*-3 ratio in GFB is around 1.53 while the ratio in grain-fed beef is about 7.65 (Daley et al., 2010; Pighin et al., 2016). It has been reported that when the amount of grain in the feed is increased, the concentration of *n*-3 PUFAs decreases and the concentrations of *n*-6 PUFAs increases. The length of time on feed also influences the PUFA content of meat. Klopatek et al. (2022) found that cattle grazing for 20 months and finished for 45 days on a high concentrate diet displayed a *n*-6:*n*-3 ratio of 2.5:1 compared to animals kept on pasture for 20 or 25 months without any concentrate displaying a *n*-6:*n*-3 ratio of 1.5:1. This was mainly due to a decrease in *n*-3 PUFA concentrations and it was confirmed by a *n*-6:*n*-3 ratio of 5.5:1 in animals that were fed a concentrate diet for 128 days in a feedlot. Analyzing the effects of various feedstuff on the *n*-6:*n*-3 ratio in GFB will help consumers to understand these vast differences and select the healthiest GFB products. Beef from cattle fed diets rich in grass and other forages also have about 2 to 3

times higher concentrations of CLA *c*9*t*11 and TVA than grain-fed cattle (Leheska et al., 2008;Alfaia et al., 2009). This is mainly due to a more favorable rumen pH which allows for more efficient microbial biohydrogenation (French et al., 2000;Kraft et al., 2008).

Phytochemicals are also variables of interest that differ based on the production system. GFB contains higher amounts of common antioxidants including 3 times more α-tocopherol (vitamin E) and 1.5 to 10 times more β-carotene than grain-finished beef (Duckett et al., 2009a;Pighin et al., 2016;Bronkema et al., 2019;Logan et al., 2020). Although intrinsic biological factors such as breed and age can affect carcass fat color, grass-fed beef usually has a yellower fat, mainly due to carotenoids found in the lush green forages they are grazing on (Dunne et al., 2009). Yellow carcass fat is generally related to healthier FA profiles and higher antioxidant content (Daley et al., 2010). Even though direct comparison of phenolic compounds in grass-finished and grain-finished beef has not yet been reported in the literature, differences in phenolics were observed in milk based on grass or concentrate diets (Besle et al., 2010;Prache et al., 2020). Furthermore, some findings suggest that cattle finished on forages might showcase higher phenolic content and diversity in their meat (Provenza et al., 2019;van Vliet et al., 2021a;van Vliet et al., 2021b). When cattle graze on phytochemically diverse mixture of plants, the sensory and biochemical characteristics of their carcasses are modified (Alothman et al., 2019;Provenza et al., 2019;van Vliet et al., 2021b). For instance, a study comparing inflammatory responses of subjects after consuming kangaroo meat (eating a mixture of phytochemically diverse plants) or beef meat (fed a high-grain diet) showed that people who consumed the kangaroo meat had lower inflammatory responses (Arya et al., 2010). However, it is important to note that the generic term "grass-fed" or "grass-finished" does not reflect phytochemical diversity of the feed. There are

many variations that exist among grass-fed and grass-finished diets, and these differences can greatly influence the nutritional properties of beef (**Figure 1**).

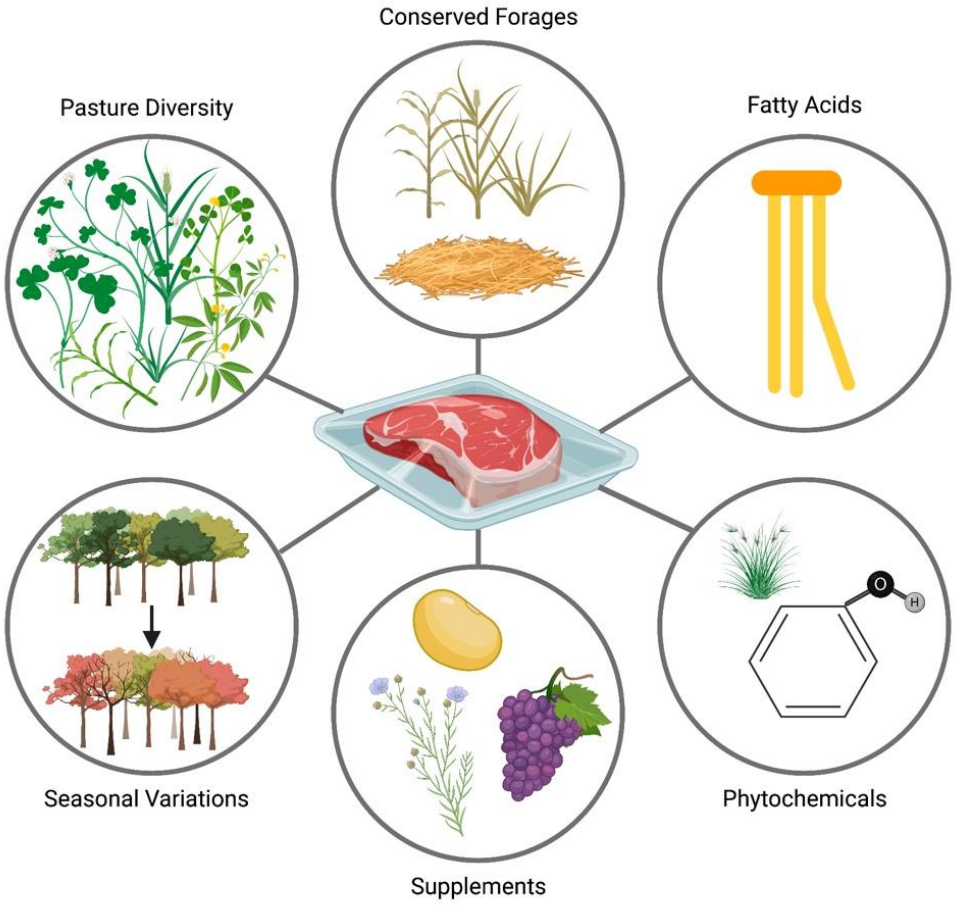

Figure 1. Effects of pasture diversity, seasonal variations, feed conservation, and supplementation on phytochemicals and fatty acids of beef. Cattle finished in different seasons exhibit different fatty acid profiles and phytochemical content. Supplementing cattle diets with flaxseed, algae, or other conserved forages can also affect the nutritional quality of beef.

1.4 How variations in cattle diet influence meat nutritional quality

1.4.1 Regenerative agriculture and pasture diversity

Increasing demand for GFB and the use of regenerative agriculture practices go hand in hand (Spratt et al., 2021;van Vliet et al., 2021b). Regenerative agriculture can be defined as a practice that links soil health and livestock management to farm profitability, human, animal, and ecosystem health, as well food system sustainability (Spratt et al., 2021). Regenerative agriculture might also be referred to as ecological agriculture, conservation agriculture, permaculture, or holistic management and focuses on restoring holistic and regenerative systems supported by ecosystems that allow healthy soils. Farmers and ranchers with livestock typically use a holistic grazing method with the purpose of increasing soil health, moisture retention, and fertility while continuously moving animals between habitats to allow optimal forage conditions (Gosnell et al., 2019). More specifically, regenerative grazing involves rest-rotation cycles: grazing periods followed by forage rest periods to allow plant recovery (Spratt et al., 2021). Regenerative agriculture is known to improve biodiversity and to enhance ecological function (Provenza et al., 2019). Grazing systems used in regenerative agriculture imitate natural ecosystems and improve plant diversity (van Vliet et al., 2021b). When compared to feedlots or monocultures, soil, animal, and human health are favored when herbivores, including cattle, graze on phytochemically diverse mixtures of grasses and trees (Provenza et al., 2019). However, diverse plant species and grazing systems have varying effects on the nutritional profile of beef **(Table 1)**.

Table 1. Effects of various feedstuff and their bioactive compounds on the nutritional profile of beef

Plant Species; Feedstuff	Bioactive Compounds in Diet	Effects on Beef	Reference
Pasture Diversity			
Alfalfa vs bermudagrass vs cowpea vs chicory vs pearl millet	Bermudagrass highest in LA Cowpea highest in C16:0, Mg, and Fe Pearl millet highest in ALA and Zn	C16:0 higher in beef finished on alfalfa Zn, Fe, and Mg higher and *n*-6 PUFAs lower in beef finished on bermudagrass α-tocopherol higher in beef finished on cowpea *n*-3 PUFAs higher in beef finished on chicory β-carotene and retinol higher in beef finished on pearl millet CLA higher in beef finished on alfalfa or pearl millet	(Schmidt et al., 2013)
Alfalfa vs pearl millet vs mixed pastures (bluegrass, orchardgrass, tall fescue, and white clover)	-	C16:0, *n*-6, and *n*-3 PUFAs higher in beef fed alfalfa Total MUFAs and Zn higher and *n*-6:*n*-3 ratio lower in beef fed pearl millet C18:0, α-tocopherol, β-carotene, Mg, and Fe higher in beef fed mixed pastures	(Duckett et al., 2013)
Birdsfoot trefoil vs meadow brome	-	C16:0, C18:0, total MUFA, LA, ALA, and CLA higher in beef fed birdsfoot trefoil *n*-6:*n*-3 ratio lower in beef fed birdsfoot trefoil	(Chail et al., 2016)
Seasonal Variations			
Fall: sorghum, oat/pea/triticale silage, soybean hulls, cane molasses, perennial grasses, baleage Spring: oat/pea silage, alfalfa, cane molasses, soybean hulls, baleage, perennial grasses, barley, wheat, sorghum silage	-	C16:0, C18:0, LA, ALA, EPA, DPA, DHA, CLA, Fe, Zn, and α-tocopherol higher in cattle finished in spring *n*-6:*n*-3 ratio and β-carotene higher in cattle finished in fall	(Jain et al., 2020)

LA: linoleic acid; C16:0: palmitic acid; Mg: magnesium; Fe: iron; ALA: α-linolenic acid; Zn: zinc; *n*-6: omega-6; PUFAs: polyunsaturated fatty acids; *n*-3: omega-3; CLA: conjugated linoleic acid; MUFAs: monounsaturated fatty acids; *n*-6:*n*-3 ratio: omega-6:omega-3 ratio; C18:0: stearic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; SH: soybean hulls; TVA: *trans*-vaccenic acid; C14:0: myristic acid.

Table 1. (cont'd)

Plant Species; Feedstuff	Bioactive Compounds in Diet	Effects on Beef	Reference
Supplementation: Conserved Forages			
Fresh grass vs grass silage	Grass and grass silage had similar FA profile	Lower PUFAs, LA, ALA, and CLA from beef finished on silage	(Fredriksson Eriksson and Pickova, 2007)
Supplementation: Soybean Hulls			
Orchardgrass and soybean hulls pellets vs tall fescue and soybean hulls pellets vs tall fescue	-	Higher C16:0, lower C18:0, no change in CLA, and less grassy flavor in beef fed either forage diet supplemented with SH Higher *n*-6 PUFAs in beef fed orchardgrass and SH Higher *n*-3 PUFAs and lower *n*-6:*n*-3 ratio in beef fed tall fescue without SH	(Baublits et al., 2006)
Varying amounts of soybean hulls and soybean meal (8-41%) prior to being finished on forages for 150 days	High amounts of fiber in soybean hulls	No observed differences in TVA, CLA, *n*-6 PUFAs, *n*-3 PUFAs, and *n*-6:*n*-3 ratio	(Duckett et al., 2009a)
Supplementation: Grape Byproducts			
Dried grape pomace and pelleted total mixed ration vs pelleted total mixed ration	Polyphenols present in dried grape pomace	LA, ALA, CLA, total *n*-3 PUFAs, and total PUFAs higher in beef fed finishing diets with dried grape pomace	(Tayengwa et al., 2021)
Supplementation: Flaxseed			
Mixed forage and ground flaxseed vs mixed forage and ground corn and soybean meal vs mixed forage	Flaxseed diet had significantly greater concentrations of ALA than corn and soybean meal diet and forage diet with no supplement	No observed differences in C14:0, C16:0, and total PUFAs among the three groups ALA and total *n*-3 PUFAs highest and *n*-6:*n*-3 ratio lowest in beef fed forage diet with flaxseed	(Kronberg et al., 2011)
Supplementation: Algae			
Total mixed ration vs total mixed ration with 2% seaweed	-	Beef from cattle fed diet with seaweed had more C18:0, ALA, and total *n*-3 PUFAs, less C14:0, and a lower *n*-6:*n*-3 ratio	(Hwang et al., 2014)

The “grass-fed” label does not reflect the phytochemical diversity of the diet (Provenza et al., 2019). Therefore, large variations are seen among grass-finished beef (Bronkema et al., 2019). Not all GFB graze on the same type of pastures. Forages including grass and clover contain high concentrations of ALA (50-75% of total FAs), which is the essential FA that can be synthesized into beneficial EPA and DHA (Scollan et al., 2014;Bronkema et al., 2019). Chloroplasts contain high levels of PUFAs, explaining why green plants have high concentrations of ALA (Elgersma et al., 2013). Orchardgrass, tall fescue, and perennial ryegrass have 2, 4, and 7 more mg of ALA per g of dry matter compared to alfalfa respectively (Dierking et al., 2010). Schmidt et al. (2013) reported concentrations of main FAs in alfalfa, bermudagrass, chicory, pearl millet, and cowpea. Bermudagrass contained 7% more ALA than cowpea and pearl millet, while pearl millet and chicory contained 8% more LA than alfalfa and bermudagrass. Other forage mixtures including pearl millet, bluegrass, and clovers increased the *n*-3 content of beef by more than 2% compared to beef fed a concentrate diet (Duckett et al., 2013). Adding different varieties of plants like red clover can help increase levels of ALA and LA (Scollan et al., 2006). It appears that nutritional profiles of plants differ based on the leaf-to-stem ratio, with leaves containing more *n*-3 PUFAs than stems as Elgersma et al. (2005) found a positive relation between proportion of leaf blades and C18:3. In general, an increase in *n*-3 PUFAs is observed in diverse pastures compared to perennial ryegrass and lowland pastures.

There is an increasing interest in botanically diverse pastures as cattle feed. However, the information available in the literature remains scarce (Scollan et al., 2014). Different plant species have varying effects on the nutritional quality of beef products. The subcutaneous fat *n*-6:*n*-3 ratio of cattle grazing on cicer milkvetch was greater compared to cattle grazing on meadow bromegrass or treated in feedlot. The lower subcutaneous fat *n*-6:*n*-3 ratio was found in animals grazing on

birdsfoot trefoil (Allen, 2021). It is important to note that unsaturated FAs are toxic to rumen bacteria and therefore undergo extensive rumen biohydrogenation. LA and ALA are hydrogenated to the extent of 70-95% and 85-100% respectively (Lock et al., 2006). Based on this, increasing PUFA concentrations in the diet could lead to more biohydrogenation and formation of stearic acid, but these rates also depend on other factors such as rumen pH, plant secondary metabolites, and the impact of plant cell walls on the availability of free FAs for biohydrogenation (Lock et al., 2006;Fredriksson Eriksson and Pickova, 2007;Jenkins et al., 2008;Lee et al., 2018). These findings emphasize the importance of defining plant species in pasture and how they affect rumen biohydrogenation and beef nutrient profiles.

Herbivores and plants work synergistically, leading plants to produce a wide array of phytochemicals. These phytochemicals accumulate in meat and milk when animals graze on these diverse pastures, but these metabolites remain underdiscussed when assessing the nutritional quality of meat (van Vliet et al., 2021b). Plant diversity and grazing are important elements of regenerative agriculture; they play major roles in soil and environmental health, as well as contributing health-enhancing phytonutrients for animals and humans (Provenza et al., 2015). However, factors other than pasture diversity contribute to the nutritional properties of cattle feeds.

1.4.2 Seasonal variations

The diversity of production systems reflects differences in nutritional profiles of beef. It has been reported that significant nutritional differences are seen among GFB. However, there are more factors affecting the quality of beef including season, geography, and climate (Mathews and Johnson, 2013;Bronkema et al., 2019;Jain et al., 2020). These variations seen in beef are due to variations in feeds. Factors such as plant maturity and development, cutting date, soil, weather,

and light exposure play major roles in the nutritional composition of feeds (Dewhurst et al., 2001;Khan et al., 2009;Garcia et al., 2016).

Generally, grasses decline in FA quality faster than legumes or grains, highlighting the importance of seasonal variations (Kilcher, 1981;Glasser et al., 2013). Nutritional quality of feeds varies with plant growth and maturity, as well as the leaf-to-stem ratio (Boufaïed et al., 2003;Glasser et al., 2013). Different growth periods have been identified in fresh grass between May and September in temperate Northern Hemisphere areas. These growth periods are further subdivided into the primary growth, and the first, second, and third regrowth. Based on these cycles, it was found that total FAs and ALA are higher during the primary growth before strongly declining during the second regrowth, which is a stemmy regrowth period, and increasing again during the last regrowth cycle, which is a leafy regrowth period. The opposite trend was true for LA (Bauchart et al., 1984). These growth and regrowth periods emphasize the importance of plant growth and the leaf-to-stem ratio when assessing the nutritional quality of forages since forage lipids are mainly of leaf origin (Boufaïed et al., 2003). Total fat in grasses is usually higher in early spring before gradually declining, while concentrations of the SFA palmitic acid gradually increase throughout the season (Mir et al., 2006). Concentrations of LA, as well as MUFAs such as C16:1 and C18:1 usually follow the same pattern as palmitic acid, while the beneficial *n*-3 PUFA ALA decreases over time (Garcia et al., 2016). Following the gradual decrease of ALA, increasing concentrations are seen in the late season because of regrowth vegetation cycles (Glasser et al., 2013). It is important to notice that throughout the season, forages have a more beneficial FA profile compared to grains since seeds are higher in *n*-6 PUFAs while leaves are higher in *n*-3 PUFAs (Butler, 2014). Forages are also the largest natural source of vitamins for ruminants, but concentrations vary based on species and maturity. A study comparing α-tocopherol and β-

carotene in grasses and legumes found that the highest levels of vitamins were found in the fall and were based on regrowth cycles (Danielsson et al., 2008).

Temperature and weather affect the quality of forages. Higher temperatures seen during the summer months negatively affect the quality of feeds by increasing plant maturation and cell wall lignification (Revello-Chion et al., 2011). Regarding precipitation, rainfall promotes grass quality and productivity (Mir et al., 2006;Revello-Chion et al., 2011). On the other hand, water deficit decreases forage quality by reducing the proportion of leaves. This is because nutrients migrate to the roots, decreasing the important leaf-to-stem ratio (Revello-Chion et al., 2011). Furthermore, precipitation directly affects the FA biosynthesis in forages; lipid biosynthesis is decreased or even inhibited under water stress (Gigon et al., 2004).

Seasonal differences in feeds ultimately affect the nutritional composition of beef. Jain et al. (2020) reported that cattle finished in the spring exhibit higher levels of *n*-3 (including ALA, EPA, DPA, DHA) and *n*-6 PUFAs, stearic acid, and oleic acid. The *n*-6:*n*-3 ratio is also significantly lower in the spring compared to the fall. The higher levels of *n*-3 PUFAs in the spring are most likely due to higher *n*-3 levels in spring forages but also higher levels of antioxidants protecting *n*-3 PUFAs from oxidation and biohydrogenation. Sodium, phosphorus, and β-carotene were reported to be significantly higher in the fall, while magnesium, potassium, iron, zinc, selenium, and α-tocopherol were higher in the spring. Even if feed composition is usually self-reported by producers, it has been found that mineral levels in forages are higher in the spring rather than summer and fall (Jain et al., 2020).

Fatty acid content in beef is also affected by the region and the climate since they affect cattle growth and weight. For instance, Southern regions experience hot weather most of the year with some short periods of weather changes, while regions of the Midwest for example experience

drastic weather changes based on seasons that last a few months each. Heat stress might be linked to differences in FA profiles (Jain et al., 2020; Steiner et al., 2015).

Seasonal variations are important to consider in the production of GFB. A well-managed grazing system taking into consideration feed nutritional differences based on season and weather conditions is crucial to determine optimal finishing phases to yield the healthiest nutrient profile in beef.

1.4.3 Supplementation: Effects of different supplementary feeds in U.S. grass-fed beef

Providing only fresh forage to grass-fed cattle can become difficult for producers, especially since fresh grass is not always readily available in some regions and seasons. During the winter for instance, producers rely on hay or haylage, as well as non-starchy feeds like alfalfa rations, wheat, or oat straws (Gwin, 2009). According to the USDA, "hay, haylage, baleage, silage, crop residue without grain, and other roughage sources" may be added to grass-fed cattle diets, but these feeds are nutritionally different from fresh forage (Food Safety and Inspection Service, 2019). Further, in some cases, additional supplementary feeds such as soybean hulls (SH) or grapeseed extracts may be added to forage-fed beef to improve beef quality and utilize byproducts of other industries (Kiesling, 2013;Muñoz-González et al., 2019). It is important to note that the addition of feed supplements described in this section varies by labeling organizations and does not necessarily reflect what is permitted in "grass-fed" labels outside of the U.S. such as certification by A Greener World in the United Kingdom or by the Pasture-fed Cattle Assurance System in Australia (A Greener World;PCAS). Thus, grass-fed cattle diets in the U.S. can be composed of an array of feeds which may lead to differences in nutritional profiles, particularly FA and phytochemical content, of GFB.

1.4.3.1 Conserved forages

Grasses and legumes can be conserved by drying or fermentation. Hay is prepared by cutting and quickly drying grasses or legumes until they reach less than 20% moisture (Allen et al., 2011). Forages are spread into a field and raked until dry before storing (Tripathi et al., 1995). Silage, haylage, and baleage are preserved by fermentation in an oxygen-free environment in which bacteria convert sugar from forages into organic acids such as lactic acid; this lowers the pH and prevents spoilage (Tripathi et al., 1995). Silage refers to forages that are fermented at a high-moisture content (roughly >50% moisture) in an air-tight environment such as a silo. Haylage refers to a low-moisture silage (roughly 35-55% moisture) that is made after forages are cut and wilted (Allen et al., 2011;U.S. Department of Agriculture, 2021). Lastly, baleage, or round bale silage, refers to forage that is cut, wilted, and fermented in tightly wrapped bales (American Grassfed Association, 2022). These preservation methods have implications for the nutritional quality of the feed and thus may impact ruminant products.

Conserved forages often have reduced nutritional quality compared to fresh forages. Drying or fermenting forages decreases antioxidant and phenolic concentrations (Owens et al., 1997;Butler, 2014). In the process of making hay or silage, 80% of the carotenoid content is lost (Pickworth et al., 2012). Further, the wilting of forages for drying or ensiling results in oxidation of PUFAs, particularly ALA. In this process, lipolysis is catalyzed by plant lipases, releasing PUFAs from plant membranes. These free PUFAs are then oxidized with exposure to air by lipoxygenases, and some products of this process may be lost as components of volatile organic compounds, thus reducing PUFA content of plant tissues (Kalač and Samková, 2010). This loss of PUFAs is often accompanied by an increase in the relative amount of palmitic acid, given that SFAs are less susceptible to oxidation (Van Ranst et al., 2009;Kalač and Samková, 2010). Fresh

grass contains higher concentrations of ALA, LA, and oleic acid compared to hay (Daley et al., 2010;Butler, 2014;Jain et al., 2020). Moreover, in a review of fresh and conserved forages, fresh perennial ryegrass contained 71.8% ALA, 8.8% LA, and 11.4% palmitic acid compared to perennial ryegrass hay with 55.9% ALA, 14.0% LA, and 15.8% palmitic acid, and perennial ryegrass silage with 52.2% ALA, 13.4% LA, and 21.2% palmitic acid (Kalač and Samková, 2010). Changes in FA profiles of feeds can alter FA metabolism in the rumen and therefore the FA content of beef products (Buccioni et al., 2012;Glasser et al., 2013).

Regardless, the magnitude of the change in the nutritional quality of forages is dependent on the method and quality of preservation (Glasser et al., 2013). Tripathi et al. (1995) noted that the process of haymaking is particularly susceptible to shattering and dropping of leaves, the most nutritious part of the plant. In comparison, silage, haylage, and baleage making are much less susceptible to leaf loss (Tripathi et al., 1995). Further, compared to ensiled forages, haymaking is more susceptible to nutrient loss due to sunlight and inclement weather (Tripathi et al., 1995;Coblentz and Akins, 2018). A meta-analysis of reported FA profiles of forages assessed the relationship between preservation methods and changes in the FA profile (Glasser et al., 2013). Turning fresh forage into hay did not impact the LA content, but it caused a decrease in total fat, total FAs, and ALA. At most, ALA decreased by 17%, and it was observed that this decrease was greater under poor haymaking conditions. Haymaking, especially under poor conditions such as wet weather, was found to be the second most deleterious factor affecting the ALA content of forages following the cutting date when compared to other preservation, vegetation stage, and fertilization factors (Glasser et al., 2013).

Ensiled forages have many advantages compared to dry hay. In general, ensiling does not greatly impact the FA profile, but instead the extensive lipolysis involved in ensiling leads to an

increase in the free FA content (Kalač and Samková, 2010;Glasser et al., 2013). This increase in free FA content may impact biohydrogenation in the rumen of cattle given that lipolysis must occur prior to biohydrogenation (Van Ranst et al., 2009). The impact of turning fresh forages and legumes into silage differs among unwilted silages, wilted silages, and haylages. In a meta-analysis of reported forage FA profiles, total fat content was increased in unwilted and wilted silages, while total FAs were only increased in unwilted silages. Wilted silages and haylages had 5% lower ALA content, while ensiling without wilting did not impact the ALA content compared to their fresh counterparts (Glasser et al., 2013). Though ensiling forages protects FAs from oxidation, aeration of ensiled forages prior to feeding exposes the free FAs to oxygen, inducing oxidation (Kalač, 2011). Exposing grass silages to air for 24 hours lowered the PUFA and total FA content and increased the proportion of palmitic acid (Khan et al., 2009). However, the oxidation of FAs is generally still greater in hay which has a longer exposure to air (Kalač, 2011). These results indicate that ensiled forages, compared to hay, may be a more desirable supplementary feed for grass-fed production systems.

However, it is important to note that the composition of the feed itself may influence beef nutrient profile to a greater extent than the feed's preservation method. Butler (2014) highlighted the importance of feed composition by noting differences in beef nutrient profile among types of silages provided: grass silages led to enhanced beef CLA content, while clover and legume silages led to enhanced *n*-3 content. Maize silages, not permitted in GFB, led to increased beef *n*-6 content (Butler, 2014). On the other hand, Glasser et al. (2013) found that forage vegetation stage and conservation method had a greater impact on nutrient profile compared to differences among forage species. Some studies reviewed by Glasser et al. (2013) noted an increase in *n*-3 content with a greater proportion of grasses and a decrease in *n*-3 content with a greater proportion of

legumes, but these differences were not as large as those observed due to preservation method (Lourenço et al., 2007b;Steinshamn and Thuen, 2008). Further, while grass species can influence the FA content of grass silages, plant maturity at harvest caused the most variation, predominantly in *n*-3 content (Khan et al., 2012). Based on this, there are important differences in feed composition, plant maturity, and preservation method to take note of when considering incorporating conserved forages into GFB systems.

There is limited evidence demonstrating how feeding conserved forages impacts the nutritional quality of GFB. A review of studies comparing various fresh pasture and silage diets concluded that the FA profile of beef finished on fresh grass was more favorable, including greater *n*-3 PUFAs and CLA, compared to beef finished on grass silage; however, many of the studies included in this review compared diets containing both forages and concentrates (Kalač, 2011). A study conducted by Fredriksson Eriksson and Pickova (2007) compared the FA and α-tocopherol content of exclusively grass-fed cattle finished on fresh grass in September compared to exclusively grass-fed cattle finished on grass silage in February. Though they found that the grass and grass silage diets had a similar FA profile, beef finished on silage had lower PUFA and significantly lower LA, ALA, and CLA. The authors suggested that the higher PUFA content in beef from the fresh grass group may be because the cell wall limits the biohydrogenation of FAs in fresh grass as compared to the free FAs in grass silage. Further, the authors suggested that the higher plant secondary metabolite content in fresh grass compared to grass silage in the alpine region included in the study may limit biohydrogenation (Fredriksson Eriksson and Pickova, 2007). Similarly, red clover silages are found to increase PUFA content in meat compared to grass silages (Lourenço et al., 2007a;Lee et al., 2009;Van Ranst et al., 2009). It is thought that red clover silages have reduced lipolysis and thus less free FAs available for biohydrogenation due to lipase-

inhibiting compounds like polyphenol oxidase (PPO) found in red clover, though evidence suggests the reduction in lipolysis and PUFA biohydrogenation may occur independently of PPO activity (Van Ranst et al., 2009;Lee et al., 2018). More research comparing finishing cattle on fresh grass compared to conserved forages or fresh grass diets supplemented with conserved forage is needed to better understand how conserved forages alter the nutritional profile of beef.

1.4.3.2 Soybean hulls

Soybean hulls are another supplement to GFB used by some producers during the finishing phase (Bronkema et al., 2019). Soybean hulls refer to the seed coats of soybeans that are removed in the process of soybean crushing (Poore et al., 2002). Soybean hulls are mostly composed of fiber with low amounts of lignin and are known to have high potential digestibility for ruminants without lowering ruminal pH (Poore et al., 2002;Pugh, 2003). There are mixed results in the current literature about the effects of SH supplementation on the nutritional profile of GFB. In one study, there were no observed differences in CLA, TVA, *n*-3 PUFAs, *n*-6 PUFAs, and the *n*-6:*n*-3 ratio among cattle fed varying amounts of SH prior to forage finishing for 150 days (Duckett et al., 2009a;Bronkema et al., 2019). However, in another study, cattle fed fescue or orchard grass supplemented with SH had greater total fat, lower *n*-3 PUFAs, and a greater *n*-6:*n*-3 ratio compared to cattle fed only fescue. It is important to note that the *n*-6:*n*-3 ratio was still below four, CLA did not decrease, and the intensity of grassy flavor decreased in the beef supplemented with SH (Baublits et al., 2006). According to sensory studies conducted in Chicago and San Francisco, only about 23% of consumers preferred the taste of GFB as opposed to grain-fed beef (Gwin, 2009). Thus, a reduction in the intensity of grassy flavor by SH supplementation may increase palatability of GFB to consumers. In another study, CLA concentrations and *n*-3 PUFAs were greater and the *n*-6:*n*-3 ratio was lower in cattle fed a SH supplement compared to cattle fed a corn supplement

(Kiesling, 2013). CLA and *n*-3 PUFAs were also increased and the *n*-6:*n*-3 ratio decreased in lambs when SH were included as a replacement for corn (Costa et al., 2012a). There was not a comparison group to cattle or lambs fed purely forages in the aforementioned studies, but the results indicate that SH could be a better supplement than corn. It is hypothesized that since SH contain significant amounts of fiber and maintain ruminal pH at optimal levels, more biohydrogenation can occur, leading to greater amounts of CLA and TVA (Kiesling, 2013). Further studies need to be done to clearly elucidate the effects of SH supplementation and the mechanisms for these effects. However, SH are not permitted by some organizations providing grass-fed labels, including the American Grassfed Association, so producers need to keep this in mind when considering supplements for grass-fed cattle (American Grassfed Association, 2022).

1.4.3.3 Grape pomace and grapeseed extract

Increased levels of UFAs found in GFB might render meat more subject to biohydrogenation and oxidation. To avoid this, cattle feeds can be supplemented with waste or byproducts from the food industry that possess antioxidative capabilities. The winemaking industry, for example, generates large amounts of waste and byproducts including grape pomace and grapeseed extracts (Brenes et al., 2008). The valorization of these byproducts would reduce the environmental impact of winemaking and would add functional ingredients to meat (Muñoz-González et al., 2019). These byproducts contain significant amounts of bioactive compounds such as antioxidants, phenolic compounds, and fiber. Grape pomace and grapeseed extracts contain high levels of polyphenols including anthocyanins, proanthocyanins, and flavanols (Brenes et al., 2008;Arola-Arnal et al., 2013;Muñoz-González et al., 2019). Adding these functional ingredients to feeds instead of adding them during the processing stages allows these compounds to remain bioavailable and to be metabolized by the animal (Antonini et al., 2020). Natural antioxidants like

grape pomace or grapeseed extracts can exhibit better antioxidative properties than conventional antioxidants like butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) (Kumar et al., 2015). Thus, adding grape pomace or grapeseed extracts to cattle feeds could be beneficial.

In rats, grapeseed extracts feeding led to a dose-dependent increase in muscle polyphenol content (Serra et al., 2013). In addition, rats fed grapeseed polyphenols had significantly greater adipose tissue accumulation of flavanols and their metabolites (Margalef et al., 2015). When these byproducts were added to monogastric animal feeds, the meat had higher levels of α-tocopherol, PUFAs, and less lipid peroxidation (Muñoz-González et al., 2019). A study on the effects of grape pomace concentrate in chickens concluded that the polyphenols found in grape pomace concentrate were absorbed in high enough amounts to modulate antioxidant activity in chicken muscle tissue (Brenes et al., 2008). Other studies evaluating the effects of grapeseed extracts in birds suggested that grape polyphenols and their metabolites might be absorbed and remain in active tissues (Muñoz-González et al., 2019). These findings suggest that supplementing cattle feed with grape pomace or grapeseed extracts could help improve the shelf-life of beef products and help to maintain higher levels of PUFAs in beef (Serra et al., 2013).

Several studies have investigated the effect of adding grapeseed extract to ground beef on lipid oxidation. Oxidative stability is commonly measured by the thiobarbituric acid reactive substances (TBARS) value. In one study, beef patties supplemented with grapeseed extract had mean TBARS values of approximately 0.59 mg MDA/kg compared to 2.94 mg MDA/kg for beef patties without grapeseed extract. The upper limit of rancidity acceptable to consumers is around 2 mg MDA/kg (Gomez et al., 2014). Adding grapeseed extract also kept TBARS values relatively steady. Beef samples without grapeseed extract had increasing levels of TBARS over time, from 0.57 mg MDA/kg to about 3.24 mg MDA/kg. However, beef samples with grapeseed extract

stayed relatively constant around 0.53 mg MDA/kg (Gomez et al., 2015). It is important to note that in these studies, grapeseed extract was added directly to ground beef samples.

The effect of grape pomace or grapeseed extract on the FA profile of beef is not well-known. One recent study observed that adding dried grape pomace to the finishing diet of beef cattle significantly increased CLA, *n*-3 PUFAs, and total PUFAs compared to the control. Supplementing dried grape pomace also decreased aldehydes, ketones, and alcohols in beef as well without significant changes in sensory properties albeit a modest reduction in tenderness compared to controls (Tayengwa et al., 2021). These results are promising but limited. Future research on the effects of grape pomace and grapeseed extract on the nutritional profile and sensory attributes of beef is needed.

Similar to grapeseed extract, cherry has also been investigated for its impact on lipid stability in beef. Britt et al. (1998) found that, like grapeseed extract, adding cherry to ground beef patties decreased rates of oxidation and kept TBARS values under the upper limit of rancidity. Since adding these products to beef directly produced positive results, future studies should investigate the effects of adding grapeseed extract or cherry tissue to cattle feed on the lipid stability of the beef produced.

1.4.3.4 Flaxseed

Flaxseed is another supplement used by some GFB producers. Flaxseed oil is a significant source of ALA (45-52%) and antioxidants including α-tocopherol and phenolic compounds (Pouzo et al., 2016). Because of these natural properties, flaxseed supplementation is a potential way to increase concentrations of *n*-3 PUFAs and improve the oxidative stability of beef. There have been several studies investigating the effects of flaxseed supplementation on the FA profile of beef fed fresh forages, conserved forages, and concentrate. Mapiye et al. (2013) found that beef from cattle

fed red clover silage with flaxseed had about double the proportions of ALA (1.59% vs. 0.68%) and total *n*-3 PUFAs (2.04% vs 1.10%), and about 5 times more TVA (6.37% vs 1.11%) in intramuscular fat compared to beef from cattle that were fed the control diet without flaxseed. Beef from cattle fed the flaxseed diet also contained less myristic acid and palmitic acid (Mapiye et al., 2013). Another study also found that beef from cattle fed grass hay or barley silage supplemented with flaxseed had greater ALA, total *n*-3 PUFAs, and TVA as well as less palmitic acid than cattle fed just grass hay or barley silage (Nassu et al., 2011). Kronberg et al. (2011) reported that beef from cattle fed forage diets with flaxseed had a significantly lower *n*-6:*n*-3 ratio compared to beef from cattle fed forage diets with corn and soybean meal and beef from cattle fed forage diets with no supplements (2.34:1 vs. 3.63:1 vs. 3.41:1). However, they did not observe differences in myristic acid and palmitic acid (Kronberg et al., 2011). While there are variations in the extent of differences, especially regarding SFAs, it is widely agreed that flaxseed supplementation increases ALA concentrations and total *n*-3 PUFAs in beef.

Regarding oxidative stability, a study conducted by Pouzo et al. (2016) found that adding low amounts of flaxseed to pasture diets improved lipid stability of beef. Interestingly, adding high amounts of flaxseed had deleterious effects on lipid stability. It is hypothesized that the low amount of flaxseed provided enough antioxidants to offset the increase in lipid peroxidation caused by elevated *n*-3 PUFA levels, leading to greater oxidative stability (Pouzo et al., 2016). This is an avenue that has not been extensively studied, so further research is needed to better understand the effects of varying amounts of flaxseed supplementation on the oxidative stability of beef.

1.4.3.5 Algae

Consumption of fish high in long-chain *n*-3 PUFAs is low in the American diet. Therefore, there has been an interest in supplementing cattle feeds with marine ingredients such as algae to

increase the *n*-3 content of beef (Glover et al., 2012;Morais et al., 2020). Seaweed, a macroalgae, are a supplement of interest because of their high concentrations of phenolic compounds, pigments, carotenoids, PUFAs, and minerals such as calcium, potassium, and iodine (Schmid et al., 2018;Morais et al., 2020). Algae can synthesize ALA and LA as well as the long-chain *n*-3 PUFAs, EPA and DHA, and generally have an *n*-6:*n*-3 ratio around 1:1 (Schmid et al., 2018). Seaweed are fast growing, have a high biomass yield, and do not compete with other crops for arable land or fresh water. However, there is wide variation in nutritional composition among different seaweeds, and they are susceptible to heavy metal bioaccumulation (Morais et al., 2020). Despite this, seaweed have been shown to have beneficial effects when it is added to cattle feed.

For instance, feeding seaweed to cattle may address the challenge of increasing the *n*-3 content of beef caused by biohydrogenation (Stamey et al., 2012). Generally, 85 to 100% of ALA is hydrogenated in the rumen if left unprotected (Glover et al., 2012). While there is a lack of evidence demonstrating whether seaweed supplementation improves the FA profile of GFB, it was found that grain-fed cattle supplemented with seaweed produced beef with more ALA, total *n*-3 PUFAs, and stearic acid, less myristic acid, and a lower *n*-6:*n*-3 ratio compared to the control diet (Hwang et al., 2014). Smith (2017) demonstrated that supplementing grass-fed cattle with algae resulted in higher *n*-3 PUFA concentrations compared to grain-fed cattle supplemented with algae. Further, animals fed only grass can consume more algae, resulting in an increased intake of *n*-3 PUFAs, and meat with more EPA and DHA per serving (Smith, 2017). It is important to note that the efficacy of feeding marine ingredients high in *n*-3 PUFAs depends on the strength of the algal cell wall and the acidity of the rumen. A lower ruminal pH results in greater breakdown of algal cell walls and thus greater loss of *n*-3 PUFAs to biohydrogenation (Smith, 2017). Due to their high antioxidant content, seaweed may act to prevent oxidation in beef products, similar to grape

byproducts and flaxseed (Morais et al., 2020). Overall, there is limited evidence demonstrating the efficacy of seaweed as a cattle feed supplement including its impact on the nutritional composition of GFB (Morais et al., 2020;Costa et al., 2021). Additional research should focus on the potential of these marine organisms as grass-fed cattle feed supplements.

1.5 Challenges

While GFB products have many advantages, there are some challenges to consider. GFB is usually produced on a much smaller scale than conventional products. These products are mostly sold in local farms and farmers markets, which makes it harder for producers to reach their customers despite growing purchasing interest (Gwin, 2009;Mathews and Johnson, 2013). This limitation partially explains why conventional production systems are used on a larger scale. Production systems based on forage diets take longer to finish cattle than conventional systems due to a less energy-concentrated diet (Gwin, 2009;Mathews and Johnson, 2013;Hayek and Garrett, 2018). Finding efficient genotypes for grass-finishing is another challenge that producers need to consider. Doyle et al. (2021) pointed out that early maturing genotypes might be more suitable for grass-finishing due to their higher potential for fat deposition at a younger age whereas late maturing genotypes might be more suitable for a grass and concentrate system. U.S. customers are accustomed to having affordable and year-round-available beef in supermarkets. Grass-fed products are usually more expensive, not widely available in single-serving packs in supermarkets, and not available on a year-round basis (Gwin, 2009;Gwin et al., 2012). Along with convenience and affordability, U.S. customers prefer the tenderness, juiciness, marbling, and milder flavor of conventional beef compared to GFB (Gwin, 2009;Mathews and Johnson, 2013).

Producers who wish to finish their cattle on grass face challenges including having insufficient grass and land to grow pastures (Hayek and Garrett, 2018). Depending on the region,

fresh grass may not be available all year long for grazing (Duckett et al., 2009a;Jain et al., 2020). Therefore, producers must adapt and find ways to feed their cattle during seasons when fresh pastures are not available while still respecting the labeling definitions for grass-fed or finished beef. For this reason, the supplement options that we mentioned in this review might be helpful to overcome the lack of fresh grass.

Increasing *n*-3 PUFAs in beef is an important way to improve the nutrient profile to favor human health, but this comes with a set of challenges. Fatty acids are subject to oxidation which limits the shelf-life of meat and can result in undesirable, rancid flavors (Kumar et al., 2015). Increased levels of PUFAs in meat can lead to increased lipid peroxidation if not accompanied by adequate antioxidant content (Pighin et al., 2016;Pogorzelska-Nowicka et al., 2018;Saini and Keum, 2018). Grazing on antioxidant-rich, diverse pastures might provide adequate antioxidant levels (van Vliet et al., 2021b). Grapeseed extract and flaxseed supplementation, both important sources of antioxidants, are promising ways to increase *n*-3 PUFAs and improve the oxidative stability of GFB, but further research is needed in order to comprehensively evaluate the effects of these supplements.

1.6 Recommendations

To produce beef that has the greatest potential to benefit consumer health, nutrition recommendations indicate the importance of increasing *n*-3 PUFA content, reducing *n*-6 PUFA content, and increasing CLA content (Woods and Fearon, 2009;Butler, 2014;Vannice and Rasmussen, 2014). Farmers and ranchers need thorough information on feeding practices and awareness of variations based on season and feed ingredients used. If permitted by the relevant grass-fed and grass-finished standards, cattle fed a botanically diverse pasture mixture managed in a rotational grazing manner, supplemented with phytochemically-rich ingredients such as grape

byproducts, flaxseed, or algae would produce beef products high in health-enhancing nutrients such as phenolic compounds, *n*-3 PUFAs, and CLA. Season and weather should also be considered to assess plant's growth and re-growth cycles and leaf-to-stem ratios. In temperate climates, finishing cattle in the spring compared to the fall produces beef with higher beneficial bioactive compounds. Feeds are of higher nutritional quality either during the early or late grazing season. Grazing management should be adapted to give pastures adequate recovery, and the symbiotic relationship between ruminants and pastures should be supported. When fresh forages cannot be fed, conserved ingredients with the highest nutritional potential should be used. High quality ensiled forages such as silage or baleage are typically preferred to hay because of reduced leaf loss. While not always permitted in GFB, SH supplementation can decrease the intensity of the "grassy" flavor of GFB while having neutral or positive effect on the nutrient profile. Testing of FA and antioxidant content of feeds is also encouraged to ensure the highest nutritional quality. Early maturing steers might have an advantage over late maturing genotypes due to their potential for greater fat deposition at a younger age which may reduce the finishing period before slaughter (Doyle et al., 2021). These recommendations based on the information provided in this review would lead to healthier beef products not only for human health, but also for soil, animal, and environmental health.

1.7 Conclusions

World-leading organizations recommend reducing red meat consumption However, differences exist among red meats when comparing grass-fed and GFB. The human health recommendations often neglect the beef production system employed. GFB nutrient profile is typically more consistent with nutritional recommendations as it is higher in beneficial *n*-3 PUFAs and phytochemicals. Variations in nutritional profiles exist among pasture-raised beef, resulting in

unequal pasture-raised products and misleading labels. This suggests a need for a "truth in label" based on validation tests and labeling of the FA content of GFB.

This review highlighted the benefits of producing and consuming GFB, but also emphasized the need for standardization. Rotational grazing systems carried out on botanically rich pastures reinforce the symbiotic relationship between ruminants and landscapes, leading to healthier animals, environment, and humans. Nevertheless, it is critical to determine the effects of different ingredients allowed in GFB on meat nutritional quality. Seasonal differences and supplementation affect the healthfulness of GFB and need to be reported to give consumers a representative idea of the nutritional profile of the products they are consuming.

Future research should focus on assessing and comparing the nutritional profiles of commonly used feeds allowed in GFB production. New efforts should be directed towards developing metabolomic methods to better identify and quantify bioactive compounds that are not well reported in the literature yet (e.g., FA isomers in ruminants and phytochemicals such as phenolic compounds). The effects of phenolic-rich waste and byproducts from the food industry on meat should be assessed. We also propose that a standardized grass-fed label is implemented, mentioning the production system utilized including the diet. Addressing these research and production gaps will lead to improved grass-fed cattle management and production, with the hope of improving human health.

CHAPTER 2: EVALUATION OF FATTY ACID AND ANTIOXIDANT VARIATION IN A COMPLEX PASTURE SYSTEM AS COMPARED TO STANDARD CATTLE FEED IN THE GREAT LAKES REGION

This chapter has been published in:

Krusinski, L., Maciel, I.C.F., Sergin, S., Goeden, T., Ali, H., Kesamneni, S., Jambunathan, V., Cassida, K.A., Singh, S., Medina-Meza, I.G., Rowntree, J.E., and Fenton, J.I. (2022). Evaluation of fatty acid and antioxidant variation in a complex pasture system as compared to standard cattle feed in the Great Lakes Region. *Front. Sustain. Food Syst.* 6, 945080. doi: 10.3389/fsufs.2022.945080

2.1 Abstract

As the demand for grass-fed ruminant products keeps increasing, more data is needed to assess the nutritional value of feedstuffs, especially pastures. In addition, global climate change adds another challenge to the management of grasslands with projections of changing temperature and precipitation patterns. Consequently, the variations in bioactive compounds such as fatty acids and antioxidants in feeds will be harder to predict. Therefore, it is critical to report region and time-specific results of the nutritional value of feeds intended for ruminant nutrition. The objectives of this study were to compare the antioxidant and fatty acid content of commonly used feedstuffs including a complex pasture mixture from the Great Lakes Region and a traditional grain-based diet, and to assess the variations of these bioactive compounds in the pasture over the course of two grazing seasons. Weather parameters including temperature and rainfall were recorded for the length of the study. Feed samples were collected between June and September 2019 and 2020 and analyzed for nutrient composition, chlorophyll A and B, carotenoids, and total phenols. Fatty acids were analyzed by GC-MS. Correlations were reported to analyze the relationship between individual plant species, antioxidants, and fatty acids. We observed higher antioxidant parameters in the pasture compared to the grain diet. Total polyunsaturated fatty acids were higher in the pasture including α-linolenic acid while the grain diet was higher in *n*-6 polyunsaturated fatty acids

including linoleic acid. The *n*-6:*n*-3 ratio was more beneficial in the pasture and was 50 to 90 times higher in the grain diet. Variations in the fatty acid profile of the pasture were observed and varied between 2019 and 2020. Plant growth cycles, climatic conditions, and grazing methods were hypothesized to cause these changes. Altogether, this study increased our knowledge about the nutritional value of feedstuffs and will help ranchers and researchers to better understand the variations of bioactive content based on region, season, and climatic conditions. We are hopeful that this study will contribute to making more nutritious feeds and grass-fed products for human health.

2.2 Introduction

Grass-finished beef (GFB) is growing in popularity among health and environmentally conscious consumers (Daley et al., 2010;Alothman et al., 2019). The composition of cattle feeds directly impacts the nutrient density of ruminant products (Daley et al., 2010). GFB primarily consume grass forages with hay and other supplementation, while grain-fed cattle predominately consume a diet based on corn and soy in the finishing phase (Gwin, 2009). Grain-finishing cattle is the most common practice, despite GFB having a longer shelf-life and a nutrient profile favoring human health (Gwin, 2009;Provenza et al., 2019;Jain et al., 2020). GFB typically has lower total fat, a lower *n*-6:*n*-3 ratio, and higher vitamin and mineral content (Daley et al., 2010). However, a recent survey of GFB demonstrated wide variations in the lipid and micronutrient profile. The *n*-6:*n*-3 ratio ranged from 1.8 to 28.3 (Bronkema et al., 2019). Thus, it is important to characterize the composition of cattle diets due to its impact on the nutritional profile of meat.

Grain-based diets have high starch and energy contents, reducing the biohydrogenation rates of unsaturated fatty acids (FAs) (Hatew et al., 2016;Alothman et al., 2019). Grain-based diets, primarily composed of seeds, contain higher concentrations of *n*-6 PUFA and saturated FAs (SFA)

and lower amounts of phenolic compounds and antioxidants compared to forages (Butler, 2014;Alothman et al., 2019). In grasses, there is a strong positive correlation between chlorophyll A and B and C18:3 *n*-3 (ALA) (Khan et al., 2012). Green forages also contain fat-soluble vitamins with antioxidant properties such as vitamin E and carotenoids (Elgersma et al., 2013). The consumption of complex pastures often results in higher concentrations of vitamins and minerals in GFB (Jain et al., 2020). Nutritional composition of feedstuffs depends on plant maturity and development, conservation method, cutting date, plant species, geography, climate, soil, weather, and light exposure (Dewhurst et al., 2001;Khan et al., 2009;Garcia et al., 2016).

The climate is changing across the world, largely because of anthropogenic activities (Hopkins and Del Prado, 2007;Hatfield et al., 2011;Giridhar and Samireddypalle, 2015). Climate change affects livestock productivity directly and indirectly by modifying the availability and the quality of forages (Giridhar and Samireddypalle, 2015). Key indicators of climate change such as increased mean temperatures, changes in precipitation patterns, and floods occurring more often are triggered by land-use changes (Hopkins and Del Prado, 2007). Climate change impacts grasslands by changing the composition of pastures (e.g., changes in the ratio of grasses to legumes), changing grass growth and quality, and modifying precipitation occurrence (Hopkins and Del Prado, 2007;Giridhar and Samireddypalle, 2015). To complicate this issue, the impacts are dependent on geographic location. Rising temperatures and altered precipitation may have positive or negative impacts on forage availability and quality depending on the specific location (Hatfield et al., 2011). Because of this region-specificity, there is an increased need for predictive capacity as the world's ecosystems are changing (van Oijen et al., 2018).

Part of adaptation strategies include prioritizing biodiverse pastures since pasture diversity may be crucial for the long-term resilience of ecosystems (van Oijen et al., 2018). Integrated

systems involving various plant species and herbivores may remain more productive in variable climatic conditions, and thus be better equipped to adapt to climate change (Izaurralde et al., 2011). Since changes in forage quantity are straightforward to assess, gaining more knowledge about changes in forage nutritional quality is crucial (Berauer et al., 2020). Therefore, the objectives of this study were to compare the nutrient composition, antioxidant, and FA profile of a traditional grain-based diet and a complex Michigan pasture mixture across the grazing season, and to provide region- and time-specific data to farmers and ranchers from the Great Lakes region.

2.3 Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

2.3.1 Experimental design and diet characteristics

The study was conducted at Michigan State University Upper Peninsula Research and Extension Center (latitude: 46°20'N, longitude: 86°55'W; elevation: 271 m) located in Chatham, MI. A total mixed feedlot ration (GRAIN) and a mixed-species pasture forage (PAST) were collected between June and September 2019 (n = 15 and n = 21 respectively) and June and September 2020 (n = 10 and n = 24 respectively). For 2019, GRAIN samples consisted of 20% hay, 50% dry corn, 24% high moisture corn, and 6% pellet. For 2020, GRAIN samples were constituted of 20% hay, 74% dry corn, and 6% pellet. Pellets were identical for both years and contained 36% crude protein (n536, Kalmbach Feeds, INC. Upper Sandusky, OH, USA). The botanical composition of the diets was reported by Maciel et al. (2021). The pasture consisted of an established mixed forage. The prevalent plant species on this research site included meadow fescue (*Schedonorus pratensis* (Huds.) P. Beauv.), red clover (*Trifolium pratense* L.), timothy grass (*Phleum pratense*), alfalfa (*Medicago sativa*), white clover (*Trifolium repens* L.), birdsfoot trefoil (*Lotus corniculatus*), chicory (*Cichorium intybus*), orchardgrass (*Dactylis glomerata* L.),

and dandelion (*Taraxacum oficinale* L.). The monthly botanical composition of the PAST diet is shown in **Figure 2**.

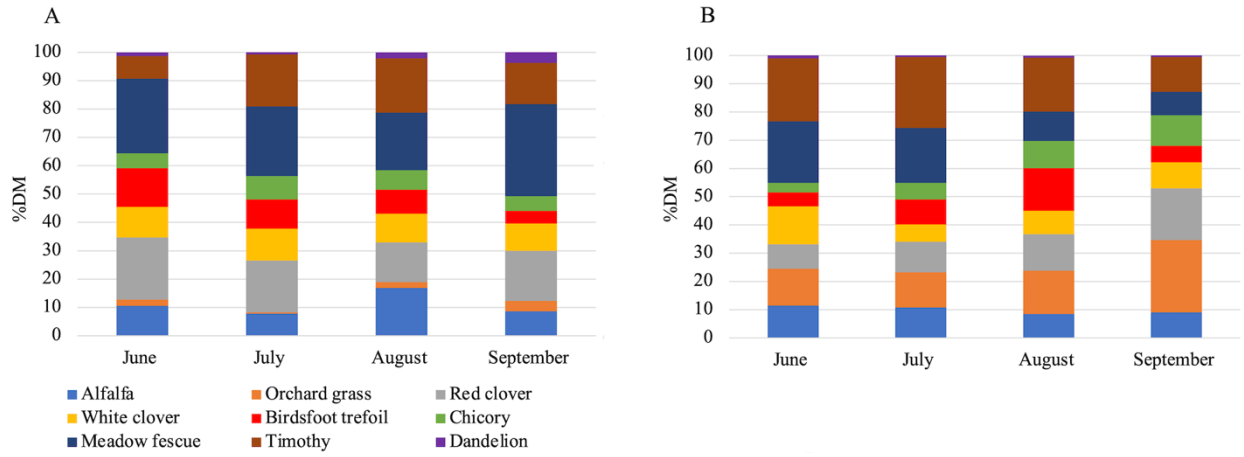

Figure 2. Monthly botanical composition of the pasture. 2019 (A) and 2020 (B) in percentage of dry matter. DM: dry matter.

2.3.2 Collection

PAST samples were collected every two weeks in each sub-paddock in pre-grazing areas, immediately before steers were allowed access to fresh forage. The grazing period lasted 80 days in 2019 and 121 days in 2020. Experimental design and animal management were shared with Maciel et al. (2021). Briefly, PAST samples were collected by randomly clipping three 0.25 m^2 quadrats to a 5 cm stubble using Gardena 8803 (Ulm, Germany) battery-operated harvest shears. GRAIN samples were sampled every two weeks from the mixers from three different pens. At the end of each month, samples collected every two weeks were mixed and composited by group. GRAIN samples were expected to have less variations over time. Therefore, we decided to composite GRAIN samples and record less time points. Botanical composition was determined monthly as described by Maciel et al. (2021).

For proximate analysis, wet weights were recorded, and samples were dried at 55 °C in a forced-air oven for 72 h and ground through a 1 mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA) before undergoing subsequent analysis. For FA and antioxidant analysis, a 30 g subsample was taken, packed in a whirl pack bag (air manually removed), and frozen at -20 °C immediately after collection when arriving in the laboratory. To ensure representative PAST samples, 10 g of each replicate was taken by thoroughly mixing the bag content before being combined. Samples spent at most 100 days at -20 °C and were then stored at -80 °C. Before analysis, samples were freeze-dried in a Harvest Right Home Freeze Dryer Large (Harvest Right, North Salt Lake, UT, USA) for 18.5 h, and ground through a 1 mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA) with dry ice. Samples were flushed under nitrogen before undergoing subsequent analysis.

Temperature, precipitation, and growing degree days (GDD) were recorded for the period of the study using the Michigan State University Enviroweather platform at the Chatham, MI weather station. GDD is a useful measurement that represents changes in temperature related to different phases of plant development and refers to the accumulation of heat during the growth of the plant. It can also be an indicator of climate change on plants (Anandhi, 2016). The Baskerville-Emin method with a base temperature of 4 °C was used to calculate GDD. 30-year normal temperature and precipitation (1991-2020) were reported using the National Centers for Environmental Information: National Oceanic and Atmospheric Administration website which records U.S. climate normals in Chatham, MI. Weather conditions were averaged to obtain monthly data for the length of the study.

2.3.3 Proximate analysis for nutritive value of diets

Proximate analysis was performed as described by Maciel et al. (2021). Feed samples were analyzed for ash, neutral detergent fiber (NDF), acid detergent fiber (ADF), crude protein (CP), and gross energy. Briefly, all nutrients were expressed as percentages of dry matter (DM), determined by drying at 105 °C in a forced-air oven for at least 8 hours. Ash content was determined after 6 hours of oxidation at 500 °C in a muffle furnace. Neutral detergent fiber was analyzed according to Mertens (2002) with the inclusion of amylase and sodium sulfite. Acid detergent fiber was analyzed according to AOAC (2000). Crude protein was determined according to Hach et al. (Hach et al., 1987). Gross energy was determined by bomb calorimeter, and net energy values were estimated according to Belyea and Ricketts (1993) using the following conversion equations:

$$\text{Net energy for PAST} = \left(1.50 - 0.0267(\text{ADF})\right) \times 2.2$$

$$\text{Net energy for GRAIN} = 0.3133 \times \left(2.86 - \frac{35.5}{100 - (1.67 \times \text{ADF})}\right) \times 2.2$$

2.3.4 Chlorophyll and carotenoid analysis

Chlorophyll A, chlorophyll B, and total carotenoids were determined as described by Lichtenthaler and Wellburn (1983). Briefly, 2 g of lyophilized feed powder was combined with 70% aqueous acetone, shaken for 30 min, and centrifuged for 20 min (2500 RPM, 4 °C). The supernatant was recovered into a new tube, and extraction was repeated twice. Carotenoid and chlorophyll content of the extracted samples were measured using a UV-Vis Double Beam Spectrophotometer (VWR, Radnor, PA, USA) in cuvettes. The absorbance was recorded at three wavelengths (663, 646, and 470 nm) and used to calculate chlorophyll A, chlorophyll B, and total carotenoids as follows:

$$\text{Chlorophyll a } (C_a) = 12.21A_{663} - 2.81A_{646}$$

$$\text{Chlorophyll b } (C_b) = 20.13A_{646} - 5.03A_{663}$$

$$\text{Total carotenoids} = \frac{1000A_{470} - 3.27C_a - 104C_b}{229}$$

2.3.5 Phenolic analysis

A modified method from Nimalaratne et al. (2011) was used to extract phenolic compounds. Briefly, 2 g of lyophilized feed powder was added to 20 mL methanol:distilled water:acetic acid (70:28:2, *v/v/v*). The tube was shaken for 30 min and then centrifuged for 20 min (2500 RPM, 4 °C). The supernatant was transferred to a new tube. A second solution of 20 mL acetone:distilled water:acetic acid (70:28:2, *v/v/v*) was added to the original tube. The original tube was shaken again for 10 min and centrifuged for 15 min (2500 RPM, 4 °C). The supernatants were combined and stored at 4 °C until analysis.

The Folin-Ciocalteu assay modified from Singleton and Rossi (1965) was used to quantify total phenolic content (TPC). A gallic acid standard curve was made from a 1 mg/mL gallic acid standard stock solution in methanol, followed by a serial dilution by a factor of two to obtain concentrations ranging from 1 mg/mL to 0.002 mg/mL. Then, 100 µL Folin-Ciocalteu reagent and 800 µL 5% sodium bicarbonate were added to the standard curve and to a 100 µL portion of supernatant. The standard curve and the samples were then heated at 40 °C for 30 min and cooled at room temperature for 10 min. Cooled samples were plated in triplicate in a 96-well plate, scanned at 765 nm, compared against the gallic acid standard curve, and reported as mg of gallic acid equivalents/g of feed.

2.3.6 Fatty acid analysis

A modified version of the microwave assisted extraction (MAE) method described by Bronkema et al. (2019) was used to extract FAs from feed samples using the CEM Mars 6

microwave digestion system, equipped with a 24-vessel rotor and GlassChem vessel set (CEM Corporation, Matthews, NC, USA). This method was also described by Sergin et al. (2021). Briefly, 400 mg of lyophilized feed sample was added to a microwave vessel with 8 mL of 4:1 (*v/v*) solution of ethyl acetate:methanol and 0.1% butylated hydroxytoluene (BHT) as an antioxidant. FAs were extracted using the following microwave parameters: 55 °C for 15 min with initial ramp of 2 min at 400 W maximum power. Vessel contents were filtered using Whatman lipid free filters (Grade 597) (Weber Scientific; Hamilton, NJ, USA) into a test tube containing 3.5 mL HPLC water. Samples were centrifuged at 2500 RPM for 6 min, and the top organic layer was transferred to a new tube and dried under nitrogen. Extracted oil was resuspended in 4:1 (*v/v*) dichloromethane:methanol with 0.1% BHT to bring each sample to 20 mg oil/mL. Dichloromethane was purchased from VWR Chemicals (Radnor, PA, USA).

For the creation of fatty acid methyl esters (FAME), a modified methylation described by Jenkins (2010) was conducted. Two mg of suspended oil (100 µL) was aliquoted from each sample, dried under nitrogen, and resuspended in toluene with 20 µg of internal standard (methyl 12-tridecenoate, U-35M, Nu-Chek Prep, Elysian, MN, USA). Two mL of 0.5 N anhydrous potassium methoxide was added and samples were heated at 50 °C for 10 min. Once cool, 3 mL of 5% methanolic HCl was added, and samples were heated at 80 °C for 10 min. Once cool, 2 mL of water and 2 mL hexane were added, samples were centrifuged (2500 RPM at room temperature for 5 min), and the upper organic phase was removed and dried to obtain FAMEs. FAMEs were suspended in 1 mL isooctane to reach a concentration of 2 mg/mL and transferred to GC-MS vials with glass inserts.

One µL of methylated sample was injected in a PerkinElmer (Waltham, MA, USA) 680/600S GC-MS in the electron impact mode (70 eV) equipped with an Agilent Technologies

(Santa Clara, CA, USA) HP-88 column (100 m, 0.25 mm ID, 0.2 µM film thickness) for FAME quantification. Injection temperature was set at 250 °C, and the GC temperature parameters were as follows: initial temperature at 80 °C for 4 min; ramp 13.0 °C/min to 175 °C; hold 27 min; ramp 4.0 °C/min to 215 °C; hold 35 min modified from Kramer et al. (2008) previously used for improved separation of FA isomers in beef and dairy products. Helium was used as the carrier gas at a flow rate of 1 mL/min. The MS data were recorded in full scan mode (mass range of m/z 70-400 amu). MS transfer line and ion source temperature were set at 180 °C.

For identification of FAMEs, data analysis was conducted using MassLynx V4.1 SCN 714 (Waters Corporation, Milford, MA, USA). FAs were identified by retention time and EI mass fragmentation in comparison to that of our reference standard. Our GC-MS reference standard was created by using the Supelco 37 Component FAME Mix (Sigma-Aldrich, St. Louis, MO, USA). FAs were analyzed using extracted ion chromatograms of the respective quantitative ions. FAs not included in the reference standard were identified according to elution order reported in literature and confirmed by the EI mass fragmentation (Kramer et al., 2008). Identification of retention times, mass fragmentation, and quantitative ions used are outlined in **Appendix Table A1**. For quantification of FAMEs, we utilized a standard curve including our reference and internal standards. The internal standard peak area and analyte peak area relative to the standard curve were used to calculate each FAME concentration. FAs were reported as g/100 g FA quantified.

2.3.7 Statistical analysis

Data from the carotenoid and phenolic analyses were analyzed for their statistical significance using Prism v7.0d for Mac OS X (GraphPad Software, La Jolla, CA, USA) to perform unpaired t-tests. Data from the fatty acid analysis were analyzed using RStudio v1.4.1103. Individual nutrients were analyzed separately for the two years after checking for year effect.

Treatment significance was checked using one-way analysis of variance (ANOVA) and mean comparison was performed using Tukey's HSD, correcting for multiple comparisons. When included, sampling date was the fixed factor in the ANOVA. Values below the lower limit of detection were treated as zeroes in analysis. Statistical significance for all analyses was set at $p < 0.05$. Correlations between individual plant species, weather, antioxidants, and selected FAs were assessed using Pearson correlations and graphically displayed using the R package *corrplot*. Only results with $p < 0.05$ were shown in the correlation matrix. Weather conditions, antioxidants, and FAs were averaged by month to match the monthly botanical composition data. August 2019 was removed since no sample collection occurred at that time.

2.4 Results

2.4.1 Weather conditions and nutritive quality of the diets

The weather conditions for the length of the study are displayed in **Figure 3**. July was the warmest month in both years. The coldest temperatures were recorded in September. No abnormal trends regarding the average temperatures for the length of the study were observed. Regarding precipitation, notable differences were seen between 2019 and 2020. The highest levels of precipitation were seen in September 2019 and in July 2020. The lowest levels of precipitation were observed in July 2019 and in September 2020. On average, precipitation levels were higher in 2020 compared to 2019.

The nutritive values of the diets are shown in **Table 2**. Dry matter was higher for the GRAIN diet compared to PAST. Overall values for DM were higher in 2020 compared to 2019. Crude protein, NDF, ADF, and gross energy values were higher for the PAST diet in both years. Total FAs were higher in the GRAIN diet in both years compared to the PAST diet.

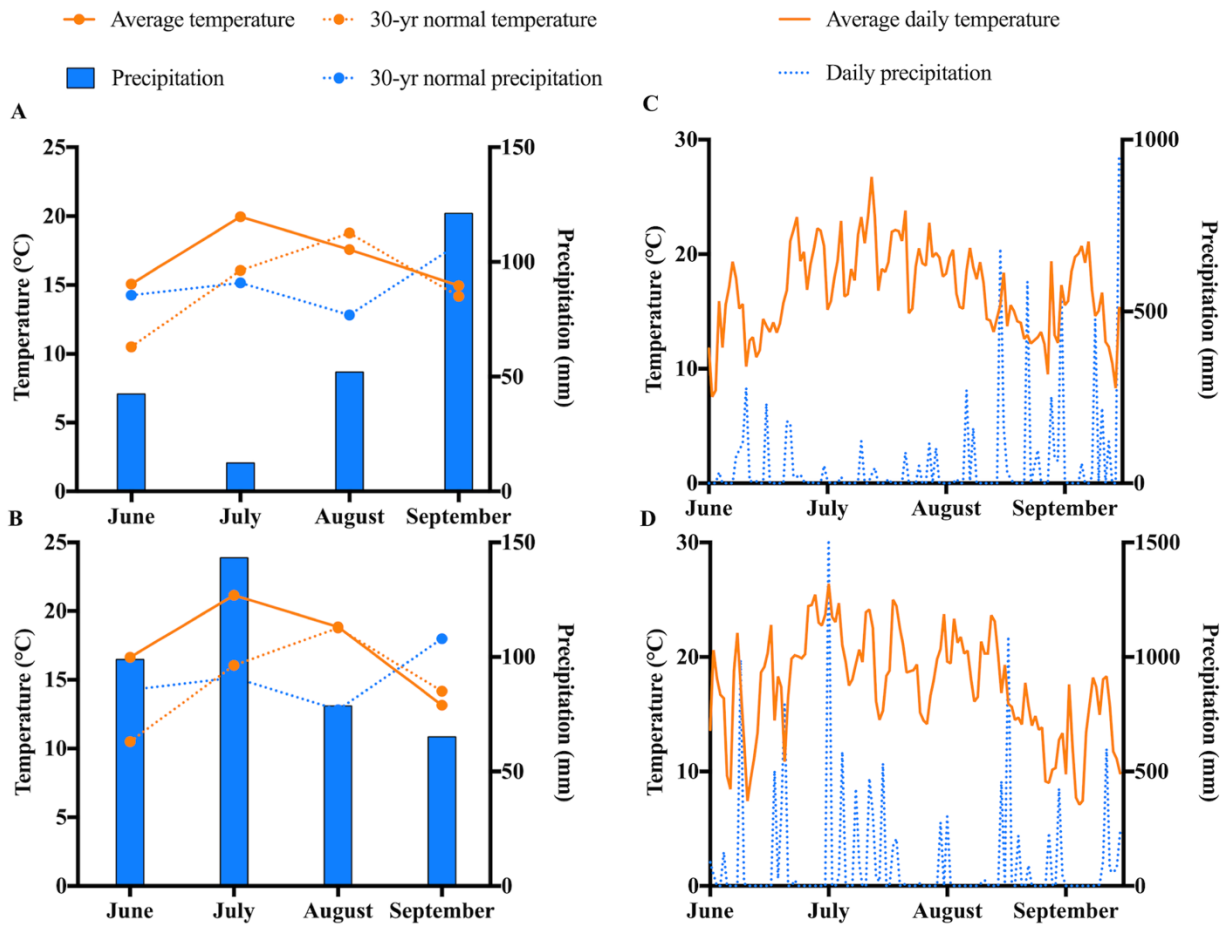

Figure 3. Weather trends at the experimental site in Chatham, MI for the duration of the study. Monthly weather in (A) 2019 and (B) 2020. Daily weather in (C) 2019 and (D) 2020.

Table 2. Nutritive value of the diets

	2019		2020	
	PAST	**GRAIN**	**PAST**	**GRAIN**
Dry matter, %	20.54 ± 5.35	76.11 ± 1.76	22.11 ± 3.01	85.32 ± 1.09
Ash*	7.12 ± 0.89	4.44 ± 0.39	6.14 ± 1.03	3.17 ± 0.60
Crude protein*	11.54 ± 2.42	9.84 ± 0.99	15.03 ± 2.85	9.38 ± 0.83
NDF[1]*	52.21 ± 7.69	21.31 ± 1.60	51.49 ± 4.54	20.71 ± 2.33
ADF[2]*	34.99 ± 6.19	10.24 ± 1.16	31.98 ± 3.01	9.87 ± 1.50
Gross energy, Mcal/kg	4.41 ± 0.05	4.22 ± 0.04	4.52 ± 0.06	4.33 ± 0.03
Net energy, Mcal/kg[3]	1.24 ± 0.36	1.68 ± 0.01	1.42 ± 0.18	1.68 ± 0.01
Total FA[4], g/kg	6.10 ± 1.59	14.13 ± 2.98	6.14 ± 1.52	20.25 ± 3.70

Data reported as mean ± standard deviation, '*' reported as % dry matter
[1]NDF: neutral detergent fiber; [2]ADF: acid detergent fiber; [3]FA: fatty acid;
[4]estimated from ADF values.

2.4.2 Chlorophyll, carotenoid, and phenolic content of the diets

The phytochemical composition of the diets is depicted in **Table 3**. Significant differences of all antioxidant parameters assessed in this study demonstrated the rich antioxidant profile found in PAST compared to GRAIN. Chlorophyll A and chlorophyll B were found in higher concentrations in PAST samples compared to GRAIN samples in both years ($p < 0.001$). Chlorophyll B levels increased from 2019 to 2020 in PAST samples (28.30 vs. 111.10 mg/g, respectively). Total carotenoids were also higher in PAST samples compared to GRAIN samples in both years ($p < 0.001$). Total phenols were found in higher amounts in PAST samples in 2019 (4.44 vs. 2.91 mg GAE/g, $p = 0.009$) and in 2020 (7.73 vs. 3.07 mg GAE/g, $p = 0.001$) compared to GRAIN samples.

Table 3. Antioxidant profile of pasture and grain samples

2019			
	PAST	**GRAIN**	***p*-value[1]**
Chlorophyll A, µg/g	117.00 ± 12.00	34.40 ± 3.12	< 0.001
Chlorophyll B, µg/g	28.30 ± 3.53	10.60 ± 1.26	< 0.001
Total carotenoids, µg/g	60.30 ± 2.58	10.40 ± 0.80	< 0.001
Total phenols, mg GAE[2]/g	4.44 ± 1.01	2.91 ± 0.35	0.009
2020			
	PAST	**GRAIN**	***p*-value**
Chlorophyll A, µg/g	190.10 ± 3.49	17.02 ± 2.67	< 0.001
Chlorophyll B, µg/g	111.10 ± 13.99	19.85 ± 3.13	< 0.001
Total carotenoids, µg/g	55.02 ± 4.69	14.50 ± 1.37	< 0.001
Total phenols, mg GAE/g	7.73 ± 0.86	3.07 ± 0.11	0.001

Data reported as mean ± standard deviation. [1]*p*-values indicate results of the unpaired t-test and were considered significant at $p < 0.05$. [2]GAE: gallic acid equivalent.

2.4.3 Fatty acid profiles of the diets

The FA profiles of the PAST and GRAIN diets are shown in **Table 4**. Total SFAs were higher in PAST compared to GRAIN in both years, but the difference was significant in 2020 (19.74 vs. 15.08 g/100 g FA, $p < 0.001$). More specifically, C8:0 through C15:0 were all significantly higher in PAST compared to GRAIN in both years of the study. Palmitic acid (C16:0) was significantly higher in GRAIN in 2019 but was significantly higher in PAST in 2020. Stearic acid (C18:0) was significantly higher in PAST in 2020 (1.61 vs. 1.43 g/100 g FA, $p = 0.016$). Longer chain SFAs (C20:0 – C24:0) were all found in higher concentrations in PAST in both years.

Total MUFAs were significantly higher in GRAIN samples compared to PAST in both years ($p < 0.001$). More precisely, C16:1 *n*-7 was higher in PAST in 2019, but no significant difference was observed in 2020. Regarding C16:1 *n*-9, PAST contained higher levels in both years

compared to GRAIN ($p < 0.001$). The higher total MUFA content in GRAIN is due to significantly higher levels of C18:1 *n*-9 observed compared to PAST in 2019 and in 2020 ($p < 0.001$).

When assessing total PUFAs found in the diets, PAST displayed significantly higher concentrations compared to GRAIN in 2019 ($p < 0.001$) and in 2020 ($p < 0.001$). Linoleic acid (LA - C18:2 *n*-6) was found in significantly higher levels in GRAIN samples compared to PAST in 2019 ($p < 0.001$) and in 2020 ($p < 0.001$). On the other hand, ALA (C18:3 *n*-3) was significantly higher in PAST in 2019 ($p < 0.001$) and in 2020 ($p < 0.001$). Finally, the *n*-6:*n*-3 ratio was almost 50 times higher in GRAIN in 2019 (10.77 vs. 0.22, $p < 0.001$) and 90 times higher in GRAIN in 2020 (21.63 vs. 0.25, $p < 0.001$) compared to PAST.

Table 4. Fatty acid profiles of the diets

	2019			2020		
	PAST	**GRAIN**	***p*-value[1]**	**PAST**	**GRAIN**	***p*-value**
C8:0	0.07 ± 0.03	0.01 ± 0.01	< 0.001	0.24 ± 0.36	0.05 ± 0.02	0.014
C12:0	0.25 ± 0.11	0.04 ± 0.01	< 0.001	0.17 ± 0.10	0.02 ± 0.01	< 0.001
C14:0	0.72 ± 0.38	0.07 ± 0.01	< 0.001	0.61 ± 0.21	0.08 ± 0.02	< 0.001
C15:0	0.14 ± 0.05	0.03 ± 0.00	< 0.001	0.12 ± 0.05	0.03 ± 0.01	< 0.001
C16:0	13.73 ± 1.66	14.55 ± 0.23	0.037	14.48 ± 1.28	12.74 ± 0.60	< 0.001
C16:1 *n*-7	0.18 ± 0.14	0.09 ± 0.01	0.004	0.18 ± 0.17	0.10 ± 0.06	0.062
C16:1 *n*-9	0.90 ± 0.14	0.13 ± 0.03	< 0.001	1.20 ± 0.22	0.14 ± 0.03	< 0.001
C17:0	0.17 ± 0.06	0.06 ± 0.00	< 0.001	0.22 ± 0.05	0.07 ± 0.01	< 0.001
C18:0	1.86 ± 0.59	1.89 ± 0.07	0.810	1.61 ± 0.32	1.43 ± 0.09	0.016
C18:1 *n*-7	0.23 ± 0.10	0.53 ± 0.03	< 0.001	0.78 ± 0.30	0.49 ± 0.07	< 0.001
C18:1 *n*-9	4.10 ± 1.79	22.45 ± 0.78	< 0.001	2.36 ± 1.24	21.99 ± 0.35	< 0.001
C18:2 *n*-6	13.25 ± 2.34	53.97 ± 0.85	< 0.001	14.82 ± 3.04	59.33 ± 1.25	< 0.001
C18:3 *n*-3	62.35 ± 6.92	5.24 ± 1.10	< 0.001	60.93 ± 5.69	2.87 ± 0.66	< 0.001
C20:0	0.54 ± 0.21	0.50 ± 0.03	0.416	0.70 ± 0.15	0.30 ± 0.07	< 0.001
C22:0	0.60 ± 0.26	0.17 ± 0.02	< 0.001	0.89 ± 0.18	0.19 ± 0.03	< 0.001
C24:0	0.76 ± 0.28	0.25 ± 0.03	< 0.001	0.71 ± 0.15	0.19 ± 0.03	< 0.001
Total SFA[2]	18.92 ± 3.05	17.56 ± 0.3	0.055	19.74 ± 2.01	15.08 ± 0.82	< 0.001
Total MUFA[3]	5.41 ± 1.91	23.22 ± 0.75	< 0.001	4.52 ± 1.32	22.72 ± 0.34	< 0.001
Total PUFA[4]	75.66 ± 4.72	59.22 ± 0.54	< 0.001	75.75 ± 2.98	62.20 ± 0.82	< 0.001
Total *n*-6[5]	13.26 ± 2.34	53.98 ± 0.85	< 0.001	14.82 ± 3.04	59.33 ± 1.25	< 0.001
Total *n*-3[6]	62.40 ± 6.92	5.24 ± 1.10	< 0.001	60.93 ± 5.69	2.87 ± 0.66	< 0.001
n-6:*n*-3 ratio[7]	0.22 ± 0.07	10.77 ± 2.47	< 0.001	0.25 ± 0.08	21.63 ± 4.61	< 0.001
Total OCFA[8]	0.32 ± 0.11	0.08 ± 0.01	< 0.001	0.34 ± 0.08	0.10 ± 0.02	< 0.001

Data reported as mean ± standard deviation in g/100 g FA. [1]*p*-values indicate results of one-way ANOVA and were considered significant at $p < 0.05$. [2]Total SFA: saturated FAs 8:0-24:0 (even and odd); [3]Total MUFA: monounsaturated FAs 16:1-18:1 (even and odd); [4]Total PUFA:18:2 *n*-6 + 18:3 *n*-3; [5]Total *n*-6: 18:2 *n*-6; [6]Total *n*-3: 18:3 *n*-3; [7]*n*-6:*n*-3 ratio: total *n*-6/total *n*-3; [8]Total OCFA: odd-chain FAs 15:0 + 17:0.

2.4.4 Variations in the fatty acid profile of pasture over time

We assessed the changes of the fatty acid profile of PAST by sampling date in 2019 and 2020. These results are displayed in **Table 5**. In 2019, total FA was significantly highest on June 10 (9.29 g/kg feed) before decreasing and remaining constant throughout the season. When looking at SFAs in 2019, lauric acid (C12:0), myristic acid (C14:0), and stearic acid (C18:0) were all significantly higher on September 3 and significantly lower on June 10. They gradually increased from June 10 to September 3 before dropping again on September 18. Regarding MUFAs in 2019, C18:1 *n*-7 and C18:1 *n*-9 were both significantly higher on September 3 and lower on June 25. They also gradually increased from June 25 to September 3 before drastically dropping on September 18. Finally, we can observe that LA (C18:2 *n*-6) was higher on September 3 and lower in June and on September 18 while ALA (C18:3 *n*-3) was significantly higher in June and on September 18 and was lower on September 3. These two PUFAs followed opposite trends.

In 2020, total FA was significantly highest on August 25 (7.74 g/kg feed) and lowest on July 15 (4.18 g/kg feed). Saturated FAs in 2020 followed a different trend than in 2019. Lauric acid (C12:0), palmitic acid (C16:0), and stearic acid (C18:0) were all higher on July 15. Regarding MUFAs, C16:1 *n*-9 was significantly higher on June 3 (1.55 g/100 g FA) before decreasing and going back up in July. The other MUFA, C18:1 *n*-9, was significantly higher on July 1 (5.01 g/100 g FA) and remained constant throughout the other sampling dates. LA (C18:2 *n*-6) was higher in July and remained lower during the other months. On the other hand, ALA (C18:3 *n*-3) followed the opposite trend by being at the lowest concentrations in July while being higher and constant during the other months.

Table 5. Fatty acid profile of pasture by sampling date

Date	C12:0	C14:0	C16:0	C16:1 *n*-7	C16:1 *n*-9	C18:0	C18:1 *n*-7	C18:1 *n*-9	C18:2 *n*-6	C18:3 *n*-3	Total FA
					g/100 g FA						g/kg feed
2019											
June 10	0.11 ± 0.01 ^{d}	0.30 ± 0.01 ^{d}	12.09 ± 0.11 ^{c,d}	0.08 ± 0.01 ^{b}	1.01 ± 0.06 ^{a}	1.03 ± 0.09 ^{c}	0.17 ± 0.03 ^{b,c}	2.67 ± 0.15 ^{b,c}	11.66 ± 1.41 ^{b,c}	69.59 ± 1.66 ^{a}	9.29 ± 1.03 ^{a}
June 25	0.15 ± 0.01 ^{c,d}	0.43 ± 0.03 ^{c,d}	12.88 ± 0.69 ^{b,c}	0.07 ± 0.06 ^{b}	0.84 ± 0.08 ^{a,b}	1.44 ± 0.11 ^{b,c}	0.13 ± 0.01 ^{c}	2.29 ± 0.46 ^{c}	11.34 ± 0.38 ^{b,c}	68.26 ± 1.52 ^{a}	5.84 ± 0.45 ^{b}
July 1	0.18 ± 0.03 ^{c,d}	0.54 ± 0.02 ^{c,d}	15.11 ± 0.21 ^{a}	0.13 ± 0.04 ^{b}	0.94 ± 0.12 ^{a}	1.88 ± 0.11 ^{b}	0.16 ± 0.02 ^{b,c}	3.75 ± 0.11 ^{a,b,c}	12.92 ± 0.72 ^{b,c}	62.27 ± 1.12 ^{a,b}	5.90 ± 0.72 ^{b}
July 16	0.27 ± 0.04 ^{b}	0.68 ± 0.06 ^{b,c}	15.54 ± 0.67 ^{a}	0.12 ± 0.10 ^{b}	1.01 ± 0.10 ^{a}	1.82 ± 0.09 ^{b}	0.22 ± 0.07 ^{b,c}	4.15 ± 0.72 ^{a,b,c}	14.29 ± 0.66 ^{a,b}	59.67 ± 1.83 ^{b,c}	5.38 ± 0.35 ^{b}
July 31	0.37 ± 0.02 ^{a}	0.95 ± 0.10 ^{b}	15.03 ± 0.23 ^{a}	0.28 ± 0.02 ^{a,b}	0.86 ± 0.06 ^{a}	2.49 ± 0.29 ^{a}	0.29 ± 0.04 ^{a,b}	5.42 ± 1.14 ^{a,b}	15.97 ± 1.47 ^{a}	55.1 ± 2.71 ^{b,c}	4.78 ± 0.42 ^{b}
Sept 3	0.42 ± 0.06 ^{a}	1.47 ± 0.24 ^{a}	14.29 ± 0.52 ^{a,b}	0.41 ± 0.20 ^{a}	0.64 ± 0.04 ^{b}	2.75 ± 0.34 ^{a}	0.41 ± 0.10 ^{a}	6.96 ± 2.37 ^{a}	16.10 ± 1.66 ^{a}	52.76 ± 5.52 ^{c}	5.06 ± 0.13 ^{b}
Sept 18	0.23 ± 0.02 ^{b,c}	0.70 ± 0.08 ^{b,c}	11.19 ± 0.76 ^{d}	0.20 ± 0.06 ^{a,b}	0.99 ± 0.07 ^{a}	1.63 ± 0.18 ^{b}	0.21 ± 0.05 ^{b,c}	3.43 ± 0.77 ^{b,c}	10.48 ± 1.05 ^{c}	68.82 ± 3.08 ^{a}	6.45 ± 1.64 ^{b}
p-value[1]	< 0.001	< 0.001	< 0.001	0.005	< 0.001	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001

Data reported as mean ± standard deviation in g/100 g FA for fatty acids and g/kg of feed for total FAs.

[1]*p*-values indicate results of one-way ANOVA. Means within a column that have different letters are significantly different according to Tukey's HSD test ($p < 0.05$).

Table 5. (cont'd)

Date	C12:0	C14:0	C16:0	C16:1 *n*-7	C16:1 *n*-9	C18:0	C18:1 *n*-7	C18:1 *n*-9	C18:2 *n*-6	C18:3 *n*-3	Total FA
					g/100 g FA						g/kg feed
2020											
June 3	0.10 ± 0.04 [b]	0.39 ± 0.10 [b]	14.61 ± 0.22 [a,b]	0.27 ± 0.04	1.55 ± 0.11 [a]	1.37 ± 0.08	0.76 ± 0.13	1.77 ± 0.07 [b]	14.05 ± 1.07 [b]	62.80 ± 1.74 [a]	6.49 ± 0.79 [a,b,c]
June 16	0.09 ± 0.01 [b]	0.45 ± 0.04 [b]	13.69 ± 0.90 [b]	0.23 ± 0.21	1.12 ± 0.07 [a,b]	1.59 ± 0.37	0.90 ± 0.13	1.85 ± 0.34 [b]	13.72 ± 1.09 [b]	63.51 ± 2.70 [a]	5.99 ± 0.61 [a,b,c]
July 1	0.16 ± 0.04 [b]	0.66 ± 0.04 [a,b]	16.20 ± 0.57 [a]	0.23 ± 0.21	0.96 ± 0.16 [b]	1.71 ± 0.09	0.78 ± 0.19	5.01 ± 1.30 [a]	20.05 ± 3.41 [a]	51.44 ± 5.44 [b]	6.42 ± 1.71 [a,b,c]
July 15	0.37 ± 0.13 [a]	0.89 ± 0.14 [a]	16.14 ± 1.12 [a]	0.15 ± 0.25	1.03 ± 0.20 [b]	2.10 ± 0.05	1.07 ± 0.23	3.11 ± 0.78 [b]	17.64 ± 1.87 [a,b]	53.70 ± 3.91 [b]	4.18 ± 1.26 [c]
July 28	0.17 ± 0.08 [a,b]	0.59 ± 0.28 [a,b]	13.97 ± 0.63 [a,b]	0.09 ± 0.15	1.17 ± 0.19 [a,b]	1.38 ± 0.49	0.43 ± 0.37	1.49 ± 0.12 [b]	12.51 ± 1.31 [b]	65.96 ± 2.40 [a]	7.74 ± 0.67 [a,b]
Aug 11	0.13 ± 0.12 [b]	0.57 ± 0.05 [a,b]	14.30 ± 1.47 [a,b]	0.25 ± 0.21	1.17 ± 0.28 [a,b]	1.64 ± 0.29	0.65 ± 0.57	2.18 ± 0.41 [b]	12.99 ± 3.47 [b]	62.63 ± 3.37 [a]	5.38 ± 0.86 [a,b,c]
Aug 25	0.13 ± 0.01 [b]	0.42 ± 0.04 [b]	13.21 ± 0.40 [b]	0.20 ± 0.18	1.24 ± 0.07 [a,b]	1.51 ± 0.31	0.76 ± 0.12	1.89 ± 0.47 [b]	14.12 ± 1.40 [b]	64.14 ± 2.09 [a]	7.94 ± 1.14 [a]
Sept 8	0.19 ± 0.02 [a,b]	0.89 ± 0.06 [a]	13.69 ± 0.61 [b]	0.00 ± 0.00	1.33 ± 0.16 [a,b]	1.57 ± 0.23	0.93 ± 0.12	1.56 ± 0.16 [b]	13.45 ± 0.76 [b]	63.28 ± 0.98 [a]	4.94 ± 0.85 [b,c]
p-value	0.005	< 0.001	0.002	0.572	0.016	0.114	0.25	< 0.001	0.004	< 0.001	0.005

2.4.5 Correlations between individual plant species, weather, antioxidants, and fatty acids

Pearson correlations were calculated for individual plant species, weather, antioxidants, and selected FAs (**Figure 4**). Alfalfa was positively correlated with phenolics while timothy was positively correlated with temperature, GDD, LA, the *n*-6:*n*-3 ratio, and palmitic acid. Meadow fescue was positively correlated with oleic acid but negatively correlated with chlorophyll B. Orchard grass was negatively correlated with meadow fescue and carotenoids while being positively correlated with chlorophyll B. Temperature was positively correlated with GDD and palmitic acid while GDD was positively correlated with the *n*-6:*n*-3 ratio and palmitic acid. Chlorophyll A was negatively correlated with stearic acid while chlorophyll B was negatively correlated with carotenoids and oleic acid. LA was positively correlated with the *n*-6:*n*-3 ratio and palmitic acid, and the *n*-6:*n*-3 ratio was positively correlated with palmitic acid. Stearic acid was positively correlated with oleic acid. ALA was negatively correlated with LA, the *n*-6:*n*-3 ratio, and palmitic acid.

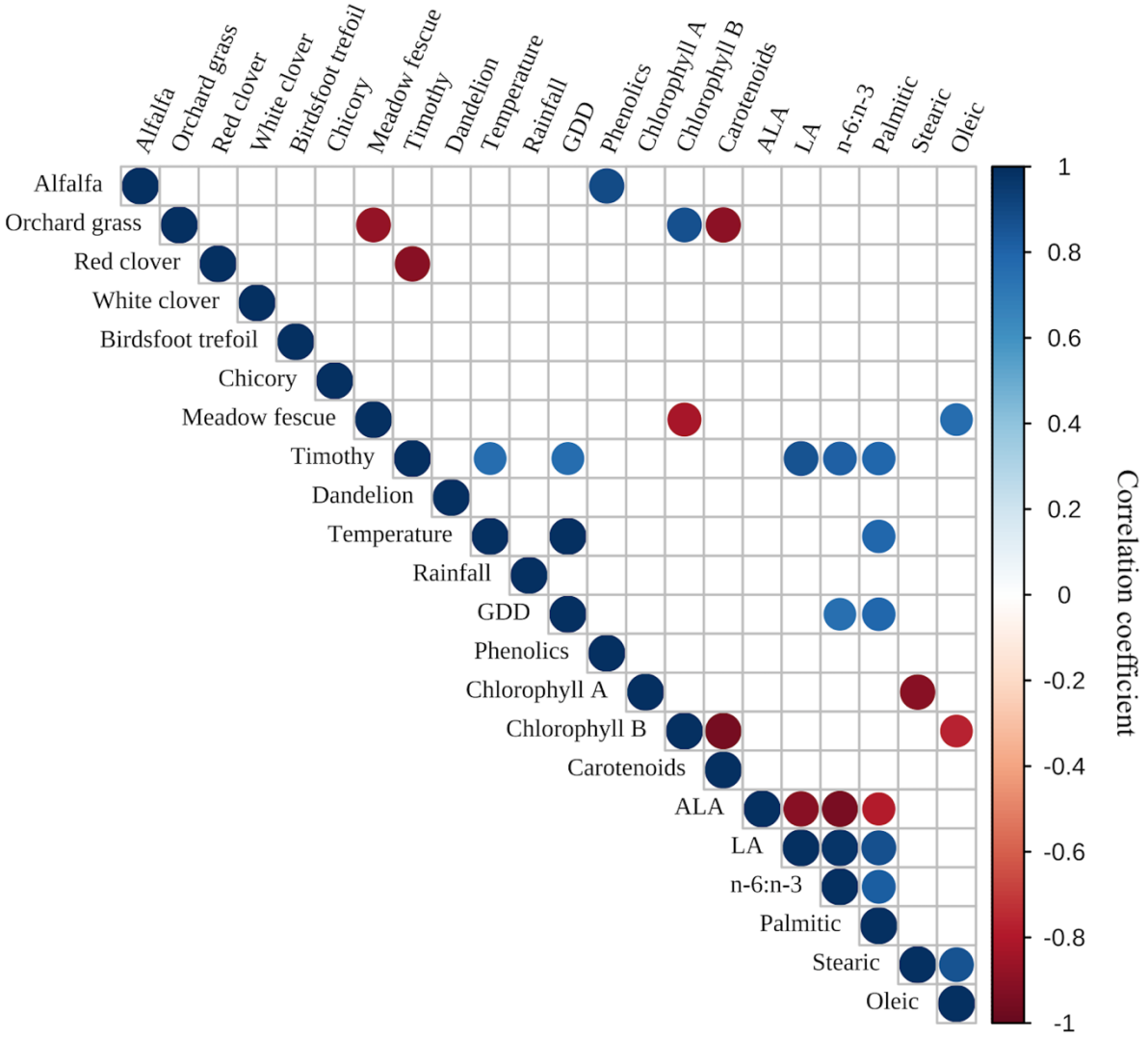

Figure 4. Pearson correlations between individual plant species, weather, and selected fatty acids. Pearson correlation matrix displays r correlation coefficients represented as circles. Blue shades denote positive r correlation coefficients, while red shades denote negative r correlation coefficients. Only results with $p < 0.05$ are displayed, thus, empty boxes were not significant. ALA: α-linolenic acid; GDD: growing degree days; LA: linoleic acid.

2.5 Discussion

2.5.1 Rich antioxidant profile of pasture

In the present study, all bioactive compounds assessed were higher in PAST compared to GRAIN for both years. The higher carotenoid content found in PAST is in accordance with what was reported by Daley et al. (2010) and Pickworth et al. (2012) stating that lush green forages are usually higher in carotenoids and give carcass fat a yellow color. The higher carotenoid levels observed in PAST compared to GRAIN can be attributed to the wide array of plant species found in the pasture (Daley et al., 2010). Carotenoid levels are highly variable due to the seasonal nature of plant growth (Daley et al., 2010). Pickworth et al. (2012) emphasized the need to provide more data for carotenoids in commonly used feedstuff; vitamin A equivalents from feed collected in different states varied largely probably due to growing conditions and plant maturity. In our study, carotenoid concentrations were higher in 2019 compared to 2020 in PAST but were higher in 2020 compared to 2019 for GRAIN, emphasizing the need to collect more time and feed specific data.

Large variations in chlorophyll content between 2019 and 2020 were observed in this study. For example, chlorophyll B in PAST raised from 28.30 to 111.10 mg/g between 2019 and 2020. These differences may be due to changes in temperature and precipitation. Islam et al. (2021) found that low and high temperatures reduce chlorophyll levels in wheat and barley grasses. Additionally, the authors hypothesize that chlorophyll concentrations might increase because of chemical changes during photosynthesis. Inversely, lower and higher temperatures can affect chloroplast enzymes and may lower the chlorophyll content of grasses (Islam et al., 2021). The assessment of chlorophyll concentrations in grasses is important since there is a strong positive relationship between chlorophyll A and B and α-linolenic acid (C18:3 n-3) or total FA content (Khan et al., 2012).

The large plant diversity found in complex pastures usually results in more biochemically rich feeds with varying amounts of bioactive compounds such as terpenoids, carotenoids, and phenolic compounds that may be reflected in ruminant products (Reynaud et al., 2010).

We also reported a higher total phenol content in PAST samples in this study. These findings are consistent with results reported in the literature comparing total phenolic content of grass and red sorghum (Tejerina et al., 2012;Xiong et al., 2020). Niroula et al. (2019) previously reported that the total phenolic content and the antioxidant activity are lower in seeds compared to sprouts and grasses. Assessing the phenolic content and the antioxidant activity of feedstuffs for ruminants is an important step since they have the potential to increase volatile FA production that can decrease rumen ammonia and methane production, reduce lipid oxidation, and increase anti-microbial action (Kalantar, 2018). Further, polyphenol intake impacts protein digestion by inhibiting protein degradation in the rumen and thereby increasing protein availability in the intestinal tract (Bonanno et al., 2011). One limitation of the present study is that we did not assess antioxidant activity directly, but rather measured bioactive compounds with antioxidant potential.

2.5.2 Fatty acid profiles of the diets

Our findings in the present study indicate a beneficial FA profile in PAST samples compared to GRAIN. On a g/100 g FA basis, PAST displayed significantly higher levels of total SFAs compared to GRAIN in 2020. The difference was not statistically significant in 2019. The largest differences were seen in the short chain SFAs (C8:0 – C15:0) and C24:0. These results are in agreement with similar results published by Glasser et al. (2013) and Rhee et al. (2000). Interestingly, the concentrations of palmitic acid (C16:0) were higher in GRAIN compared to PAST in 2019. This goes against data published in the literature (Rhee et al., 2000;Garcia et al., 2008;Glasser et al., 2013) and our 2020 results that found higher levels in grasses compared to

grain feeds. These variations can be explained by differences in diet compositions and climatic conditions.

The higher total MUFA content in GRAIN compared to PAST in both years of the present study is supported by various studies (Garcia et al., 2008;Khan et al., 2012;Glasser et al., 2013). Regarding the difference in C18:1 *n*-9, Khan et al. (2012) suggest that this MUFA increases in grains due to the growth of ears and the accumulation of FA in these ears. This is also true for the accumulation of C18:2 *n*-6 in grain (Khan et al., 2012), leading to higher levels of LA as confirmed by our results. On the other hand, we found higher levels of C18:3 *n*-3 in PAST compared to GRAIN in both years. The content of ALA in grasses is highly dependent on the leaf-to-stem ratio, with this PUFA accumulating in the leaf tissue of fresh pasture (Mir et al., 2006;Revello-Chion et al., 2011;Khan et al., 2012;Butler, 2014;Alothman et al., 2019).

The potential to modify the FA profile of animal products to favor human health requires the determination of the FA profile of feeds (Boufaïed et al., 2003;Mir et al., 2006). Great emphasis is put on reducing the *n*-6:*n*-3 ratio in grazing ruminant products to favor human health (Butler, 2014). To do so, higher *n*-3 PUFA concentrations are needed. In the current study, our results show that PAST concentrations of C18:3 *n*-3 were 12 times greater than GRAIN in 2019 and 20 times greater in 2020. Generally, forages contain high levels of this *n*-3 PUFA (50-75% of total FA) (Dewhurst et al., 2006). Consequently, we also reported a highly beneficial *n*-6:*n*-3 ratio in PAST compared to GRAIN in both years.

The beneficial FA profile of PAST reported in the current study could potentially be reflected in grazing ruminant products. Additional research is needed to determine to what extent the FA profile of the cattle diet coincides with the nutritional profile of resulting animal products.

One limitation in our work is the lack of species-specific FA profile, which does not allow us to isolate which plant species contribute positively to a beneficial FA profile.

2.5.3 Fatty acid profile of pasture varies over time

Time-specific data about the nutritional value of feeds is critical for ranchers and producers to understand the complex variations occurring in these systems. Climatic changes have important implications for grasslands and the increase in dramatic seasonal variability add more challenges for farmers, scientists, and policy-makers (Hopkins and Del Prado, 2007). In the current study, we emphasize the trends of some FAs over time and how they differ year to year. In 2019, we found that total FA was higher in the early season before decreasing and remaining constant. This observation is in agreement with results reported by Revello-Chion et al. (2011) who noticed that total FA concentrations decreased during the growing season. The SFAs C12:0, C14:0, and C18:0, as well as the C18:1 MUFAs all gradually increased from the early all the way to the late season before drastically dropping between September 3 and September 18. These FAs showed a similar trend than reported by Garcia et al. (2016). Interestingly, LA and ALA followed opposite trends in 2019, with LA gradually increasing from June to September before dropping on September 18 and ALA gradually declining from June to September before spiking back up on September 18. Boufaïed et al. (2003) noted that high proportions of C18:3 were observed during the vegetative growth before declining and recovering by the beginning of the fall. The authors hypothesized that this pattern can be explained by the changes in leaf proportion. Garcia et al. (2016) reported similar opposite trends between C18:3 *n*-3 and C18:2 *n*-6, citing the changes in leaf-to-stem ratio as a reason why ALA decreases over time while LA increases. The sudden increase in C18:3 *n*-3 concentrations between September 3 and September 18 2019 can be explained by growth periods. Glasser et al. (2013) found an increase of C18:3 in September, corresponding to the regrowth

vegetation cycle. Bauchart et al. (1984) found that C18:3 levels in alpine grass were higher during the primary growth period before strongly declining during the stemmy regrowth and increasing again during the last leafy regrowth. The authors also noted the opposite trend followed by C18:2. It appears that the different growth periods modify the leaf-to-stem ratio, and that chloroplast lipid (found in green, leafy plants) are high in ALA (Elgersma et al., 2013). It is also interesting to note that late September experienced the most rainfall during the study period.

The second year of the experimental period (2020) did not follow the same patterns as 2019. This emphasizes the importance of having time- and region-specific data. For instance, total FA concentrations did not follow any statistically notable trend but were higher in late August and lower on July 15. We also observed that most SFAs, MUFAs, and C18:2 *n*-6 were all having higher concentrations in early and mid-July. On the other hand, ALA (C18:3 *n*-3) remained somewhat constant throughout the season but decreased dramatically on July 15. When comparing these trends to the weather trends for 2020, we can see that July experienced the most rainfall and the highest temperatures that year. Revello-Chion et al. (2011) reported that the high temperatures and water deficits can reduce forage nutritional quality by reducing the leaf-to-stem ratio. Water deficit can also directly affect FA concentrations by decreasing or even inhibiting the biosynthesis of many lipids and altering the phospholipid and galactolipid levels, resulting in a decrease in the membrane lipid contents (Gigon et al., 2004). It was reported that FA concentrations vary with rainfall and temperature, and that high rainfall promotes grass quality and productivity (Mir et al., 2006;Revello-Chion et al., 2011).

The current study was conducted under realistic grazing conditions. The action of cattle on the pasture plays a role in the variation of FAs. Meľuchová et al. (2008) reported that C16:0, C18:2 *n*-6, and C18:3 *n*-3 concentrations in a pasture grazed by ewes followed similar trends compared

to our study. Palmitic acid (C16:0) and LA (C18:2 *n*-6) increased from May to July before decreasing in September. On the other hand, ALA (C18:3 *n*-3) followed the opposite trend by declining from May to July before going back up in September.

Our results showing the variations of major FAs in pastures throughout the season are important for grassland management and ruminant nutrition. The need for these specific data is increasing with high seasonal variations and severe climatic events. Oliveira et al. (2020) reported that management of pasture lands requires mapping spatial and temporal trends; there is a critical need for geospatial data with measurable management attributes and that for pasture data to be better described and reported. Future research should focus on collecting data in grasslands for longer periods of time and on providing specific nutritional value data for each species found in pastures. More data will also be needed in events of dramatic climatic conditions such as droughts, typhoons, and floods as these unfortunate events will occur more frequently in the future (Hopkins and Del Prado, 2007;Escarcha et al., 2018).

2.5.4 Relationship between plant species, antioxidants, and fatty acids

ALA, LA, and palmitic acid usually contribute up to 93% of total FAs in most forage plants (Meľuchová et al., 2008). Dewhurst et al. (2001) found little compositional differences between FAs found in fescue, but noted large variations in FA composition in chicory (which is higher in total FAs). Boufaïed et al. (2003) also noted that individual FAs vary widely between cultivars. Meanwhile, interests in investigating the FA composition of botanically diverse pastures recently emerged (Howes et al., 2015). Because diverse pastures are composed of multiple plant species, it is important to have a better understanding of the effects of each individual species on FAs. Surprisingly, we only noted a few significant correlations between individual plant species and selected FAs. We observed that meadow fescue was positively correlated with oleic acid. This

finding is in accordance with what was reported by Arvidsson et al. (2013); fescue contained higher concentrations of oleic acid compared to timothy. Timothy was positively correlated with LA, the *n*-6:*n*-3 ratio, and palmitic acid. Timothy was found to have higher LA content and lower ALA content compared to fescue (Dewhurst et al., 2001;Arvidsson et al., 2013), but the positive correlation between timothy and palmitic acid is surprising. The positive correlation between alfalfa and phenolics was also unexpected. Kagan et al. (2015) reported that usually red clover is higher in soluble phenolic compounds than alfalfa. Most of the significant correlations were observed between individual FAs. As expected, ALA was negatively correlated with LA, the *n*-6:*n*-3 ratio, and palmitic acid. Simultaneously, LA and the *n*-6:*n*-3 ratio were positively correlated with palmitic acid. To our surprise, chlorophyll was not significantly correlated to ALA. Dierking et al. (2010) reported that chlorophyll was most closely correlated to ALA. The lack of correlation observed in our study could be due to different plant species found in the complex pasture mixture. These results highlight the importance of understanding the nutritional quality of botanically diverse pastures since individual plant species have varying effects on antioxidants and FAs.

2.6 Conclusions

In this study, we investigated the antioxidant and FA profiles of a total mixed ration diet and a complex Michigan pasture. We also investigated the variations in FA content of the pasture over time and we discussed possible explanations for these variations based on growth cycles, temperature, rainfall, and grazing conditions. Additionally, we correlated the pasture composition to weather, antioxidants, and FAs. To our knowledge, no other studies reported correlations between individual plant species found in diverse pastures and specific FAs. We observed that the pasture displayed a higher antioxidant content and a more beneficial FA profile compared to the grain diet. Furthermore, we identified trends in the FA profile of the pasture that will provide

farmers, ranchers, and scientists with valuable data for grassland management and further modeling of pasture areas. With climatic events being more severe and more frequent, region- and time-specific data on the nutritional quality of forages is needed. In addition, the demand for grass-fed ruminant products keeps increasing. Therefore, a better understanding of the variations in nutritional quality of feedstuffs is critical to better understand how feeds affect the nutritional value of grass-fed meats. In conclusion, the results provided in the current study will provide guidance to farmers, ranchers, and researchers to better assess variations in feedstuffs and to develop optimal feeding methods for grass-fed ruminant products to favor human health.

CHAPTER 3: FATTY ACID AND MICRONUTRIENT PROFILE OF LONGISSIMUS LUMBORUM FROM RED ANGUS AND RED ANGUS X AKAUSHI CATTLE FINISHED ON GRASS OR GRAIN

This chapter has been published in:

Krusinski, L., Maciel, I.C.F., Sergin, S., Goeden, T., Schweihofer, J.P., Singh, S., Rowntree, J.E., and Fenton, J.I. (2022). Fatty acid and micronutrient profile of longissimus lumborum from Red Angus and Red Angus x Akaushi cattle finished on grass or grain. *Foods* 11, 3451. doi: 10.3390/foods11213451

3.1 Abstract

Cattle diet and breed modify the nutritional profile of beef. The objective of this study was to compare the fatty acid (FA) and micronutrient profiles of Red Angus (RA) and RA x Akaushi (AK) crossbreed steers fed either a grass or grain diet. This two-year study randomly assigned steers to the diets using a 2x2 factorial experiment. FAs and micronutrients were analyzed. Diet effect was the strongest with grass-finished beef being higher in *n*-3 polyunsaturated FAs ($p < 0.001$), conjugated linoleic acid ($p < 0.05$), vaccenic acid ($p < 0.05$), iron ($p < 0.001$), and vitamin E ($p < 0.001$) compared to grain-finished beef. Breed effects were observed for lauric and myristic acids ($p < 0.05$), selenium ($p < 0.05$), and zinc ($p < 0.01$) with AK containing more of these compounds than RA. Diet x breed effects were non-existent. These results indicate that diet has a stronger influence than breed on modifying the nutritional profile of beef. Because of a more favorable FA and antioxidant profile, consumption of grass-finished beef could benefit human health.

3.2 Introduction

Meat is an important part of the diet of many cultures and is consumed for its taste and nutritional properties (Pighin et al., 2016;Melendrez-Ruiz et al., 2019). Beef consumption increases with population and per capita income (Godfray et al., 2018). World meat production was estimated at 328 Mt in 2020, and is expected to reach 374 Mt in 2030 (OECD/FAO, 2021).

Beef is highly nutrient-dense and provides significant amounts of protein, fat, zinc, iron, selenium, and B vitamins (Omaye and Omaye, 2019). Beef is also an important source of conjugated linoleic acid (CLA), and depending on the production system can provide beneficial omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs) mainly as α-linolenic acid (ALA) which is a precursor for eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) (Scollan et al., 2014). Cattle diet and breed are two factors that modify the nutritional profile of beef (Pighin et al., 2016). Feeding cattle a diet of either grass or grain or selecting for different breeds can alter the fatty acid (FA) and micronutrient content of beef (Garcia et al., 2008;Scollan et al., 2014).

Cattle diet significantly impacts the nutritional profile of beef, leading to variations in the FA profile (Berthelot and Gruffat, 2018;Lenighan et al., 2019;Prache et al., 2020). The nutrient profile of grass-finished beef (GFB), compared to grain-finished beef, is generally more consistent with health recommendations, especially regarding *n*-3 PUFAs and phytochemicals (Vannice and Rasmussen, 2014;Omaye and Omaye, 2019;van Vliet et al., 2021b). GFB is usually higher in CLA, *n*-3 PUFAs, vitamin E and β-carotene, and has a more favorable *n*-6:*n*-3 ratio compared to conventional beef (Duckett et al., 2009a;Pighin et al., 2016;Bronkema et al., 2019;Logan et al., 2020). On the other hand, grain-finished beef contains more *cis*-monounsaturated FAs (MUFAs), especially oleic acid (C18:1 *c*9) (Scollan et al., 2006;Klopatek et al., 2022). The nutritional profile of GFB may vary with different plant species, supplemental feeds, regions, and season of slaughter (Duckett et al., 2013;Bronkema et al., 2019;Krusinski et al., 2022d).

Crossbreeding is a common practice used to produce calves for fattening and finishing while benefiting from hybrid vigor (Gregory and Cundiff, 1980). Breeds impact the FA profile of meat, proving that genetic selection can improve the nutritional quality of beef (Malau-Aduli et

al., 2000;Garcia et al., 2008). For instance, Japanese Black Wagyu contains more MUFAs than other breeds including Holstein, Japanese Brown, Charolais, and Angus (Sturdivant et al., 1992;Zembayashi et al., 1995). When Wagyu steers were crossed with Angus, crossbred steers produced meat higher in MUFAs than Angus when fed a diet high in roughage (May et al., 1993). Chung et al. (2006) hypothesized that Angus steers could equal the MUFA concentrations of Wagyu steers if fed a corn-based diet to a typical U.S. endpoint slaughter weight. Their results showed that beef from Wagyu consistently contained more MUFAs and oleic acid than Angus. Akaushi is one of four Japanese beef breeds that can be called Wagyu (Motoyama et al., 2016). Akaushi is known for its high marbling capabilities and high MUFA content (Sturdivant et al., 1992;Lunt et al., 1993;Zembayashi et al., 1995). Angus is the most common breed in the U.S. and is known for its high feeding efficiency (Lunt et al., 1993;Liu et al., 2021).

Numerous studies investigated the effects of finishing systems on the FA profile of beef from identical or different cattle breeds (Malau-Aduli et al., 1997;Warren et al., 2008a;Warren et al., 2008b;Costa et al., 2012b;Horcada et al., 2016;Horcada et al., 2020). Most of these studies reported that while diet influences the FA profile of beef, there were strong genetic effects. However, Warren et al. (2008b) reported that diet had the biggest effect on meat quality.

In the present study, we compared the FA and micronutrient profiles of Red Angus steers (moderate fat breed) and Red Angus x Akaushi crossbreed steers (high fat breed) fed either a complex pasture mixture or a standard grain-based diet. With the increasing demand for healthier beef, there is an urgent need to understand the effects of different diets, breeds, and their interaction on the nutritional profile of beef.

3.3 Materials and methods

The Michigan State University Institutional Animal Care and Use Committee approved the research protocols for the use of animals and procedures (IACUC #201800155).

3.3.1 Experimental design

The experimental design for this study was reported by Maciel et al. (2021). Briefly, this study was conducted over a period of two years (2019 and 2020) at the Michigan State University Upper Peninsula Research and Extension Center (UPREC) located in Chatham, MI (latitude: 46°20'N, longitude: 86°55'W; elevation: 271 m). Sixty steers (n = 60) and 44 steers (n = 44) (14-20 months old) were randomly allocated using a 2x2 factorial experiment in 2019 and 2020, respectively. Two beef breeds were used in this experiment: Red Angus (RA) and Red Angus x Akaushi crossbred (AK). Animals were randomly assigned to one of two finishing systems: a complex pasture mixture (GRASS) or a total mixed feedlot ration (GRAIN). The goal was to have three groups for each finishing system (five animals of each breed in each group). Animals were stratified randomly and assigned to one of the groups for each breed in each finishing system. This design was followed in 2019. As clarified by Maciel et al. (2021), less steers were available in 2020 for the GRAIN group due to a low number of male births. Thus, 15 RA and 15 AK were assigned to GRASS, and seven RA and seven AK were assigned to GRAIN for the second year of the study. For GRAIN, two groups were made: one with four animals of each breed, and a second group with three animals of each breed. This was accounted for in the statistical model as described in the Statistical Analysis section. The nutritive value of the experimental diets is displayed in **Table 6** and the botanical composition of the diets is shown in **Figure 5**. For full details about the composition of the diets, see Maciel et al. (2021). An extensive study on the FA and antioxidant profile of the diets used was previously published (Krusinski et al., 2022a).

Table 6. Nutritive value of experimental diets (% of total fatty acids)[1]

	2019		2020	
	Grass	**Grain**	**Grass**	**Grain**
C16:0	13.73	14.55	14.48	12.74
C18:0	1.86	1.89	1.61	1.43
C18:1 *n*-7	0.23	0.53	0.78	0.49
C18:1 *n*-9	4.10	22.45	2.36	21.99
C18:2 *n*-6 (LA)[2]	13.25	53.97	14.82	59.33
C18:3 *n*-3 (ALA)[3]	62.35	5.24	60.93	2.87
∑ SFA[4]	18.92	17.56	19.74	15.08
∑ MUFA[5]	5.41	23.22	4.52	22.72
∑ PUFA[6]	75.66	59.22	75.75	62.20
∑ *n*-6[7]	13.25	53.98	14.82	59.33
∑ *n*-3[8]	62.35	5.24	60.93	2.87
n-6:*n*-3 ratio[9]	0.22	10.77	0.25	21.63
Dry matter (DM) (%)	20.54	76.11	22.11	85.32
Ash[*]	7.13	4.59	6.14	3.17
Crude protein[*]	11.54	9.83	15.03	9.38
Neutral detergent fiber (NDF)[*]	52.21	21.22	51.49	20.71
Acid detergent fiber (ADF)[*]	34.99	10.24	31.98	9.87
Energy (cal/g)	4407.44	4223.98	4516.28	4328.79

[1]Reported according to Krusinski et al. (2022a); values expressed as means; [2]LA: linoleic acid; [3]ALA: α-linolenic acid; [4]∑ SFA = all saturated FAs 10:0 through 24:0 (even and odd); [5]∑ MUFA = all monounsaturated FAs 16:1-18:1; [6]∑ PUFA = LA + ALA; [7]∑ *n*-6 = LA; [8]∑ *n*-3 = ALA; [9]*n*-6:*n*-3 ratio = ∑ *n*-6/∑ *n*-3; [*]expressed as % dry matter (DM).

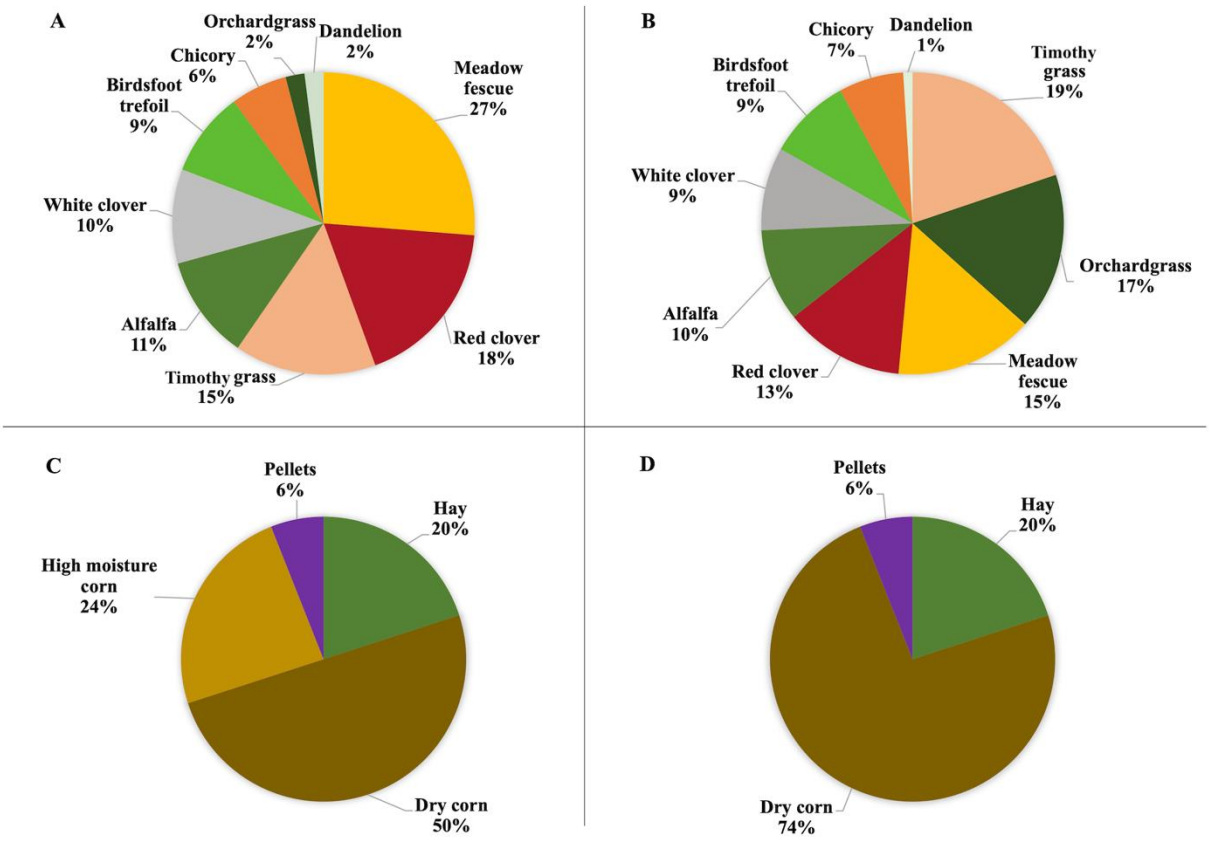

Figure 5. Botanical composition of the diets for 2019 and 2020. (A) 2019 GRASS, (B) 2020 GRASS, (C) 2019 GRAIN, (D) 2020 GRAIN. GRASS: complex pasture mixture; GRAIN: conventional grain diet (pellets contained 36% crude protein, hay was made of orchard grass).

3.3.2 Sample collection

All animals for each year were slaughtered on the same day at a commercial slaughter plant under United Stated Department of Agriculture (USDA) supervision. Body performance and carcass traits were reported by Maciel et al. (2021). Meat samples were collected from the longissimus lumborum (between 11th and 13th rib) on the left side of the carcass. Samples were then transported in a cooler on ice to the Michigan State University Meat Laboratory. A steak was cut into 1x1 cm cubes, flash frozen with liquid nitrogen, and stored into Whirl-Pak bags after manually removing all the air. Samples were then stored at -80 °C until further analysis.

3.3.3 Fatty acids analysis

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

FAs were analyzed following the protocol by Sergin et al. (2021). A microwave-assisted extraction method was used as described by Bronkema et al. (2019). FAs from minced meat samples were extracted using the CEM Mars 6 microwave digestion system equipped with a 24-vessel rotor and a GlassChem vessel set (CEM Corp., Matthews, NC, USA). Briefly, 400 mg of meat was added to a glass vessel with 8 mL of a 4:1 (*v/v*) ethyl acetate/methanol solution with 0.1% BHT and was microwaved as follows: 55 °C for 15 min (initial ramp of 2 min at 400 W). Once removed from the microwave, samples were filtered using Whatman qualitative filter paper (grade 597) into a test tube containing 3.5 mL of HPLC-grade water. Samples were then centrifuged at 2500 RPM for 6 min in order to separate the organic and aqueous layers. The top organic layer was transferred into a new test tube and dried under nitrogen gas. The oil was resuspended in a 4:1 (*v/v*) dichloromethane/methanol solution with 0.1% BHT. The concentration of each sample was 20 mg of oil/mL.

The method published by Jenkins (2010) was adapted for the creation of FA methyl esters (FAMEs). For each sample, 2 mg of suspended oil (100 µL) was aliquoted, dried under nitrogen, and resuspended in toluene with 20 µg of an internal standard (methyl 12-tridecenoate, U-35M, Nu-Chek Prep, Elysian, MN, USA). Subsequently, 2 mL of 0.5 N anhydrous potassium methoxide was added to the samples. Samples were then heated at 50 °C for 10 min. Samples were allowed to cool down to room temperature, and 3 mL of 5% methanolic HCl was added. Samples were heated at 80 °C for 10 min. Once the samples were at room temperature, 2 mL of water and 2 mL of hexane were added. Samples were centrifuged at 2500 RPM for 5 min, and the upper organic layer was removed and dried under nitrogen to obtain FAMEs. The resulting FAMEs were then

resuspended in 1 mL of isooctane to obtain a concentration of 2 mg/mL. Samples were transferred to gas chromatography-mass spectrometry (GC-MS) vials with glass inserts. Samples were stored at -20 °C until further analysis.

For the quantification of FAMEs, the PerkinElmer (Waltham, MA, USA) 680/600S GC-MS in the electron impact mode (70 eV) equipped with an Agilent Technologies (Santa Clara, CA, USA) HP-88 column (100 m, 0.25 mm ID, 0.2 μM film thickness) was used. The injection temperature was set at 250 °C, and 1 μL of sample was injected twice (20:1 split) using two different GC parameters (175 °C and 150 °C). The temperature settings were as follows: initial temperature at 80 °C for 4 min; ramp 13 °C/min to 175 °C; hold 27 min; ramp 4 °C/min to 215 °C; hold 35 min, and then an initial temperature at 80 °C for 4 min; ramp 13 °C/min to 150 °C; hold 47 min; ramp 4 °C/min to 215 °C; hold 35 min. A third injection for each sample followed in splitless mode (0.75 min splitless hold time, 40 mL/min flow exiting the vent). This method was modified from Kramer et al. (2008) created for improved separation of FA isomers in beef products. Helium was used as the carrier gas at a flow rate of 1 mL/min. The MS data were recorded in full scan mode (mass range of m/z 70-400 amu). The MS transfer line and ion source temperature were set at 180 °C.

The identification of FAMEs was performed using MassLynx V4.1 SCN 714 (Waters Corporation, Milford, MA, USA). FAs were identified by retention time and EI mass fragmentation in comparison to our reference standard created by using the Supelco 37 Component FAME Mix with mead acid, docosatetraenoic acid, *n*-3 DPA, *n*-6 DPA, and palmitelaidic acid purchased from Cayman Chemical (Ann Arbor, MI, USA). The CLA reference standard UC-59M (Nu-Chek Prep, Elysian, MN, USA) was used to identify CLA isomers. FAs not included in the reference standard were identified according to elution order reported by Kramer et al. (2008) and

confirmed by the EI mass fragmentation. Beef FAs were reported according to Vahmani et al. (2017). Note that C18:1 4*t* and C18:1 5*t* were below the limit of detection, and C18:2 9*c*,15*t*, C18:2 9*c*,12*t*, and C18:2 9*t*,12*c* were not reported as they were not distinctly separated from the C18:2 11*t*,15*c* peak. Eicosatetraenoic acid (C20:4 *n*-3) was not included in our reference standard and could not be reported. Quantification of FAMEs was performed using a standard curve including the reference and internal standards. The internal standard peak area and analyte peak area relative to the standard curve were used to calculate each FAME concentration. FAs were reported in mg/100 g of beef in this manuscript and in percent of total FAs in **Appendix Tables A2 and A3**.

3.3.4 Vitamin E and mineral analysis

Vitamin E and minerals were analyzed by a commercial laboratory (Diagnostic Center for Population and Animal Health, Michigan State University, East Lansing, MI, USA). Vitamin E was measured according to Rettenmaier and Schüep (1992). Briefly, 1 g of beef was mechanically homogenized in 5 mL of water. The solution was frozen to aid in the lysing of cells. After thawing, a measured aliquot was pipetted for extraction. Ethanol was added to precipitate the protein, and hexane was added to extract fat-soluble vitamins. After centrifugation, a measured portion of the hexane layer was removed and evaporated under reduced pressure in a vortexing chamber (10 min, 35 °C, 300 mBar vacuum). The remaining matter was solubilized in a measured portion of chromatographic mobile phase and placed in vials. A six-point calibration curve was made using the following standard: vitamin E solution (Sigma-Aldrich, St. Louis, MO, USA) diluted to working concentrations with ethanol containing BHT followed by serial dilutions (range was 50 μg/mL to 0.2 μg/mL). Samples were analyzed chromatographically using a Waters Acquity system and Water Empower Pro Chromatography Manager software (Water Corporation, Milford, MA, USA). The elution was isocratic using a mobile phase of acetonitrile:methylene chloride:methanol

(70:20:10, *v/v/v*) and a Symmetry C18, 1.7 µm, 2.1x50 mm analytical column (Waters Corporation, Milford, MA, USA). The flow rate was set at 0.5mL/min and the detection was done by UV absorption at 292 nm.

Minerals were analyzed according to Wahlen et al. (2005). Beef tissues were dried and digested overnight in a 95 °C oven, using 10x the dry tissue mass of nitric acid. Digested samples were diluted with water to 100x the dried tissue mass. Elemental analysis used an Agilent 7900 Inductivity Coupled Plasma–Mass Spectrometer (ICP-MS) (Agilent Technologies Inc., Santa Clara, CA, USA). An aliquot of each sample and calibration standard were diluted 25-fold with a solution containing 0.5% EDTA and Triton X-100, 1% ammonium hydroxide, 2% butanol, 5 ppb of scandium, and 7.5 ppb of germanium, rhodium, indium, and bismuth as internal standards (Inorganic Ventures, Christiansburg, VA, USA). Concentrations were calibrated using a six-point linear curve of the analyte-internal standard response ratio. Bovine liver and mussel standards (National Institute of Standards and Technology, Gaithersburg, MD, USA) were used as controls. Additionally, a second source calibration check standard was used (Alfa Aesar, Tewksbury, MA, USA).

3.3.5 Statistical analysis

The statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Two-way analysis of variance (ANOVA) was performed. The fixed effects in the statistical model were diet, breed, and the two-way interaction between diet and breed. Random effects included year and pen nested within year, diet, and breed (please see mathematical model below). The experimental unit for this model was each pen. The interaction term was considered first. If not significant, individual effects of diet and breed were considered. Post-hoc analysis was performed using the least squares means method. No transformation was needed. Equal variance

assumption was satisfied (checked with Levene's test) which assumed the homogeneity of variance. Results were considered significant at $p < 0.05$. Data are shown as mean ± standard error of mean (SEM).

To correct for the lower number of animals assigned to the GRAIN diet in 2020, some adjustments were made to the statistical model. Year was treated as random effect (and analyzed together instead of each year separately) and each pen was considered the experimental unit leading to combined replicates.

$$Y_{ijkl} = \mu + Y_i + P_j(Y_i\ D_k\ B_l) + D_k + B_l + (D_k*B_l) + \varepsilon_{ijkl}$$

Where:

Y = response variable

μ = mean

Y_i = year

$P_j(Y_i\ D_k\ B_l)$ = pen nested within year, diet, and breed

D_k = diet

B_l = breed

(D_k*B_l) = interaction between diet and breed

ε_{ijkl} = error term

3.4 Results

3.4.1 Saturated, branched-chain, and monounsaturated fatty acid content of beef

The saturated FA (SFA), branched-chain FA (BCFA), and MUFA content of beef are listed in **Table 7**. No significant differences by diet, breed, or diet x breed interaction were noted regarding total FA content ($p > 0.05$). Total SFA content did not significantly differ based on diet, breed, or diet x breed. Regarding individual SFAs, C12:0 and C14:0 were significantly higher in

beef from AK compared to RA ($p < 0.05$). C16:0 and C18:0 did not show significant differences by diet, breed, or diet x breed interaction ($p > 0.05$). The only diet effects observed were for C15:0 and C19:0; they were significantly higher in GRASS compared to GRAIN ($p < 0.05$).

Regarding total BCFAs, no significant diet, breed, or diet x breed effects were observed ($p > 0.05$). C14:0 *iso*, C15:0 *iso*, C15:0 *anteiso*, and C17:0 *iso* were all significantly higher in beef from GRASS compared to GRAIN ($p < 0.05$).

There were no significant differences observed for the total MUFA content of beef ($p > 0.05$). Total *cis*-MUFAs showed no significant effects ($p > 0.05$). When assessing individual *cis*-MUFAs, C16:1 9*c* was significantly higher in beef from GRAIN compared to GRASS ($p < 0.01$). C16:1 11*c* showed a similar pattern: it was higher in beef from GRAIN compared to GRASS ($p < 0.01$). C18:1 14*c* and C18:1 15*c* were significantly higher in beef from GRASS ($p < 0.05$), while 20:1 11*c* was significantly higher in GRAIN ($p < 0.05$). Total *trans*-MUFAs displayed a significant diet effect; GRASS was higher than GRAIN ($p < 0.05$). C16:1 9*t* was significantly higher in beef from GRASS compared to GRAIN ($p < 0.001$). The same was true for C18:1 11*t* ($p < 0.05$), C18:1 13,14*t* ($p < 0.01$), C18:1 15*t* ($p < 0.05$), and C18:1 16*t* ($p < 0.05$). No significant breed or diet x breed effects were observed ($p > 0.05$).

Table 7. Mean concentrations of saturated and monounsaturated fatty acids in beef by diet, breed, and diet x breed interaction (mg per 100 g meat)[1]

Fatty acid	Diet (D)		Breed (B)		Significance of ANOVA[4]		
	GRASS	GRAIN	RA[2]	AK[3]	D	B	D x B
∑ SFA[5]	892.85 ± 514.91	1118.49 ± 521.82	856.66 ± 518.08	1154.67 ± 518.08	NS	NS	NS
C10:0	1.54 ± 0.27	1.54 ± 0.30	1.40 ± 0.27	1.68 ± 0.27	NS	NS	NS
C12:0	1.23 ± 0.67	1.32 ± 0.68	0.97 ± 0.67	1.58 ± 0.67	NS	*	NS
C13:0	0.13 ± 0.06	0.10 ± 0.06	0.10 ± 0.06	0.12 ± 0.06	NS	NS	NS
C14:0	40.44 ± 25.03	67.50 ± 25.60	41.06 ± 25.29	66.87 ± 25.29	NS	*	NS
C15:0	9.92 ± 5.18	4.15 ± 5.27	6.04 ± 5.22	8.03 ± 5.22	*	NS	NS
C16:0	507.08 ± 309.05	767.23 ± 312.85	540.29 ± 310.79	734.02 ± 310.79	NS	NS	NS
C17:0	26.57 ± 14.31	12.17 ± 14.56	16.88 ± 14.33	21.85 ± 14.33	NS	NS	NS
C18:0	302.69 ± 158.95	263.28 ± 161.50	248.01 ± 160.12	317.97 ± 160.12	NS	NS	NS
C19:0	1.32 ± 0.83	0.50 ± 0.84	0.80 ± 0.83	1.01 ± 0.83	*	NS	NS
C20:0	1.27 ± 0.68	0.77 ± 0.70	0.83 ± 0.69	1.20 ± 0.69	NS	NS	NS
C22:0	0.68 ± 0.14	0.75 ± 0.14	0.68 ± 0.14	0.75 ± 0.14	NS	NS	NS
∑ BCFA[6]	34.37 ± 15.25	18.00 ± 15.60	21.69 ± 15.41	30.68 ± 15.41	NS	NS	NS
C14:0 *iso*	0.76 ± 0.38	0.17 ± 0.39	0.38 ± 0.39	0.56 ± 0.38	*	NS	NS
C15:0 *iso*	3.65 ± 1.94	1.20 ± 1.98	2.06 ± 1.96	2.79 ± 1.96	*	NS	NS
C15:0 *anteiso*	2.60 ± 1.32	0.63 ± 1.35	1.38 ± 1.33	1.86 ± 1.33	*	NS	NS
C16:0 *iso*	2.79 ± 1.88	2.39 ± 1.90	2.07 ± 1.89	3.11 ± 1.89	NS	NS	NS
C17:0 *iso*	10.75 ± 3.56	3.84 ± 3.68	6.27 ± 3.61	8.32 ± 3.61	*	NS	NS
C17:0 *anteiso*	11.91 ± 4.88	7.69 ± 5.03	7.95 ± 4.95	11.65 ± 4.95	NS	NS	NS
C18:0 *iso*	1.89 ± 1.31	2.11 ± 1.33	1.60 ± 1.32	2.40 ± 1.32	NS	NS	NS

[1]Values reported as means ± SEM (standard error of mean). [2]RA: Red Angus; [3]AK: Red Angus x Akaushi; [4]NS: not significant; $p > 0.05$; $*p < 0.05$; $**p < 0.01$ $***p < 0.001$.
[5]∑ SFA = all saturated FAs (10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 22:0); [6]∑ BCFA = sum of all branched chain FAs (*iso*14:0, *iso*15:0, *anteiso*15:0, *iso*16:0, *iso*17:0, *anteiso*17:0, *iso*18:0); [7]∑ MUFA = all monounsaturated FAs (14:1, 16:1, 17:1, 18:1, 20:1); [8]∑ *c*MUFA = 14:1, 17:1, sum of *c*16:1, *c*18:1, and *c*20:1; [9]∑ *t*MUFA = sum of *t*16:1 and *t*18:1; [10]∑ FA = sum of all FAs.

Table 7. (cont'd)

Fatty acid	Diet (D)		Breed (B)		Significance of ANOVA		
	GRASS	GRAIN	RA	AK	D	B	D x B
∑ MUFA[7]	893.35 ± 459.75	1109.48 ± 468.21	833.75 ± 463.63	1169.07 ± 463.63	NS	NS	NS
∑ *c*MUFA[8]	819.76 ± 434.62	1093.68 ± 442.53	792.82 ± 438.25	1160.62 ± 438.25	NS	NS	NS
C14:1 9*c*	7.85 ± 7.10	20.80 ± 7.24	11.16 ± 7.16	17.49 ± 7.16	**	NS	NS
C16:1 9*c*	75.85 ± 28.21	152.90 ± 29.77	92.42 ± 28.94	136.33 ± 28.94	**	NS	NS
C16:1 10*c*	3.50 ± 0.60	5.81 ± 0.70	3.86 ± 0.65	5.45 ± 0.65	*	NS	NS
C16:1 11*c*	1.68 ± 0.61	4.32 ± 0.66	2.43 ± 0.63	3.57 ± 0.63	**	NS	NS
C17:1 9*c*	11.13 ± 5.57	10.59 ± 5.67	9.42 ± 5.61	12.30 ± 5.61	NS	NS	NS
C18:1 9*c*	682.04 ± 379.00	849.55 ± 385.08	637.07 ± 381.78	849.51 ± 381.78	NS	NS	NS
C18:1 11*c*	22.88 ± 10.87	30.89 ± 11.08	22.50 ± 10.96	31.27 ± 10.96	NS	NS	NS
C18:1 12*c*	3.41 ± 1.34	4.09 ± 1.41	3.31 ± 1.37	4.20 ± 1.37	NS	NS	NS
C18:1 13*c*	3.99 ± 1.42	6.16 ± 1.49	4.00 ± 1.45	6.15 ± 1.45	NS	NS	NS
C18:1 14*c*	0.67 ± 0.08	0.29 ± 0.09	0.44 ± 0.08	0.52 ± 0.08	*	NS	NS
C18:1 15*c*	1.38 ± 0.54	0.67 ± 0.56	0.81 ± 0.55	1.24 ± 0.55	*	NS	NS
C20:1 9*c*	1.86 ± 0.33	2.17 ± 0.38	1.74 ± 0.35	2.29 ± 0.35	NS	NS	NS
C20:1 11*c*	3.51 ± 0.66	6.08 ± 0.77	3.97 ± 0.71	5.61 ± 0.71	*	NS	NS
∑ *t*MUFA[9]	73.59 ± 25.39	17.05 ± 26.44	41.56 ± 25.59	49.08 ± 25.59	*	NS	NS
C16:1 9*t*	3.49 ± 0.18	1.30 ± 0.21	2.30 ± 0.20	2.48 ± 0.20	***	NS	NS
C16:1 10-12*t*	9.01 ± 2.97	4.83 ± 3.08	6.07 ± 3.02	7.78 ± 3.02	NS	NS	NS
C18:1 6-8*t*	1.79 ± 0.40	0.96 ± 0.44	1.25 ± 0.42	1.50 ± 0.42	NS	NS	NS
C18:1 9*t*	2.33 ± 0.55	2.04 ± 0.59	1.96 ± 0.57	2.42 ± 0.57	NS	NS	NS
C18:1 10*t*	2.24 ± 0.65	1.48 ± 0.69	1.68 ± 0.66	2.04 ± 0.66	NS	NS	NS
C18:1 11*t*	34.52 ± 13.35	1.10 ± 13.99	16.43 ± 13.34	19.19 ± 13.34	*	NS	NS
C18:1 12*t*	4.47 ± 2.28	2.09 ± 2.33	2.74 ± 2.30	3.82 ± 2.30	NS	NS	NS
C18:1 13,14*t*	8.57 ± 2.73	1.45 ± 2.87	4.84 ± 2.79	5.19 ± 2.79	**	NS	NS
C18:1 15*t*	2.53 ± 0.46	0.91 ± 0.52	1.72 ± 0.49	1.72 ± 0.49	*	NS	NS
C18:1 16*t*	4.63 ± 1.99	1.14 ± 2.07	2.69 ± 2.02	3.08 ± 2.02	*	NS	NS
∑ FA[10]	1962.72 ± 1033.46	2376.72 ± 1049.38	1840.80 ± 1040.75	2498.64 ± 1040.75	NS	NS	NS

3.4.2 Polyunsaturated fatty acids and biohydrogenation intermediate products

The PUFA, CLA, and atypical dienes (AD) content of beef are displayed in **Table 8**. There were no significant effects on total PUFA content ($p > 0.05$). There was a significant diet effect on total *n*-3 PUFAs, with GRASS containing significantly more *n*-3 PUFAs than GRAIN (34.70 mg per 100 g difference; $p < 0.001$). More specifically, ALA, EPA, DPA ($p < 0.001$), and DHA ($p < 0.05$) were all significantly higher in beef from GRASS compared to GRAIN. The sum of EPA+DHA was equal to 7.49 mg per 100 g of meat for beef from GRASS and 1.96 mg per 100 g of meat for beef from GRAIN (**Figure 6**). The ALA content was 24.63 and 3.14 mg per 100 g of meat for beef from GRASS and GRAIN, respectively. Regarding total *n*-6 PUFAs, there was a significant diet effect with beef from GRAIN containing more *n*-6 PUFAs than GRASS ($p > 0.01$). All individual *n*-6 PUFAs were significantly higher in beef from GRAIN compared to GRASS. The *n*-6:*n*-3 ratio was significantly higher in beef from GRAIN compared to GRASS (8.36 vs. 1.61; $p < 0.001$). Total conjugated linolenic acid (CLnA) (C18:3 9*c*,11*t*,15*t* and C18:3 9*c*,11*t*,15*c*) content was significantly higher in beef from GRASS compared to GRAIN ($p < 0.05$). Total ADs were not significantly different based on diet, breed or diet x breed interaction ($p > 0.05$). C18:2 11*t*,15*t* ($p < 0.05$), C18:2 9*t*,12*t* ($p < 0.05$), and C18:2 11*t*,15*c* ($p < 0.01$) were all higher in beef from GRASS compared to GRAIN. C18:2 9*c*,15*c* was significantly higher in beef from GRAIN compared to GRASS ($p < 0.01$). Total CLA content was significantly higher in beef from GRASS compared to GRAIN ($p < 0.05$). C18:2 11*t*,13*c* ($p < 0.01$) and C18:2 11*t*,13*t* ($p < 0.05$) were significantly higher in beef from GRASS as opposed to GRAIN.

Table 8. Mean concentrations of polyunsaturated fatty acids in beef by diet, breed, and diet x breed interaction (mg per 100 g meat)[1]

Fatty acid	Diet (D)		Breed (B)		Significance of ANOVA[4]		
	GRASS	GRAIN	RA[2]	AK[3]	D	B	D x B
∑ PUFA[5]	113.13 ± 32.84	112.12 ± 33.15	108.30 ± 32.98	116.95 ± 32.98	NS	NS	NS
∑ *n*-3[6]	45.34 ± 12.02	10.64 ± 12.20	27.47 ± 11.94	28.49 ± 11.94	***	NS	NS
C18:3 *n*-3 (ALA)[7]	24.63 ± 7.80	3.14 ± 7.93	13.18 ± 7.82	14.59 ± 7.82	***	NS	NS
C20:3 *n*-3	0.31 ± 0.09	0.07 ± 0.09	0.16 ± 0.09	0.22 ± 0.09	**	NS	NS
C20:5 *n*-3 (EPA)[8]	6.94 ± 1.36	1.61 ± 1.38	4.49 ± 1.35	4.07 ± 1.35	***	NS	NS
C22:5 *n*-3 (DPA)[9]	12.90 ± 2.97	5.34 ± 3.12	9.14 ± 2.93	9.10 ± 2.93	***	NS	NS
C22:6 *n*-3 (DHA)[10]	0.55 ± 0.13	0.35 ± 0.13	0.45 ± 0.13	0.44 ± 0.13	*	NS	NS
∑ *n*-6[11]	66.91 ± 20.44	101.36 ± 20.69	80.31 ± 20.55	87.96 ± 20.55	**	NS	NS
C18:2 *n*-6 (LA)[12]	48.07 ± 16.84	69.08 ± 17.06	54.30 ± 16.94	62.86 ± 16.94	*	NS	NS
C18:3 *n*-6	0.29 ± 0.02	0.38 ± 0.03	0.33 ± 0.03	0.35 ± 0.03	*	NS	NS
C20:2 *n*-6	0.38 ± 0.08	0.58 ± 0.09	0.45 ± 0.08	0.52 ± 0.08	*	NS	NS
C20:3 *n*-6	3.20 ± 1.09	5.63 ± 1.11	4.34 ± 1.10	4.49 ± 1.10	***	NS	NS
C20:4 *n*-6	13.44 ± 2.88	21.02 ± 2.91	17.68 ± 2.89	16.79 ± 2.89	***	NS	NS
C22:4 *n*-6	1.53 ± 0.51	4.68 ± 0.54	3.23 ± 0.50	2.98 ± 0.50	***	NS	NS
n-6:*n*-3 ratio[13]	1.61 ± 0.39	8.36 ± 0.41	5.04 ± 0.39	4.92 ± 0.39	***	NS	NS
C20:3 *n*-9	0.88 ± 0.17	1.10 ± 0.17	0.98 ± 0.17	0.98 ± 0.17	NS	NS	NS

[1]Values reported as means ± SEM (standard error of mean). [2]RA: Red Angus; [3]AK: Red Angus x Akaushi; [4]NS: not significant; $p > 0.05$; $*p < 0.05$; $**p < 0.01$ $***p < 0.001$.
[5]∑ PUFA = LA + ALA + GLA + Eicosadienoic + Eicosatrienoic + DGLA + Mead + Arachidonic + EPA + DTA + DPA *n*-3 + DHA; [6]∑ *n*-3 = ALA + EPA + DHA + DPA *n*-3 + Eicosatrienoic; [7]ALA: α-linolenic acid, [8]EPA: eicosapentaenoic acid: [9]DPA: *n*-3 docosapentaenoic acid, [10]DHA: docosahexaenoic acid; [11]∑ *n*-6 = LA + GLA + Eicosadienoic + DGLA + Arachidonic + DTA; [12]LA: linoleic acid; [13]*n*-6:*n*-3 ratio = ∑ *n*-6/∑ *n*-3; [14]∑ CLnA = sum of conjugated linolenic acid isomers (*c*9, *t*11, *t*15 18:3 + *c*9, *t*11, *c*15 18:3); [15]∑ Atypical Dienes (AD) = sum of non-conjugated linoleic acid isomers (*t*11, *t*15 18:2 + *t*9, *t*12 18:2 + *c*9, *t*14/c9, *t*13 18:2 + *t*11, *c*15 18:2 + *c*9, *t*16 18:2 + *c*9, *c*15 18:2 + *c*12, *c*15 18:2); [16]∑ CLA = sum of conjugated linoleic acid isomers (*c*9, *t*11/*t*7, *c*9 18:2 + *t*11, *c*13 18:2 + *t*11, *t*13 18:2 + *t,t* 18:2).

Table 8. (cont'd)

Fatty acid	Diet (D)		Breed (B)		Significance of ANOVA		
	GRASS	GRAIN	RA	AK	D	B	D x B
∑ CLnA[14]	0.38 ± 0.15	0.26 ± 0.15	0.29 ± 0.15	0.34 ± 0.15	*	NS	NS
C18:3 9*c*,11*t*,15*t*	0.12 ± 0.02	0.03 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	*	NS	NS
C18:3 9*c*,11*t*,15*c*	0.26 ± 0.14	0.23 ± 0.14	0.23 ± 0.14	0.27 ± 0.14	NS	NS	NS
∑ AD[15]	19.86 ± 9.06	11.70 ± 9.22	13.60 ± 9.14	17.95 ± 9.14	NS	NS	NS
C18:2 11*t*,15*t*	5.01 ± 2.01	2.11 ± 2.07	3.02 ± 2.04	4.10 ± 2.04	*	NS	NS
C18:2 9*t*,12*t*	0.85 ± 0.14	0.17 ± 0.16	0.50 ± 0.14	0.52 ± 0.14	*	NS	NS
C18:2 9*c*,14*t*/9*c*,13*t*	2.72 ± 1.05	1.76 ± 1.08	1.89 ± 1.06	2.59 ± 1.06	NS	NS	NS
C18:2 11*t*,15*c*	5.39 ± 1.96	0.40 ± 2.04	2.58 ± 1.97	3.21 ± 1.97	**	NS	NS
C18:2 9*c*,16*t*	4.28 ± 3.19	4.37 ± 3.20	3.71 ± 3.19	4.94 ± 3.19	NS	NS	NS
C18:2 9*c*,15*c*	1.18 ± 0.53	2.80 ± 0.55	1.70 ± 0.54	2.28 ± 0.54	**	NS	NS
C18:2 12*c*,15*c*	0.42 ± 0.30	0.25 ± 0.30	0.28 ± 0.30	0.39 ± 0.30	NS	NS	NS
∑ CLA[16]	5.70 ± 0.72	2.70 ± 0.84	3.78 ± 0.78	4.62 ± 0.78	*	NS	NS
C18:2 9*c*,11*t*/9*c*,7*t*	4.54 ± 0.67	2.18 ± 0.78	2.97 ± 0.70	3.75 ± 0.70	NS	NS	NS
C18:2 11*t*,13*c*	0.47 ± 0.07	0.18 ± 0.08	0.31 ± 0.07	0.34 ± 0.07	**	NS	NS
C18:2 11*t*,13*t*	0.36 ± 0.14	0.20 ± 0.14	0.28 ± 0.14	0.28 ± 0.14	*	NS	NS
C18:2 *t*,*t*	0.33 ± 0.11	0.25 ± 0.11	0.28 ± 0.11	0.30 ± 0.11	NS	NS	NS

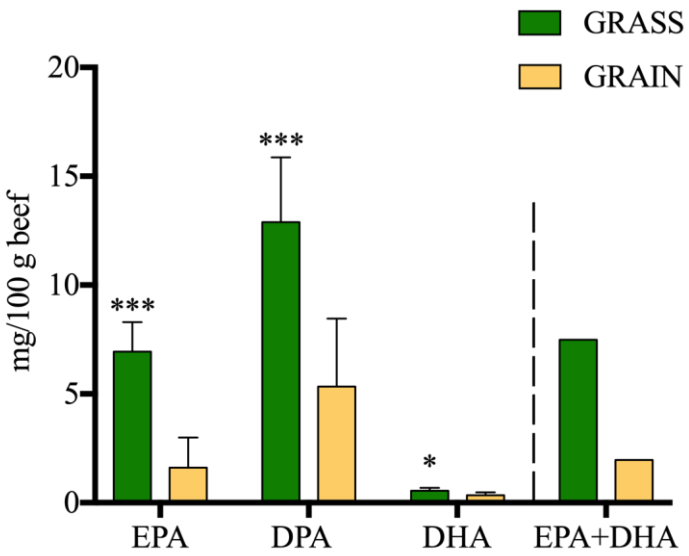

Figure 6. Long-chain *n*-3 polyunsaturated fatty acid content of beef (mg/100 g beef) by diet. EPA: eicosapentaenoic acid; DPA: *n*-3 docosapentaenoic acid; DHA: docosahexaenoic acid; EPA+DHA: sum of eicosapentaenoic acid and docosahexaenoic acid. '*' denotes statistical significance ('*' $p < 0.05$, '***' $p < 0.001$).

3.4.3 Vitamin E and mineral content of beef

The vitamin E and mineral content of beef is displayed in **Table 9**. No diet x breed effect was noted for any of the micronutrients. Copper ($p < 0.01$), iron ($p < 0.001$), and molybdenum ($p < 0.001$) were all significantly more abundant in beef from GRASS compared to GRAIN. Manganese was higher in beef from GRAIN vs. GRASS ($p < 0.05$). Selenium showed a breed effect and was present in higher quantity in AK compared to RA ($p < 0.05$). Diet and breed effects were observed for zinc; beef from GRASS contained more zinc than GRAIN ($p < 0.05$), and AK contained more zinc than RA ($p < 0.01$). Finally, vitamin E was significantly higher in beef from GRASS compared to GRAIN ($p < 0.001$).

Table 9. Diet, breed, and interaction effects on micronutrient content of beef (mg per 100 g of beef)[1]

Micronutrient	Diet (D)		Breed (B)		Significance of ANOVA[4]		
	GRASS	GRAIN	RA[2]	AK[3]	D	B	D x B
Copper	0.17 ± 0.01	0.08 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	**	NS	NS
Iron	6.76 ± 0.10	4.85 ± 0.12	5.90 ± 0.15	5.71 ± 0.15	***	NS	NS
Manganese	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	*	NS	NS
Molybdenum	0.02 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	***	NS	NS
Selenium	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	NS	*	NS
Zinc	13.61 ± 0.36	11.83 ± 0.32	12.20 ± 0.26	13.47 ± 0.26	*	**	NS
Vitamin E	3.42 ± 0.13	1.69 ± 0.06	2.62 ± 0.18	2.50 ± 0.18	***	NS	NS

[1]Values reported as means ± SEM (standard error of mean). [2]RA: Red Angus; [3]AK: Red Angus x Akaushi; [4]NS: not significant; $p > 0.05$; $*p < 0.05$; $**p < 0.01$ $***p < 0.001$.

3.5 Discussion

3.5.1 Saturated fatty acids

The U.S. Dietary Guidelines for Americans 2020-2025 recommend limiting saturated fat consumption to 10% of daily caloric intake (U.S. Department of Agriculture and U.S. Department of Health and Human Services, 2020). This recommendation contributed to the perception that red meat consumption should be limited due to its high SFA content (Casperson et al., 2020). SFAs promote inflammation and increase low-density lipoprotein (LDL) cholesterol, which is linked to coronary heart diseases. However, this varies by specific SFAs (Billingsley et al., 2018). In the present study, no significant differences in total SFA content were observed between diets and breeds. These findings are in agreement with previous studies reporting no difference in SFA content between grass-fed and grain-fed beef (Alfaia et al., 2009;Duckett et al., 2013). Myristic and palmitic acids have the strongest LDL cholesterol-raising effects compared to other SFAs (FAO, 2010;Calder, 2015). In the present study, myristic acid was higher in AK compared to RA. May et al. (1993) found no significant difference in myristic acid content between Angus and

Wagyu beef but the authors reported a higher level of palmitic acid in Angus beef. Stearic acid (C18:0) has a neutral effect on LDL cholesterol (FAO, 2010). In the present study, no significant differences were observed. Previous studies reported that grass-finishing increased the amount of stearic acid in beef (Realini et al., 2004;Garcia et al., 2008;Duckett et al., 2009a). May et al. (1993) and Chung et al. (2006) also reported that RA contained more stearic acid than AK. Malau-Aduli et al. (1997) reported significant breed differences in the degree of FA saturation when comparing beef from Jersey and Limousin cows. It was surprising to find no significant differences in LDL-neutral stearic acid between diets and breeds. This may be due to variations in the diets and breeds, but it may also be due to studies reporting FAs in different units. In the present study, when FAs were reported in percent of total FAs, significant differences were seen in stearic acid levels which aligned with the findings of other studies cited above.

3.5.2 Branched-chain fatty acids

BCFAs are mainly SFAs with at least one branching point on their carbon chain that play important roles in the gut health of adults and newborns and are mainly found in ruminant products such as dairy and beef (Ran-Ressler et al., 2014). Additionally, BCFAs might display anti-inflammatory and anti-carcinogenic properties (Taormina et al., 2020). In the present study, total BCFAs was not significantly different based on diet or breed, but individual BCFAs were higher in beef from GRASS. Information about BCFA content in beef is sparse in the literature, but Picklo et al. (2022) reported higher levels of BCFAs in beef from cattle fed a high-forage diet as compared to a low-forage diet. Klopatek et al. (2022) also stated that a 100% forage diet would result in higher amounts of BCFAs in beef compared to a standard grain-based diet. The amount of BCFAs in beef is generally inversely related to the forage-to-concentrate ratio (Melton et al., 1982).

3.5.3 Monounsaturated fatty acids

MUFAs are known for their health benefits and their ability to mitigate noncommunicable illnesses such as cardiovascular diseases (Billingsley et al., 2018). Japanese breeds of cattle are known for their marbling and their high MUFA content, so higher levels of MUFAs in beef from AK compared to RA were expected (Sturdivant et al., 1992;May et al., 1993;Zembayashi et al., 1995;Chung et al., 2006). Maciel et al. (2021) also reported higher marbling scores in AK compared to RA. Surprisingly, no significant differences based on diet or breeds were observed in the present study. Oka et al. (2002) found that final body weight is negatively correlated with MUFA content, and Maciel et al. (2021) found no significant difference in final weight between RA and AK in the same steers used in the present study. This may explain why no differences were seen in total MUFA content between breeds. However, when reporting MUFA content as percent of total FAs, the difference between breeds was significant with beef from AK containing more than RA. MUFAs, in particular oleic acid, contribute to the palatability of beef because of fat softness and their lower melting point (Chung et al., 2006;Smith et al., 2006). We did not observe differences in oleic acid content between the two breeds, which explains the similarities in sensory attributes reported by Maciel et al. (2021). Although AK had greater marbling scores, it did not affect the beef texture and/or flavor as expected (Maciel et al., 2021). Smith et al. (2006) and May et al. (1993) reported higher amounts of oleic acid in Wagyu compared to Angus, while Choi et al. (2008) reported no significant differences in oleic acid between Angus and Wagyu crossbreeds.

Grain-finished beef is expected to be higher in total MUFAs as reported in previous studies (Leheska et al., 2008;Duckett et al., 2009a;Klopatek et al., 2022). We did not observe differences between diets for total MUFAs, but some individual *cis*-MUFAs (C16:1) were higher in beef from

GRAIN compared to GRASS. Regarding benefits for human health, *cis*-MUFAs (especially oleic acid) are of interest because of their LDL cholesterol-lowering potential (Calder, 2015). In this study, we did not find significant differences in oleic acid content between diets and breeds, although numerous studies found that oleic acid is usually higher in grain-fed beef compared to GFB (Nuernberg et al., 2005;Leheska et al., 2008;Duckett et al., 2009a;Klopatek et al., 2022).

In this study, grass-finishing increased the amount of *trans*-MUFAs in beef. Klopatek et al. (2022) reported higher levels of *trans*-MUFAs in grain-finished beef compared to GFB, while Nuernberg et al. (2005) observed higher concentrations of total *trans*-C18:1 in GFB compared to cattle fed a concentrate diet. The effects of ruminal *trans*-unsaturated FAs on human health remain uncertain. While the association of ruminal *trans*-FAs with coronary heart diseases remains unclear (Kalač and Samková, 2010), other studies reported potential negative health effects (Gebauer et al., 2015;Verneque et al., 2020). However, the health effects of *trans*-MUFAs are isomer specific (Mapiye et al., 2015;Vahmani et al., 2015). For example, vaccenic acid (C18:1 *t*11) reduces plasma triglycerides and improves immune functions while C18:1 *t*9 and *t*10 have been associated with negative health effects (Mapiye et al., 2015;Chikwanha et al., 2018). Mapiye et al. (2015) reported that finishing cattle on grass can increase vaccenic acid concentrations relative to C18:1 *t*10. In the present study, vaccenic acid was significantly higher in beef from GRASS compared to GRAIN. Our results indicate that while grass-finishing increased the total *trans*-MUFA content of beef, this difference was mainly due to an increase in beneficial vaccenic acid.

3.5.4 Polyunsaturated fatty acids

Consumer interest in health foods continues to increase, and researchers and producers are investigating ways to improve the nutritional quality of beef to contribute to reducing

noncommunicable diseases in humans (Scollan et al., 2006;Decker and Park, 2010). Scollan et al. (2006) reported that increasing the *n*-3 PUFA and CLA contents of beef while reducing SFAs and the *n*-6:*n*-3 ratio are important priorities. The Western diet is usually high in *n*-6 PUFAs and deficient in beneficial *n*-3 PUFAs (Simopoulos, 2002). Long-chain *n*-3 PUFAs are thought to have anti-inflammatory properties while *n*-6 PUFAs do not (Simopoulos, 2006). GFB contains *n*-3 PUFAs but mainly as ALA which is not efficiently converted into beneficial long-chain *n*-3 PUFAs such as EPA and DHA (Welch et al., 2010;Zhou et al., 2019). Most health benefits are linked to EPA and DHA which are related to healthier cardiovascular function (Parolini, 2019;Mendivil, 2021).

All *n*-6 PUFAs were higher in beef from GRAIN compared to GRASS, similar to the results reported in previous studies (Garcia et al., 2008;Warren et al., 2008a;Klopatek et al., 2022), and no breed effect was observed. Chung et al. (2006) reported that LA (C18:2 *n*-6) content varied based on breed and diet when comparing Angus and Wagyu fed either corn or hay. Angus was higher in LA compared to Wagyu when fed corn but was lower in LA when fed hay. GFB is known to be higher in *n*-3 PUFAs compared to conventional grain-finished beef (Garcia et al., 2008;Davis et al., 2022;Klopatek et al., 2022;Krusinski et al., 2022d;Nogoy et al., 2022). The European Union considers a "source of omega-3 fatty acids" a food that contains at least 0.3 g of ALA per 100 g serving or at least 40 mg of EPA+DHA per 100 g serving, and a "good source of omega-3 fatty acids" a food that contains at least 0.6 g of ALA per 100 g serving or at least 80 mg of EPA+DHA per 100 g serving (Commission Regulation of European Union, 2010). Based on European standards, our GFB would not qualify as a "source of omega-3 fatty acids." Even though DPA is not investigated as much as EPA and DHA for its health benefits, it has been linked to improved cognitive functions, lower blood triglycerides, lower cholesterol, lower inflammation, and lower

risks of coronary heart diseases (Byelashov et al., 2015). Our results indicate that even if GFB does not qualify as a "source" of long-chain *n*-3 PUFAs, it can still contribute to the daily intake for individuals who do not have access to marine foods (Howe et al., 2006). We did not observe significant differences in *n*-3 PUFAs between breeds in the current study. Liu et al. (2020) found no significant difference in total *n*-3 PUFA content between crossbred Angus x Simmental beef and Wagyu x Simmental beef, while Chung et al. (2006) reported that Wagyu had higher ALA content than Angus when fed to the same endpoint. Warren et al. (2008a) noted subtle differences in PUFA content by breed.

A low *n*-6:*n*-3 ratio is an important factor to prevent noncommunicable diseases (Simopoulos, 2006;Husted and Bouzinova, 2016). An optimal ratio for human health is suggested around 1:1 as found in the meat of wild animals or traditional human diets (Simopoulos, 2002;2006). In this study, the *n*-6:*n*-3 ratio only differed based on diet. Beef from GRASS had a ratio of 1.61:1 while beef from GRAIN had a ratio of 8.36:1. Grass-finishing was expected to raise the *n*-3 PUFA content of beef, consequently lowering the *n*-6:*n*-3 ratio (Alfaia et al., 2009;Daley et al., 2010;Davis et al., 2022;Klopatek et al., 2022;Krusinski et al., 2022d).

3.5.5 Biohydrogenation intermediate products

PUFAs are toxic to many rumen bacteria, which is why they undergo biohydrogenation in the rumen. LA and ALA undergo extensive biohydrogenation (70-95% and 85-100%, respectively) (Lock et al., 2006). When LA and ALA go through biohydrogenation in the rumen, multiple intermediate compounds are produced including CLnA, CLA, and AD (Jenkins et al., 2008). In the present study, beef fed GRASS contained significantly more CLnA than beef fed GRAIN. This was expected since most of the ALA found in GFB comes from fresh forage (Daley et al., 2010). Regarding AD, Klopatek et al. (2022) reported similar results that the present study

and highlighted that health effects of AD remain unknown. CLA and its precursor *trans*-vaccenic acid (TVA) are thought to have health benefits including regulating insulin resistance and blood pressure and improving lipid metabolism (Field et al., 2009;Menaa et al., 2013;Da Silva et al., 2015). CLA is also purported to have anticarcinogenic effects (Mir et al., 2004). In the present study, total CLA content was higher in beef from GRASS than beef from GRAIN, which was also reported in other studies (Garcia et al., 2008;Leheska et al., 2008). We did not find any significant differences in the concentration of biohydrogenation intermediate products based on breed. This result was surprising since it was previously reported that Wagyu contained more CLA than European and British crossbred cattle (Mir et al., 2000) and that Wagyu contained more CLA per 100 g of meat than Limousin cattle (Mir et al., 2004). The authors attributed these changes mainly to the difference of total fat content.

3.5.6 Vitamin E and minerals

The higher levels of micronutrients in GFB found in the present study are supported by the literature and may be due to GFB being leaner than grain-finished beef (Leheska et al., 2008;Warren et al., 2008b;Duckett et al., 2009a;Horcada et al., 2020). Vitamin E acts as an antioxidant and protects cells against free radicals, but it can also extend the shelf-life of meat (Daley et al., 2010). De la Fuente et al. (2009) reported significantly higher levels of vitamin E in GFB compared to conventional beef, enough to protect from oxidation. Warren et al. (2008b) previously reported that diet had the biggest impact on meat quality and found higher vitamin E levels in GFB compared to beef finished on a concentrate diet. Horcada et al. (2020) showed that GFB had a higher PUFA and antioxidant content compared to grain-finished beef, and that the higher antioxidant levels (mainly as vitamin E) found in GFB resulted in the stability of the FAs. This led the authors to recommend the consumption of GFB to benefit human health. There is a

lack of information about micronutrient content of Angus vs. Akaushi beef in the literature. Li et al. (2014) published a study comparing the mineral content of Quichuan and Wagyu x Quichuan cattle liver. Their results showed that the crossbred Wagyu x Quichuan cattle liver contained significantly more iron, zinc, selenium, and manganese than the Quichuan cattle. These findings indicate that crossbreeding with Wagyu can potentially increase the mineral content of liver and meat.

3.6 Conclusions

Our results indicate that the diet effect is more dominant than the breed in this study, meaning that the cattle diet remains the most efficient way to improve the nutritional profile of beef. This is novel since most studies in the literature report strong genetic effects. Grass-finishing improved bioactive compounds of interest when compared to grain-finishing. These include *n*-3 PUFAs, the *n*-6:*n*-3 ratio, CLnA, CLA, *trans*-vaccenic acid, copper, iron, zinc, and vitamin E (**Figure 7**). To our surprise, we did not observe many significant differences by breed and diet x breed effects. Most breed effects were observed for SFAs; AK was higher in some SFAs such as lauric and myristic acids that do not favor human health (Calder, 2015). Surprisingly, we did not find significant differences in MUFA content based on diet or breed. AK beef was higher in micronutrients such as selenium and zinc. Crossbreeding Akaushi with Red Angus did not significantly improve the nutritional profile of beef.

Our study contains some limitations; we did have a control group that was 100% Red Angus, but we did not have another control group that was 100% Akaushi. Additionally, we did not feed our animals to multiple endpoints (i.e., Japanese vs. U.S.) which may explain why we did not observe any diet x breed effects. The literature does not contain much information on the specific breeds and diets that were used in the present study, making direct comparisons difficult.

However, we believe that our work fills a gap in the literature and adds important information to the current knowledge about grass-finishing (especially on diverse pastures) and improving the nutritional quality of beef for human health. To our knowledge, this is the first paper to report a comprehensive list of FAs (including BCFAs, ADs, CLA and CLnA isomers) to compare two popular breeds and two finishing systems. More research is needed to better understand how to combine breeds and finishing systems to improve the nutrient profile of beef to favor human health.

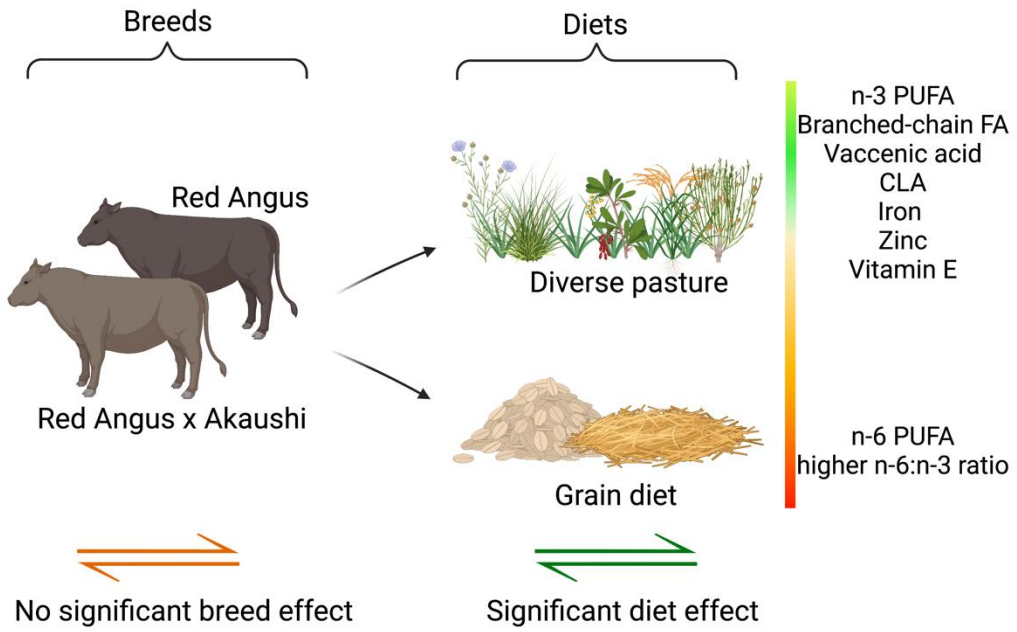

Figure 7. Breed and diet effects on the nutritional profile of beef. The diet effect was more dominant than the breed effect with grass-finished beef containing more omega-3 fatty acids, conjugated linoleic acid, iron, zinc, and vitamin E than grain-finished beef. Grain-finished beef displayed a higher *n*-6:*n*-3 ratio compared to grass-finished beef. *n*-3 PUFA: omega-3 polyunsaturated fatty acids; FA: fatty acids; CLA: conjugated linoleic acid; *n*-6 PUFA: omega-6 polyunsaturated fatty acids.

CHAPTER 4: EFFECTS OF HAY, BALEAGE, AND SOYBEAN HULLS WASTE USED AS SUPPLEMENTAL FEEDS ON THE NUTRITIONAL PROFILE OF GRASS-FINISHED BEEF

This chapter has been published in:

Krusinski, L., Maciel, I.C.F., Sergin, S., Jambunathan, V., Garg, E., Garmyn, A.J., Singh, S., Bitler, C.A., Rowntree, J.E., and Fenton, J.I. (2022). Effects of hay, baleage, and soybean hulls waste used as supplemental feeds on the nutritional profile of grass-finished beef. *Foods* 11, 3856. doi: 10.3390/foods11233856

4.1 Abstract

Grass-finished beef (GFB) has demonstrated wide nutritional variations with some GFB having a considerably higher *n*-6:*n*-3 ratio compared to grain-finished beef. To better understand these variations, the current study investigated the effects of commonly used supplemental feeds on the nutritional profile of GFB. This two-year study involved 117 steers randomly allocated to one of four diets: 1) grass + hay (G-HAY), 2) grass + baleage (G-BLG), 3) grass + soybean hulls (G-SH), and 4) baleage + soybean hulls in feedlot (BLG-SH). Feed samples were analyzed for their nutritional value, and beef samples underwent analysis for fatty acids (FAs), vitamin E, minerals, lipid oxidation, and shear force. FAs were measured by GC-MS, vitamin E was analyzed chromatographically, minerals were analyzed by ICP-MS, and lipid oxidation was measured via a thiobarbituric acid reactive substances (TBARS) assay. G-SH beef had the highest *n*-6:*n*-3 ratio ($p < 0.001$), while BLG-SH beef contained less vitamin E ($p < 0.001$) and higher TBARS values ($p < 0.001$) compared to the other groups. G-HAY beef contained more long-chain *n*-3 polyunsaturated FAs compared to the other groups ($p < 0.001$). In conclusion, G-HAY beef had the most beneficial nutritional profile, while soybean hulls increased the *n*-6:*n*-3 ratio of beef.

4.2 Introduction

The market for grass-finished beef (GFB) is growing with retail sales of pasture-raised beef increasing from $17 million in 2012 to $272 million in 2016 (Cheung et al., 2017;Provenza et al.,

2019). The consumer reasoning for these growing sales is complex. However, sustainable food production is driving food choices for savvy consumers (Stampa et al., 2020;Butler et al., 2021). GFB appeals to consumers who are interested in a healthier product for human consumption and the environmental considerations (Xue et al., 2010). Leading organizations and platforms such as the EAT Lancet commission recommend to drastically reduce red meat consumption for health and environmental reasons (Willett et al., 2019); however, the type of production system is generally ignored in this recommendation (Provenza et al., 2019). GFB remains an underexplored alternative to attain sustainability goals (Davis et al., 2022). For one, GFB is more consistent with health recommendations. Compared to conventional grain-finished beef, GFB contains more omega-3 (*n*-3) fatty acids (FAs) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), twice as much conjugated linoleic acid (CLA), and 25% more polyunsaturated FAs (PUFAs) (Van Elswyk and McNeill, 2014;Berthelot and Gruffat, 2018;Lenighan et al., 2019;Prache et al., 2020;Butler et al., 2021). GFB also contains less omega-6 (*n*-6) PUFAs, less total fat, and less cholesterol-raising saturated FAs (SFAs) (Ponnampalam et al., 2006;Alfaia et al., 2009). GFB has an *n*-6:*n*-3 ratio of 1.5:1 compared to 7.7:1 for conventional grain-finished beef (Daley et al., 2010;Pighin et al., 2016). Foods containing an *n*-6:*n*-3 ratio closer to 1:1 are recommended by human nutrition health professionals (Simopoulos, 2002;2006). Additionally, consumption of foods containing higher concentrations of phytochemicals, typically fruits and vegetables, is also a key recommendation. In fact, GFB has important antioxidant properties that also contribute to its healthfulness (van Vliet et al., 2021b;Krusinski et al., 2022d). GFB contains three times more vitamin E and 1.5-10 times more β-carotene than grain-finished beef (Duckett et al., 2009a;Pighin et al., 2016;Bronkema et al., 2019;Logan et al., 2020). Some studies suggest that grass-finishing enhances the phenolic content of beef, especially when cattle are grazing on

phytochemically biodiverse pastures (Provenza et al., 2019;van Vliet et al., 2021a;Krusinski et al., 2022d). Properly managed complex biodiverse pastures also have the merit to sequester more carbon and to enhance fresh-water systems (Godfray et al., 2018).

The nutritional composition of beef is highly dependent on the feeding system, yet these differences in nutritional quality are generally not reflected on food labels (Provenza et al., 2019;Krusinski et al., 2022d). GFB typically means that cattle were fattened solely on grass and forages before slaughter (Mathews and Johnson, 2013). According to the American Grassfed Association standards, all cattle must be pasture based meaning that grass and forage must be consumed throughout the lifetime of the animal except for milk consumed before weaning. Hay, baleage, and silages may be consumed by the animal when fresh grass and forages are not available due to inclement weather for instance (American Grassfed Association, 2022). Supplemental feeds might be needed in some regions where fresh forages are not available year-round. Season, soil composition, weather, and light exposure all play crucial roles in feed quality and availability (Dewhurst et al., 2001;Khan et al., 2009;Garcia et al., 2016;Krusinski et al., 2022d).

Interestingly, a nutritional survey of commercially available GFB published by our group highlighted important differences among beef from grass-finishing systems (Bronkema et al., 2019). The *n*-6:*n*-3 ratio varied from 1.8:1 to as high as 28.3:1 and some GFB was devoid of β-carotene. These differences were hypothesized to be due to a wide variety of feeding practices that were reflected in the nutritional profile of beef (Bronkema et al., 2019). Feeding fresh forages to cattle usually results in the most beneficial nutritional profile of beef (Bronkema et al., 2019;Krusinski et al., 2022d). However, producers may rely on conserved forages and other supplemental feeds when fresh grass is not available (Gwin, 2009). Unfortunately, conserved forages made by drying (hay) or fermentation (baleage) often display lower nutritional quality

compared to fresh forages with lower concentrations of antioxidants and phenolic compounds (Owens et al., 1997;Butler, 2014). The processing of fresh forages into conserved feeds results in oxidation of PUFAs and an increase in palmitic acid (Van Ranst et al., 2009;Kalač and Samková, 2010). These changes in the nutritional profile of feeds modify FA metabolism in the rumen, resulting in variations in the FA content of beef (Buccioni et al., 2012;Glasser et al., 2013). Although not allowed by the American Grassfed Association (American Grassfed Association, 2022), soybean hulls are also used as supplemental feed by some producers in the U.S. (Bronkema et al., 2019;Krusinski et al., 2022d). The effects of feeding soybean hulls to cattle remain controversial in the literature. Some studies found no differences in CLA, *trans* vaccenic acid (TVA), *n*-3 PUFAs, and the *n*-6:*n*-3 ratio among cattle fed soybean hulls or fresh forage in the finishing phase (Duckett et al., 2009a;Bronkema et al., 2019). On the other hand, another study reported that cattle supplemented with soybean hulls had more total fat, less *n*-3 PUFAs, and a higher *n*-6:*n*-3 ratio compared to cattle fed only fescue (Baublits et al., 2006).

Krusinski et al. (2022d) highlighted that the nutritional profile of GFB is highly variable and depends on a multitude of factors ranging from supplemental feeds to seasonal variations. The present study builds on the work of Bronkema et al. (2019) in an attempt to explain the large variations reported among GFB, especially regarding the *n*-6:*n*-3 ratio. With growing interest in assessing the nutritional impact and sustainability of food systems, determining the accurate nutritional value of foods is crucial. The objective of this study was to compare the FA and micronutrient content of GFB fed a diverse pasture mixture and commonly used supplemental feeds to better understand the effects of different feeds on the nutritional profile of GFB.

4.3 Materials and methods

The animal protocol was reviewed and approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC #202000054).

4.3.1 Experimental design, animals, and diets

This two-year study (2020 and 2021) took place at the Michigan State University Kellogg Biological Station (KBS) located in Hickory Corners, MI (latitude: 42°24'38°N, longitude: 85°22'45"W, elevation: 282 m). Sixty steers for each year were randomly allocated to one of four diets: grass supplemented with hay (G-HAY), grass supplemented with baleage (G-BLG), grass supplemented with soybean hulls (G-SH), or baleage and soybean hulls in feedlot (BLG-SH). Three groups for each diet were formed (n = 5 animals/replicate; 3 replicates/diet; 15 animals/diet) for each year. Animals were randomly stratified and allocated to one of the three groups in each diet.

In April of each year, 60 Simmental-Angus influenced feeder cattle weighing on average 387 kg (± 47 kg) were purchased from the same producer and shipped from Oklahoma to KBS. Upon their arrival at KBS, initial weights were collected, and steers were randomly stratified and assigned to the diets. Steers allocated to the three diets containing grass were kept on pasture and had *ad libitum* access to a diverse pasture mixture (GRASS) and 4.5 kg of supplemental feed (dry matter; DM) per day per head. Steers kept in the feedlot group had *ad libitum* access to baleage (BLG) and 4.5 kg of soybean hulls (SH) per day per head. GRASS was a five-species mix of alfalfa (*Medicago sativa*), red clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.), orchard grass (*Dactylis glomerata* L.), and endophyte-free tall fescue (*Festuca arundinacea*). Dry hay (HAY) was composed of alfalfa (*Medicago sativa*), orchard grass (*Dactylis glomerata* L.), and tall fescue (*Festuca arundinacea*). BLG was a mixture of alfalfa (*Medicago sativa*) and orchard

grass (*Dactylis glomerata* L.). Each subgroup for each grass-containing diet was allocated a fenced paddock. In total, each diet was allocated three paddocks, each containing five animals. Each paddock was further divided into sub-paddocks to give time to the pasture to rest and regrow. Animals were rotated three times per week within their paddocks to fresh parcels of grass. The 15 steers in the BLG-SH diet were treated as feedlot cattle and were separated into three pens each containing five animals. One animal died during the first year of the experiment, and two carcasses were misplaced by the slaughterhouse during the second year of the study, bringing the total number of animals for the entire study to 117 (n = 117).

4.3.2 Sample collection and preparation

4.3.2.1 Feed samples

Samples from grazing areas and supplemental feeds were collected every two weeks. The sample collection started in July and ended in late October of each year. No sample collection occurred between April and July 2020 because of COVID-19 restrictions. To stay consistent, the sample collection period was kept the same for 2021. GRASS samples were gathered every two weeks in each sub-paddock immediately before animals had access to the pasture (n = 63 for each year, n = 126 in total). GRASS samples were collected by randomly cutting three 0.25 m^2 quadrats to a 5 cm stubble using hand grass clipper scissors. HAY, BLG, and SH were sampled monthly before being distributed to the steers (n = 4 of each supplemental feed for each year, n = 8 of each in total). Supplemental feeds were sampled monthly instead of bi-weekly because less variations over time in the nutritional profile of these feeds were expected. For proximate analysis, wet weights were recorded, and samples were dried in a forced-air oven (72 h, 55 °C) and ground through a 1 mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA). For FA and phytochemical analysis, a 30 g sub-sample was packed in a Whirl-Pak bag and frozen at -20 °C

immediately after collection. In order to obtain representative samples, bag contents were mixed, and 10 g of each replicate was taken before being combined. Feed samples were stored at -20 °C for the length of the trial before being stored at -80 °C once they were brought back to the laboratory. Before further analysis, samples were freeze-dried in a freeze dryer (Harvest Right, North Salt Lake, UT, USA) for 18.5 h, and ground in a Wiley mill (1 mm screen) (Arthur H. Thomas, Philadelphia, PA, USA) with dry ice.

Weather conditions were reported according to Krusinski et al. (2022a) using the Michigan State University Enviroweather platform at KBS. 30-year normal temperature and precipitation (1991-2020) were reported according to the National Centers for Environmental Information: National Oceanic and Atmospheric Administration website (Gull Lake, MI meteorological station).

4.3.2.2 Meat samples

In November of each year, before going to slaughter, steers were weighed again to obtain total weight gain and average daily gain (ADG). Steers were slaughtered in a USDA facility at 18-20 months of age. Body performance and carcass traits (ribeye area, 12th rib back fat, USDA yield grade, and marbling score) were collected by trained personnel 48 h after slaughter. Simultaneously, meat samples (approximately 7.5 to 10 cm in length) were collected from the left-side longissimus lumborum (between 13th rib and first two lumbar vertebra). For FA analysis and thiobarbituric acid reactive substances (TBARS), one steak per carcass was cut into 1×1 cm cubes before being flash frozen with liquid nitrogen, put into Whirl-Pak bags, and stored at -80 °C until analysis. For Warner-Bratzler shear force (WBSF), another 2.54 cm-thick steak was cut, vacuum packed, and stored at 4 °C until 14 days postmortem. At 14 days postmortem, the steaks were frozen at -20 °C until WBSF analysis was performed.

4.3.3 Feed chemical analysis

4.3.3.1 Proximate analysis

The protocol for the feed proximate analysis was previously described by Maciel et al. (2021). Samples were dried at 105 °C in a forced-air oven for 8 h. To determine the ash content, feed samples were oxidized at 500 °C for 6 h in a muffle furnace. Neutral detergent fiber (NDF) was determined according to Mertens (2002) with the addition of amylase and sodium sulfite. The protocol described in AOAC (2000) was used to determine acid detergent fiber (ADF). Crude protein (CP) was measured according to Hach et al. (1987) and gross energy was measured by bomb calorimeter.

4.3.3.2 Phytochemical analysis

Chlorophyll A and B were determined as described previously (Lichtenthaler and Wellburn, 1983). Briefly, 2 g of lyophilized and ground feed was added to 70% aqueous acetone. The mixture was shaken for 30 min and centrifuged for 20 min (4 °C, 2,500 RPM). The upper layer was transferred to a new tube, and the extraction was repeated twice. Compounds were measured using a UV-Vis Double Beam Spectrophotometer (VWR, Radnor, PA, USA) in cuvettes. Readings were recorded at 663 and 646 nm and were used in the following equations:

$$\text{Chlorophyll A } (C_a) = 12.21A_{663} - 2.81A_{646}$$

$$\text{Chlorophyll B } (C_b) = 20.13A_{646} - 5.03A_{663}$$

To extract phenolic compounds, a modified protocol from Nimalaratne et al. (2011) was performed. First, 2 g of freeze-dried and ground feed was added to 20 mL of methanol:distilled water:acetic acid (70:28:2, *v/v/v*). The mixture was shaken for 30 min and centrifuged for 20 min (4 °C, 2,500 RPM). The supernatant was recovered and transferred to a new tube. An additional 20 mL of acetone:distilled water:acetic acid (70:28:2, *v/v/v*) was added to the original tube before

being shaken for 10 min and centrifuged for 15 min (4 °C, 2,500 RPM). Both supernatants were combined and stored at 4 °C. The Folin-Ciocalteu assay adapted from Singleton and Rossi (1965) was used to measure total phenolic content. A standard curve was made using a 1 mg/mL gallic acid stock solution in methanol. A serial dilution was performed by a factor of two to obtain concentrations ranging from 1 mg/mL to 0.002 mg/mL. Next, 100 µL of Folin-Ciocalteu reagent and 800 µL of 5% sodium bicarbonate were added to the standard curve and to 100 µL of supernatant. The standard curve and the samples were heated at 40 °C for 30 min. Samples were allowed to cool down to room temperature before being plated in triplicates in a 96-well plate. Samples were scanned at 765 nm and compared against the gallic acid standard curve. Values were reported as mg of gallic acid equivalents (GAE)/g of feed.

4.3.4 Fatty acid analysis of feed and meat

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

The FA analysis for feed and meat samples was conducted according to Sergin et al. (2021). A microwave-assisted extraction protocol was performed to extract FAs as reported by Bronkema et al. (2019) using a CEM Mars 6 microwave (CEM Corp., Matthews, NC, USA). For this step, 400 mg of ground feed sample or minced meat was added to a microwave vessel containing 8 mL of 4:1 (*v/v*) ethyl acetate:methanol solution with 0.1% BHT. The samples underwent extraction with the following settings: 55 °C for 15 min with initial ramp of 2 min at 400 W. Samples were then filtered in another tube containing 3.5 mL of HPLC water before being centrifuged (6 min, 2,500 RPM). The upper layer was removed and dried under nitrogen. To resuspend the oil, a 4:1 (*v/v*) dichloromethane:methanol solution with 0.1% BHT was used to bring the concentration of each sample to 20 mg of oil/mL.

For the creation of FA methyl esters (FAMEs), a modified version of the protocol by Jenkins (2010) was applied. Briefly, 2 mg of oil (100 μL) was resuspended in toluene with 20 μg of internal standard (methyl 12-tridecenoate, U-35M, Nu-Chek Prep, Elysian, MN, USA). Then, 2 mL of 0.5 N anhydrous potassium methoxide was added and the samples were heated at 50 °C for 10 min. Next, 3 mL of methanolic HCl (5%) was added to the samples before being heated at 80 °C for 10 min. Once cool, 2 mL of HPLC water and 2 mL of hexane were added, and samples were centrifuged for 5 min at 2,500 RPM. The top layer was moved to another tube and dried under nitrogen to obtain FAMEs. FAMEs were then resuspended in 1 mL of isooctane to get a concentration of 2 mg/mL. Samples were transferred to gas chromatography-mass spectrometry (GC-MS) vials with glass inserts.

The PerkinElmer (Waltham, MA, USA) 680/600S GC-MS in electron impact mode (70 eV) equipped with an Agilent Technologies (Santa Clara, CA) HP-88 column (100 m, 0.25 mm ID, 0.2 μM film thickness) was used for the quantification of FAMEs. For feed samples, one μL of sample was injected with the GC temperature set at 250 °C. For meat samples, one μL was injected twice (20:1 split) at two different GC temperatures (175 °C and 150 °C). The temperature settings for both feed and meat samples were as follows: initial temperature at 80 °C for 4 min; ramp 13 °C/min to 175 °C; hold 27 min; ramp 4 °C/min to 215 °C; hold 35 min, and then an initial temperature at 80 °C for 4 min; ramp 13 °C/min to 150 °C; hold 47 min; ramp 4 °C/min to 215 °C; hold 35 min. For meat samples, a third injection followed in splitless mode (0.75 min splitless hold time, 40 mL/min flow exiting the vent). This GC-MS method was adapted from Kramer et al. (2008). Helium was the carrier gas (flow rate of 1 mL/min). MS data were recorded in full scan mode (mass range of m/z 70-400 amu) and the MS transfer line and ion source temperature were set at 180 °C.

MassLynx V4.1 SCN 714 (Water Corporation, Milford, MA, USA) was used for the identification of FAMEs. FAs were identified by retention time and EI mass fragmentation compared to the reference standard containing the Supelco 37 Component FAME Mix with mead acid, docosatetraenoic acid, *n*-3 DPA, *n*-6 DPA, and palmitelaidic acid purchased from Cayman Chemical (Ann Arbor, MI, USA). CLA isomers were identified using the CLA reference standard UC-59M (Nu-Chek Prep, Elysian, MN, USA). FAs not included in the reference standard were identified by elution order and confirmed by the EI mass fragmentation (Kramer et al., 2008). FAs were quantified using a standard curve including the reference and internal standards. Each FAME concentration was calculated by using the internal standard peak area and analyte peak area compared to the standard curve. C18:1 4*t* and C18:1 5*t* were below the limit of detection, and C18:2 9*c*,12*t* and C18:2 9*t*,12*c* were not separated from the C18:2 11*t*,15*c* peak. Eicosatetraenoic acid (C20:4 *n*-3) was not included in our reference standard and was therefore not reported. FAs were reported in mg/100 g of beef in this manuscript and in percent of total FAs in **Appendix Tables A4 and A5.**

4.3.5 Vitamin E and mineral analysis

Protocols by Rettenmaier and Schüep (1992) were followed for vitamin E analysis. In brief, 1 g of beef was homogenized in 5 mL of water before being frozen. For extraction, samples were thawed, and a measured aliquot was pipetted out. To precipitate the protein, ethanol was added, and fat-soluble vitamins were extracted with hexane. After being centrifuged, part of the hexane layer was removed and dried under reduced pressure in a vortexing chamber (10 min, 35 °C, 300 mBar vacuum). What remained after evaporation was solubilized in the chromatographic mobile phase and placed in vials. A calibration curve (six points) was made as follows: a vitamin E solution (Sigma-Aldrich, St. Louis, MO, USA) diluted with ethanol (containing BHT) underwent

serial dilutions (from 50 μg/mL to 0.2 μg/mL). For the chromatography analysis, a Waters Acquity system and Water Empower Pro Chromatography Manager software (Water Corporation, Milford, MA, USA) were used. An isocratic elution was performed using a mobile phase of acetonitrile:methylene chloride:methanol (70:20:10, *v/v/v*) and a Symmetry C18, 1.7 μm, 2.1×50 mm analytical column (Waters Corporation, Milford, MA, USA). The flow rate was 0.5mL/min and the detection was performed by UV absorption at 292 nm.

Mineral analysis was performed as previously described (Wahlen et al., 2005;Krusinski et al., 2022b). Briefly, beef samples underwent drying and digestion in an oven (95 °C, overnight) using 10 times the dry tissue mass of nitric acid. A dilution with water to 100 times the dried tissue mass followed. An Agilent 7900 Inductivity Coupled Plasma–Mass Spectrometer (ICP-MS) (Agilent Technologies Inc., Santa Clara, CA, USA) was used for the analysis. A six-point calibration curve was used. Standards of bovine liver and mussels (National Institute of Standards and Technology, Gaithersburg, MD, USA) were used as controls.

4.3.6 Thiobarbituric acid reactive substances (TBARS)

The TBARS assay for food and beverages (Oxford Biomedical Research, Oxford, MI, USA) adapted for a 96-well plate reader was used. First, an eight-point standard curve was created by serial dilution ranging from 0 (only HPLC water) to 3 mg/L malondialdehyde (MDA) (MDA stock solution provided in the kit). Then, 500 mg of minced beef sample was added to 5 mL of HPLC water. Samples were homogenized to obtain a smooth solution. In a microcentrifuge tube, 250 μL of sample solution and 250 μL of the indicator solution (thiobarbituric acid (TBA) and acid solution) were mixed. The indicator solution was also added to the standard curve, and the samples and the curve were set aside for 60 min for the reaction to occur. Samples were then centrifuged at 11,000 RPM for 5 min at room temperature. The aqueous layer was removed and

plated in duplicates on a 96-well plate, next to the standard curve. Absorbance was read at 532 nm on a Bio-Tek Synergy HT spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT, USA). The standard curve was plotted and the MDA concentration for the samples (mg MDA/L) was calculated according to the manufacturer's instructions.

4.3.7 Warner-Bratzler Shear Force (WBSF)

The protocol for WBSF was previously reported (Maciel et al., 2021). Briefly, the steaks were cooked to an internal temperature of 71 °C using a preheated clamshell electric grill (George Foreman, Beachwood, OH, USA). The steaks were then cooled down overnight at 4 °C. Six to eight 1.27 cm diameter cores were cut from each steak by paying close attention to cut parallel to the muscle and fibers using a drill mounted corer. Shear force was measured using the TA-XT Texture Analyzer (Stable Micro System Ltd., UK) with a V-shaped Warner-Bratzler blade. The blade was moving down at a speed of 20 cm/min and cut the sample across the muscle fiber. The purpose of the shear force testing was to measure how much force is required to cut through cooked meat. This should be a representative measure of the ease or difficulty a consumer would have chewing a cooked steak. Most consumer prefer steaks cooked between medium rare and medium well, which is why an internal temperature of 71 °C was chosen for the analysis. The mean of the cores for each sample were used for the statistical analysis.

4.3.8 Statistical analysis

SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) was used to perform the statistical analysis. Mixed model analysis was performed to test the effect of diet on response variables. In the model, the fixed effect was diet, and the random effects were year and pen nested within year x diet. Each pen was the experimental unit. Post-hoc comparison was performed using Tukey's adjustment, and results were considered significant at $p < 0.05$. Outliers were removed for

chlorophyll A and chlorophyll B after running an outlier test. The data satisfied model's normality and equal variance assumptions. Data are shown as mean ± standard error across mean (SEM).

4.4 Results

4.4.1 Weather conditions

Weather conditions at the experimental site for the length of the study are shown in **Figure 8**. The hottest month in 2020 was July with an average of 23.96 °C. The average temperature in August, September, and October 2020 were all below the 30-year normal. Every month in 2020 was below the 30-year normal for rainfall. In 2021, August was the hottest month with an average temperature of 23.10 °C. July, September, and October 2021 were above the 30-year normal temperature. September 2021 showed unusually high rainfall with 338.87 mm compared to the 30-year normal precipitation of 88.39 mm.

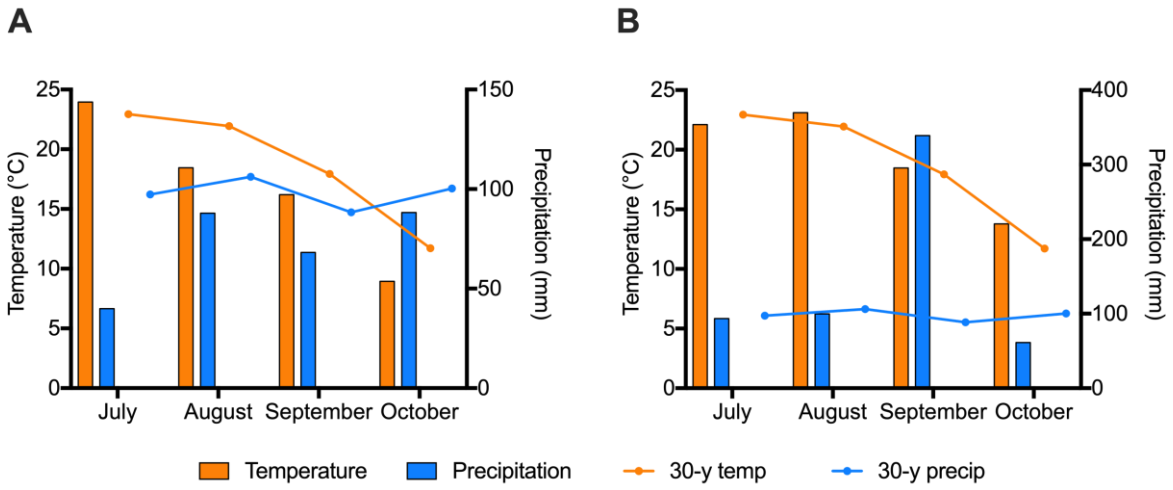

Figure 8. Monthly weather conditions at the experimental site. (A) 2020 and (B) 2021. 30-y temp: 30-year normal temperature at the experimental site; 30-y precip: 30-year normal precipitation at the experimental site.

4.4.2 Feeds

4.4.2.1 Proximate composition of feeds

The proximate composition of the feeds is displayed in **Table 10**. No significant differences were observed between feed types regarding dry matter (DM) ($p = 0.159$). GRASS and BLG had the highest values for ash ($p < 0.001$) and CP ($p < 0.001$), while SH had the lowest values for ash ($p < 0.001$) and HAY had the lowest values for CP ($p < 0.001$). Regarding NDF, SH was higher than GRASS, HAY, and BLG ($p = 0.004$). For ADF, SH was highest while GRASS was lowest ($p < 0.001$). Finally, SH had the lowest amount of energy compared to the other three feed types ($p < 0.001$).

Table 10. Mean proximate composition of the feeds

	GRASS	**HAY**	**BLG[1]**	**SH[2]**	***p*-value**
DM[3]	57.52 ± 22.47	85.82 ± 25.01	82.64 ± 25.02	89.98 ± 25.01	0.159
Ash[*]	9.20 ± 0.46 [a]	7.14 ± 0.61 [b]	8.38 ± 0.63 [a,b]	4.74 ± 0.61 [c]	< 0.001
CP[4*]	15.65 ± 0.39 [a]	7.15 ± 1.14 [c]	13.48 ± 1.19 [a,b]	9.47 ± 1.19 [b,c]	< 0.001
NDF[5*]	54.91 ± 3.30 [b]	66.19 ± 4.01 [a,b]	54.23 ± 4.04 [b]	68.29 ± 4.01 [a]	0.004
ADF[6*]	30.84 ± 0.72 [c]	37.98 ± 1.35 [b]	33.51 ± 1.39 [b,c]	51.72 ± 1.35 [a]	< 0.001
Energy[7]	4566.41 ± 50.99 [a]	4405.00 ± 90.76 [a]	4465.41 ± 50.99 [a]	3709.96 ± 90.76 [b]	< 0.001

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment). $n = 126$ for GRASS, and $n = 8$ for the other three feeds. [1]BLG: baleage; [2]SH: soybean hulls; [3]DM: dry matter (%); [*]reported in %DM; [4]CP: crude protein; [5]NDF: neutral detergent fiber; [6]ADF: acid detergent fiber; [7]Energy (cal/g).

4.4.2.2 Fatty acid composition of feeds

The FA profile of the feeds is reported in **Table 11**. Palmitic acid (C16:0) made up most of the SFA content of the feeds. HAY contained the highest concentration of C16:0, while GRASS and SH contained the lowest ($p < 0.001$). Regarding stearic acid (C18:0), SH and HAY contained the most and GRASS contained the least ($p < 0.001$). The total SFA content was significantly higher in HAY and was lower in GRASS and SH ($p < 0.001$). Total MUFA content was significantly higher in SH and lower in GRASS. BLG and HAY values were in between and were significantly different than SH and GRASS ($p < 0.001$). Regarding PUFAs, the linoleic acid (LA) content was the highest in SH and was lower in GRASS and HAY ($p < 0.001$), while the α-linolenic acid (ALA) content followed the opposite trend with GRASS containing the most and SH containing the least ($p < 0.001$). GRASS contained the highest concentration of total n-3 PUFAs and the lowest concentration of *n*-6 PUFAs. SH contained the most *n*-6 PUFAs and the least *n*-3 PUFAs ($p < 0.001$). This resulted in SH having the highest *n*-6:*n*-3 ratio and GRASS having the lowest ($p < 0.001$).

Table 11. Mean fatty acid composition of the diets (% total fatty acids)

	GRASS	HAY	BLG[1]	SH[2]	*p*-value
C10:0	0.16 ± 0.26	1.16 ± 0.38	0.14 ± 0.38	0.03 ± 0.38	0.116
C12:0	0.50 ± 0.22 [b]	1.16 ± 0.23 [a]	0.49 ± 0.23 [b]	0.10 ± 0.23 [c]	< 0.001
C13:0	0.01 ± 0.02	0.07 ± 0.03	0.02 ± 0.03	0.00 ± 0.03	0.193
C14:0	0.53 ± 0.29 [b]	2.14 ± 0.45 [a]	0.62 ± 0.45 [a,b]	0.21 ± 0.45 [b]	0.041
C15:0	0.11 ± 0.06 [b]	0.49 ± 0.09 [a]	0.33 ± 0.09 [a,b]	0.14 ± 0.09 [b]	0.010
C16:0	14.29 ± 1.98 [c]	32.16 ± 2.37 [a]	23.77 ± 2.38 [b]	14.95 ± 2.37 [c]	< 0.001
C16:1 9*c*	0.23 ± 0.02 [b]	0.51 ± 0.04 [a]	0.31 ± 0.04 [b]	0.23 ± 0.04 [b]	0.001
C16:1 7*c*	1.19 ± 0.27 [a]	1.21 ± 0.29 [a]	1.52 ± 0.29 [a]	0.11 ± 0.29 [b]	< 0.001
C17:0	0.21 ± 0.02 [c]	0.57 ± 0.03 [a]	0.36 ± 0.03 [b]	0.28 ± 0.03 [b,c]	< 0.001
C18:0	1.57 ± 0.13 [c]	3.80 ± 0.20 [a]	2.78 ± 0.20 [b]	4.33 ± 0.20 [a]	< 0.001
C18:1 9*c*	1.84 ± 0.07 [c]	3.45 ± 0.23 [b]	2.43 ± 0.24 [b,c]	12.61 ± 0.23 [a]	< 0.001
C18:1 11*c*	0.61 ± 0.03 [c]	1.04 ± 0.09 [b]	0.72 ± 0.10 [b,c]	2.43 ± 0.09 [a]	< 0.001
C18:2 *n*-6 (LA)[3]	12.22 ± 0.35 [c]	14.86 ± 0.88 [b,c]	16.42 ± 0.91 [b]	48.45 ± 0.88 [a]	< 0.001
C18:3 *n*-3 (ALA)[4]	64.66 ± 1.74 [a]	32.48 ± 2.57 [c]	46.47 ± 2.64 [b]	15.00 ± 2.57 [d]	< 0.001
C20:0	0.58 ± 0.08 [b]	2.72 ± 0.16 [a]	1.05 ± 0.16 [b]	0.41 ± 0.16 [b]	< 0.001
C20:3 *n*-3	0.08 ± 0.03	0.03 ± 0.03	0.05 ± 0.03	0.01 ± 0.03	0.052
C22:0	0.59 ± 0.16 [b,c]	1.90 ± 0.20 [a]	1.04 ± 0.21 [b]	0.29 ± 0.20 [c]	< 0.001
C24:0	0.61 ± 0.32 [b]	1.78 ± 0.36 [a]	1.40 ± 0.36 [a]	0.24 ± 0.36 [b]	0.001
∑ SFA[5]	19.17 ± 2.41 [c]	48.03 ± 3.28 [a]	31.86 ± 3.29 [b]	21.01 ± 3.28 [b,c]	< 0.001
∑ OCFA[6]	0.33 ± 0.06 [c]	1.11 ± 0.10 [a]	0.71 ± 0.10 [a,b]	0.44 ± 0.10 [b,c]	< 0.001
∑ MUFA[7]	3.87 ± 0.30 [c]	6.18 ± 0.39 [b]	4.96 ± 0.40 [b]	15.40 ± 0.39 [a]	< 0.001
∑ PUFA[8]	76.96 ± 2.72 [a]	45.79 ± 3.56 [c]	63.11 ± 3.58 [b]	63.51 ± 3.56 [b]	< 0.001
∑ *n*-6[9]	12.22 ± 0.35 [c]	14.86 ± 0.88 [b,c]	16.42 ± 0.91 [b]	48.45 ± 0.88 [a]	< 0.001
∑ *n*-3[10]	64.73 ± 1.71 [a]	32.51 ± 2.55 [c]	46.52 ± 2.62 [b]	15.02 ± 2.55 [d]	< 0.001
n-6:*n*-3 ratio[11]	0.19 ± 0.04 [c]	0.53 ± 0.07 [b]	0.38 ± 0.07 [b,c]	3.20 ± 0.07 [a]	< 0.001

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment). $n = 126$ for GRASS, and $n = 8$ for the other three feeds. [1]BLG: baleage; [2]SH: soybean hulls; [3]LA: linoleic acid; [4]ALA: α-linolenic acid; [5]∑ SFA: total saturated FAs; [6]∑ OCFA: total odd chain FAs; [7]∑ MUFA: total monounsaturated FAs; [8]∑ PUFA: total polyunsaturated FAs; [9]∑ *n*-6: LA; [10]∑ *n*-3: ALA + C20:3 *n*-3; [11]*n*-6:*n*-3 ratio: ∑ *n*-6/∑ *n*-3.

4.4.2.3 Phytochemical content of feeds

The chlorophyll A, chlorophyll B, and total phenols content of the feeds are shown in **Figure 9**. BLG and GRASS contained the highest levels of chlorophyll A. SH contained the least ($p < 0.001$). GRASS contained more chlorophyll B than HAY and SH, and BLG contained more chlorophyll B than SH ($p < 0.001$). Finally, BLG and GRASS contained more phenols than SH ($p = 0.010$).

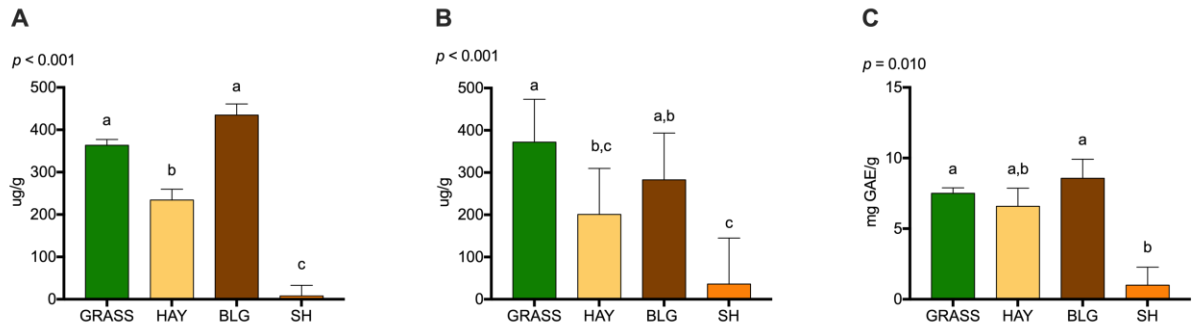

Figure 9. Phytochemical content of feeds. Chlorophyll A (A), chlorophyll B (B), and total phenols (C) found in feed samples. Values reported at means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment, $n = 126$ for GRASS, $n = 8$ for each of the other feeds). GRASS: fresh pasture; HAY: dry hay; BLG: baleage; SH: soybean hulls; GAE: gallic acid equivalent.

4.4.3 Animal performance and carcass traits

Performance and carcass traits are shown in **Table 12**. Initial weight did not differ between diet groups, which was the goal when assigning animals to each group. Final weight, total gain, and average daily gain (ADG) were all higher in the BLG-SH and G-SH groups, while they were lower in the G-BLG and G-HAY groups ($p < 0.001$). A similar trend was seen regarding hot carcass weight (HCW) with higher weights observed in the G-SH and BLG-SH groups and lower weights observed in the G-HAY and G-BLG groups ($p = 0.003$). Backfat significantly differed by diet ($p = 0.011$). BLG-SH and G-SH had more backfat compared to beef from G-BLG but did not differ from G-HAY. G-HAY had the smallest ribeye area compared to the other three groups ($p =$

0.012). Regarding USDA yield grade, G-BLG had a lower numerical yield grade than G-SH but did not differ from the remaining groups ($p = 0.032$). G-BLG had a lower marbling score than G-SH and BLG-SH but was similar to G-HAY ($p = 0.004$).

Table 12. Mean animal performance and carcass traits by diet

	G-HAY[1]	G-BLG[2]	G-SH[3]	BLG-SH[4]	*p*-value
Growth (kg)					
Initial BW[5]	388.70 ± 30.30	390.77 ± 30.33	388.77 ± 30.36	378.27 ± 30.30	0.560
Final BW	483.27 ± 8.63 [c]	493.74 ± 8.77 [b,c]	524.33 ± 8.92 [a,b]	536.31 ± 8.63 [a]	< 0.001
Total gain	94.57 ± 25.64 [b]	103.02 ± 25.65 [b]	135.77 ± 25.66 [a]	158.04 ± 25.64 [a]	< 0.001
ADG[6]	0.61 ± 0.10 [b]	0.66 ± 0.10 [b]	0.88 ± 0.10 [a]	1.03 ± 0.10 [a]	< 0.001
Carcass					
HCW[7] (kg)	281.85 ± 5.53 [c]	287.05 ± 5.62 [b,c]	311.11 ± 5.72 [a]	306.53 ± 5.53 [a,b]	0.003
Backfat (mm)	7.15 ± 0.75 [a,b]	5.91 ± 0.76 [b]	9.18 ± 0.77 [a]	9.38 ± 0.75 [a]	0.011
Ribeye area (cm^2)	68.28 ± 2.19 [b]	75.99 ± 2.20 [a]	75.57 ± 2.22 [a]	76.41 ± 2.19 [a]	0.012
USDA yield grade	2.80 ± 0.30 [a,b]	2.10 ± 0.31 [b]	3.29 ± 0.31 [a]	2.80 ± 0.30 [a,b]	0.032
Marbling score[8]	348.00 ± 11.66 [a,b]	332.55 ± 11.81 [b]	387.39 ± 11.97 [a]	392.00 ± 11.66 [a]	0.004

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment, $n = 117$). [1]G-HAY: grass and hay diet; [2]G-BLG: grass and baleage diet; [3]G-SH: grass and soybean hulls diet; [4]BLG-SH: baleage and soybean hulls diet; [5]BW: body weight; [6]ADG: average daily gain; [7]HCW: hot carcass weight; [8]Marbling score: 300-Slight-00 and 400-Small-00.

4.4.4 Beef fatty acids

4.4.4.1 Saturated and monounsaturated fatty acids

The saturated and monounsaturated FA content of beef is presented in **Table 13**. No significant differences were observed by diet for total SFAs ($p = 0.400$). Individual SFAs ranging from C10:0 to C20:0 did not differ between groups ($p > 0.05$), but C22:0 was higher in G-HAY compared to the other three groups ($p < 0.001$). No significant differences between groups were observed for total branched-chain FA (BCFA) content or for individual BCFAs ($p > 0.05$). The same trend was observed for total MUFA content and individual *cis*-MUFAs ($p > 0.05$). Regarding

trans-MUFAs, the only significant difference seen was for C16:1 9*t* which was lower in the BLG-SH group compared to the other three groups ($p = 0.003$). No differences were observed for the total FA content between groups ($p > 0.05$).

Table 13. Mean concentrations of saturated and monounsaturated fatty acids by diet (mg per 100 g beef)

	G-HAY[1]	G-BLG[2]	G-SH[3]	BLG-SH[4]	*p*-value
∑ SFA[5]	275.08 ± 40.89	272.87 ± 41.46	332.71 ± 42.05	356.22 ± 40.89	0.400
C10:0	1.75 ± 1.92	2.54 ± 1.92	3.17 ± 1.92	3.28 ± 1.92	0.163
C12:0	0.57 ± 0.31	0.60 ± 0.31	0.73 ± 0.31	0.70 ± 0.31	0.442
C13:0	0.09 ± 0.07	0.11 ± 0.07	0.12 ± 0.07	0.11 ± 0.07	0.247
C14:0	12.40 ± 2.29	12.04 ± 2.33	14.95 ± 2.36	15.98 ± 2.29	0.566
C15:0	2.00 ± 0.31	2.11 ± 0.31	1.96 ± 0.32	2.02 ± 0.31	0.988
C16:0	162.66 ± 24.15	162.29 ± 24.49	202.57 ± 24.83	220.59 ± 24.15	0.259
C17:0	4.65 ± 0.79	4.86 ± 0.81	5.52 ± 0.82	6.01 ± 0.79	0.617
C18:0	87.03 ± 14.33	84.13 ± 14.49	99.28 ± 14.66	103.06 ± 14.33	0.694
C19:0	1.91 ± 1.54	2.81 ± 1.55	2.84 ± 1.55	2.99 ± 1.54	0.270
C20:0	0.78 ± 0.35	0.67 ± 0.35	0.81 ± 0.35	0.81 ± 0.35	0.305
C22:0	1.23 ± 0.39 [a]	0.73 ± 0.39 [b]	0.79 ± 0.39 [b]	0.84 ± 0.39 [b]	< 0.001
∑ BCFA[6]	11.11 ± 1.63	11.41 ± 1.65	13.08 ± 1.68	12.29 ± 1.63	0.831
C14:0 *iso*	0.15 ± 0.06	0.19 ± 0.06	0.17 ± 0.06	0.16 ± 0.06	0.863
C15:0 *iso*	0.73 ± 0.16	0.77 ± 0.16	0.94 ± 0.16	0.84 ± 0.16	0.771
C15:0 *anteiso*	0.69 ± 0.15	0.72 ± 0.15	0.73 ± 0.15	0.67 ± 0.15	0.979
C16:0 *iso*	0.77 ± 0.25	0.77 ± 0.25	0.82 ± 0.25	0.80 ± 0.25	0.989
C17:0 *iso*	4.11 ± 0.45	4.16 ± 0.46	4.81 ± 0.47	4.21 ± 0.45	0.689
C17:0 *anteiso*	4.06 ± 0.68	4.27 ± 0.69	4.96 ± 0.70	4.96 ± 0.68	0.717
C18:0 *iso*	0.60 ± 0.19	0.54 ± 0.20	0.65 ± 0.20	0.65 ± 0.19	0.819

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment, $n = 117$). [1]G-HAY: grass and hay diet; [2]G-BLG: grass and baleage diet; [3]G-SH: grass and soybean hulls diet; [4]BLG-SH: baleage and soybean hulls diet; [5]∑ SFA = total saturated FAs; [6]∑ BCFA = total branched chain FAs; [7]∑ MUFA = total monounsaturated FAs; [8]∑ *c*MUFA = total *cis*-monounsaturated FAs; [9]∑ *t*MUFA = total *trans*-monounsaturated FAs; [10]∑ FA = all FAs.

Table 13. (cont'd)

	G-HAY	G-BLG	G-SH	BLG-SH	*p*-value
∑ MUFA[7]	313.55 ± 39.66	313.17 ± 40.22	381.51 ± 40.81	371.90 ± 39.66	0.489
∑ *c*MUFA[8]	276.91 ± 36.21	272.67 ± 36.72	342.84 ± 37.26	342.10 ± 36.21	0.358
C14:1 9*c*	2.67 ± 0.52	2.78 ± 0.53	3.36 ± 0.53	3.13 ± 0.52	0.724
C16:1 9*c*	36.73 ± 4.92	35.81 ± 5.00	45.02 ± 5.09	44.15 ± 4.92	0.444
C16:1 10*c*	4.17 ± 1.55	5.19 ± 1.55	4.51 ± 1.55	4.16 ± 1.55	0.554
C16:1 11*c*	2.00 ± 1.39	2.52 ± 1.39	2.66 ± 1.39	2.76 ± 1.39	0.328
C17:1 9*c*	3.76 ± 0.43	3.98 ± 0.43	4.35 ± 0.44	4.50 ± 0.43	0.490
C18:1 9*c*	199.97 ± 34.69	196.83 ± 35.01	252.79 ± 35.35	255.23 ± 34.69	0.309
C18:1 11*c*	11.87 ± 1.50	10.09 ± 1.51	12.75 ± 1.52	11.57 ± 1.50	0.321
C18:1 12*c*	2.14 ± 0.61	2.12 ± 0.61	2.44 ± 0.61	2.46 ± 0.61	0.560
C18:1 13*c*	2.33 ± 1.60	3.11 ± 1.61	3.64 ± 1.61	3.72 ± 1.60	0.065
C18:1 14*c*	0.91 ± 0.26	0.83 ± 0.26	0.90 ± 0.26	0.90 ± 0.26	0.878
C18:1 15*c*	1.29 ± 0.83	1.74 ± 0.83	1.81 ± 0.83	1.95 ± 0.83	0.153
C20:1 9*c*	2.82 ± 1.70	2.67 ± 1.71	3.08 ± 1.71	2.92 ± 1.70	0.718
C20:1 11*c*	6.26 ± 1.44	4.88 ± 1.44	5.51 ± 1.44	4.63 ± 1.44	0.179
∑ *t*MUFA[9]	36.64 ± 9.96	40.71 ± 9.99	38.88 ± 10.02	29.80 ± 9.96	0.479
C16:1 9*t*	6.06 ± 1.66 [a]	6.68 ± 1.66 [a]	6.11 ± 1.67 [a]	3.82 ± 1.66 [b]	0.003
C16:1 10,11,12*t*	5.49 ± 2.51	6.99 ± 2.52	6.83 ± 2.52	6.63 ± 2.51	0.373
C18:1 6-8*t*	1.56 ± 0.99	2.07 ± 0.99	1.83 ± 0.99	2.08 ± 0.99	0.181
C18:1 9*t*	1.62 ± 1.19	2.44 ± 1.19	2.51 ± 1.19	2.52 ± 1.19	0.221
C18:1 10*t*	1.35 ± 1.23	2.49 ± 1.23	2.17 ± 1.23	1.83 ± 1.23	0.149
C18:1 11*t*	13.77 ± 2.73	12.37 ± 2.77	11.73 ± 2.80	5.64 ± 2.73	0.196
C18:1 12*t*	1.36 ± 0.51	1.43 ± 0.51	1.53 ± 0.51	1.26 ± 0.51	0.755
C18:1 13,14*t*	2.92 ± 0.31	2.72 ± 0.31	2.73 ± 0.32	2.51 ± 0.31	0.832
C18:1 15*t*	1.44 ± 1.65	2.24 ± 1.65	2.60 ± 1.65	2.34 ± 1.65	0.079
C18:1 16*t*	1.36 ± 0.35	1.29 ± 0.35	1.27 ± 0.35	1.17 ± 0.35	0.918
∑ FA[10]	729.92 ± 83.69	698.46 ± 84.92	833.36 ± 86.21	840.46 ± 83.69	0.550

4.4.4.2 Polyunsaturated fatty acids and biohydrogenation intermediates

The PUFA, CLA, and atypical dienes (AD) content of beef are displayed in **Table 14**. The total PUFA content of beef was higher in the G-HAY group compared to the other three groups ($p < 0.001$). The same was true for the total *n*-3 PUFA content ($p < 0.001$). More specifically, the ALA content of beef was highest in G-HAY and lowest in BLG-SH ($p = 0.014$). All long-chain *n*-3 PUFAs including EPA, DPA, and DHA were higher in beef from the G-HAY group compared to the other three groups ($p < 0.001$). The sum of EPA+DHA was also higher in beef from G-HAY (11.59 mg/100 g of beef) compared to the other three groups, but all four groups had lower amounts of EPA+DHA compared to European Union standards to consider a food "a source of *n*-3 FAs" (Commission Regulation of European Union, 2010) (**Figure 10**). No significant differences between groups were observed for *n*-6 PUFAs ($p > 0.05$) except for C22:4 *n*-6 which was higher in beef from G-HAY compared to the other groups ($p < 0.001$). Significant differences in the *n*-6:*n*-3 ratio were also seen; the lowest ratio was seen in the G-HAY group while the highest ratio was seen in beef from G-SH ($p < 0.001$). No differences were observed between the groups for ADs and conjugated linolenic acid (CLnA) ($p > 0.05$). Significant differences were reported for the individual CLA C18:2 9*c*,11*t*/9*c*,7*t* where beef from G-HAY contained the most and beef from BLG-SH contained the least ($p = 0.015$).

Table 14. Mean concentrations of polyunsaturated fatty acids by diet (mg per 100 g beef)

	G-HAY[1]	G-BLG[2]	G-SH[3]	BLG-SH[4]	*p*-value
∑ PUFA[5]	98.31 ± 3.50 [a]	72.54 ± 3.56 [b]	76.57 ± 3.63 [b]	73.69 ± 3.50 [b]	< 0.001
∑ *n*-3[6]	47.29 ± 2.97 [a]	29.04 ± 2.99 [b]	26.57 ± 3.01 [b]	27.20 ± 2.97 [b]	< 0.001
C18:3 *n*-3 (ALA)[7]	10.63 ± 1.26 [a]	10.39 ± 1.26 [a,b]	8.62 ± 1.27 [a,b]	8.31 ± 1.26 [b]	0.014
C20:3 *n*-3	0.89 ± 0.41	0.68 ± 0.41	0.74 ± 0.41	0.70 ± 0.41	0.235
C20:5 *n*-3 (EPA)[8]	9.26 ± 0.38 [a]	5.68 ± 0.39 [b]	5.04 ± 0.39 [b]	5.00 ± 0.38 [b]	< 0.001
C22:5 *n*-3 (DPA)[9]	24.18 ± 2.82 [a]	11.09 ± 2.82 [b]	10.88 ± 2.83 [b]	11.59 ± 2.82 [b]	< 0.001
C22:6 *n*-3 (DHA)[10]	2.33 ± 0.54 [a]	1.43 ± 0.54 [b]	1.51 ± 0.54 [b]	1.60 ± 0.54 [b]	< 0.001
∑ *n*-6[11]	47.64 ± 2.84	41.67 ± 2.86	48.26 ± 2.88	44.67 ± 2.84	0.108
C18:2 *n*-6 (LA)[12]	28.23 ± 3.73	25.42 ± 3.73	30.14 ± 3.74	27.37 ± 3.73	0.121
C18:3 *n*-6	0.72 ± 0.46	0.69 ± 0.46	0.76 ± 0.46	0.76 ± 0.46	0.733
C20:2 *n*-6	1.01 ± 0.32	0.76 ± 0.32	0.84 ± 0.32	0.90 ± 0.32	0.087
C20:3 *n*-6	2.20 ± 0.31	2.21 ± 0.31	2.65 ± 0.31	2.53 ± 0.31	0.123
C20:4 *n*-6	10.43 ± 1.19	10.02 ± 1.20	10.63 ± 1.20	10.02 ± 1.19	0.903
C22:4 *n*-6	5.05 ± 1.36 [a]	2.68 ± 1.36 [b]	3.35 ± 1.36 [b]	3.29 ± 1.36 [b]	< 0.001
n-6:*n*-3 ratio[13]	1.03 ± 0.23 [c]	1.49 ± 0.23 [b]	1.89 ± 0.23 [a]	1.70 ± 0.23 [a,b]	< 0.001
C20:3 *n*-9	3.38 ± 0.93 [a]	1.87 ± 0.93 [b]	1.79 ± 0.93 [b]	1.83 ± 0.93 [b]	< 0.001

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment, n = 117). [1]G-HAY: grass and hay diet; [2]G-BLG: grass and baleage diet; [3]G-SH: grass and soybean hulls diet; [4]BLG-SH: baleage and soybean hulls diet; [5]∑ PUFA: total polyunsaturated FAs; [6]∑ *n*-3: total *n*-3 FAs; [7]ALA: α-linolenic acid; [8]EPA: eicosapentaenoic acid; [9]DPA: docosapentaenoic acid; [10]DHA: docosahexaenoic acid; [11]∑ *n*-6: total *n*-6 FAs; [12]LA: linoleic acid; [13]*n*-6:*n*-3 ratio: ∑ *n*-6/∑ *n*-3; [14]∑ CLnA: total conjugated linolenic acid isomers; [15]∑ Atypical Dienes: total non-conjugated linoleic acid isomers; [16]∑ CLA: total conjugated linoleic acid isomers.

Table 14. (cont'd)

	G-HAY[1]	G-BLG[2]	G-SH[3]	BLG-SH[4]	*p*-value
∑ CLnA[14]	1.59 ± 1.15	1.56 ± 1.15	1.76 ± 1.15	1.63 ± 1.15	0.690
C18:3 9*c*,11*t*,15*t*	0.80 ± 0.57	0.82 ± 0.57	0.91 ± 0.57	0.84 ± 0.57	0.676
C18:3 9*c*,11*t*,15*c*	0.79 ± 0.58	0.74 ± 0.58	0.85 ± 0.58	0.79 ± 0.58	0.648
∑ AD[15]	18.30 ± 6.84	17.25 ± 6.84	17.56 ± 6.85	15.84 ± 6.84	0.833
C18:2 11*t*,15*t*	4.26 ± 0.84	3.27 ± 0.84	3.22 ± 0.85	2.65 ± 0.84	0.207
C18:2 9*t*,12*t*	1.82 ± 1.09	2.27 ± 1.09	2.37 ± 1.09	2.21 ± 1.09	0.377
C18:2 9*c*,14*t*/9*c*,13*t*	2.60 ± 1.27	2.69 ± 1.27	2.78 ± 1.27	2.56 ± 1.27	0.944
C18:2 11*t*,15*c*	4.20 ± 0.82	3.48 ± 0.82	3.04 ± 0.83	2.52 ± 0.82	0.189
C18:2 9*c*,16*t*	1.90 ± 0.88	1.87 ± 0.88	2.07 ± 0.88	2.00 ± 0.88	0.715
C18:2 9*c*,15*c*	2.10 ± 1.29	2.35 ± 1.29	2.59 ± 1.29	2.39 ± 1.29	0.587
C18:2 12*c*,15*c*	1.42 ± 0.80	1.31 ± 0.80	1.48 ± 0.80	1.51 ± 0.80	0.589
∑ CLA[16]	10.45 ± 3.43	8.35 ± 3.44	9.03 ± 3.44	7.11 ± 3.43	0.107
C18:2 9*c*,11*t*/9*c*,7*t*	6.26 ± 1.00 [a]	4.41 ± 1.00 [a,b]	4.66 ± 1.00 [a,b]	3.05 ± 1.00 [b]	0.015
C18:2 11*t*,13*c*	1.72 ± 0.89	1.51 ± 0.89	1.64 ± 0.89	1.49 ± 0.89	0.570
C18:2 11*t*,13*t*	1.27 ± 0.83	1.28 ± 0.83	1.44 ± 0.83	1.36 ± 0.83	0.580
C18:2 *t*,*t*	1.20 ± 0.80	1.15 ± 0.80	1.27 ± 0.80	1.21 ± 0.80	0.799

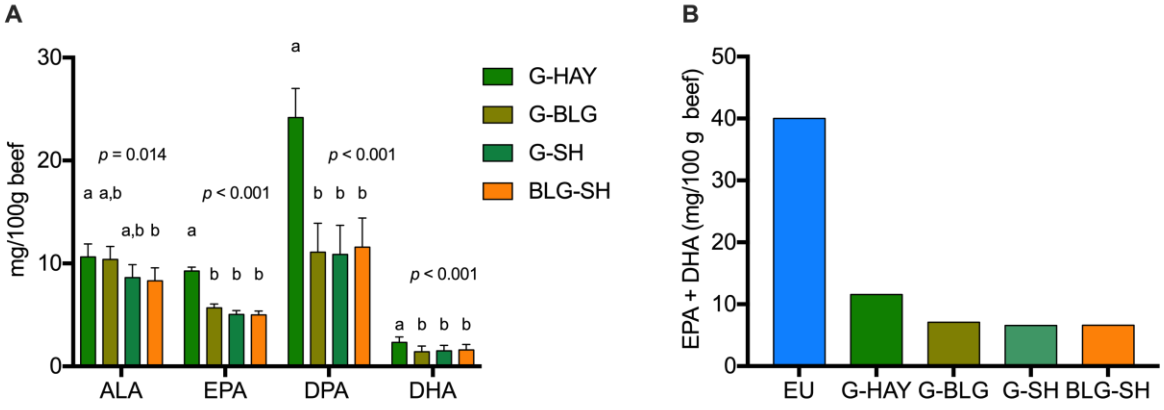

Figure 10. Long-chain *n*-3 PUFAs in beef by diet. (A) α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) content of beef by diet. (B) Sum of EPA+DHA in beef by diet compared to the European Union (EU) standard to consider a food "a source of *n*-3 PUFAs" (Commission Regulation of European Union, 2010). Data shown as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment, $n = 117$). G-HAY: grass and hay diet; G-BLG: grass and baleage diet; G-SH: grass and soybean hulls diet; BLG-SH: baleage and soybean hulls diet; EU: European Union standard for a food to be considered "a source of *n*-3 fatty acids."

4.4.5 Vitamin E and minerals in beef

The vitamin E and minerals in beef are presented in **Table 15**. Vitamin E was significantly lower in beef from the BLG-SH group compared to the other three diets ($p < 0.001$). Selenium, iron, copper, and zinc did not differ between diets ($p > 0.05$). Manganese was higher in beef from G-HAY and G-BLG, and lower in beef from BLG-SH ($p = 0.002$).

Table 15. Mean concentrations of vitamin E and minerals by diet (µg per g of beef)

	G-HAY[1]	G-BLG[2]	G-SH[3]	BLG-SH[4]	*p*-value
Vitamin E	29.93 ± 1.44 [a]	28.86 ± 1.46 [a]	25.62 ± 1.47 [a]	13.83 ± 1.44 [b]	< 0.001
Selenium	0.44 ± 0.04	0.42 ± 0.04	0.44 ± 0.04	0.45 ± 0.04	0.561
Iron	59.87 ± 7.61	59.65 ± 7.61	60.41 ± 7.62	56.94 ± 7.61	0.422
Copper	1.98 ± 0.07	2.09 ± 0.07	2.07 ± 0.07	1.93 ± 0.07	0.117
Zinc	126.31 ± 3.35	123.35 ± 3.40	123.80 ± 3.45	119.36 ± 3.35	0.545
Manganese	0.92 ± 0.02 [a]	0.90 ± 0.02 [a,b]	0.85 ± 0.02 [b,c]	0.84 ± 0.02 [c]	0.002

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment, $n = 117$). [1]G-HAY: grass and hay diet; [2]G-BLG: grass and baleage diet; [3]G-SH: grass and soybean hulls diet; [4]BLG-SH: baleage and soybean hulls diet.

4.4.6 TBARS and WBSF values of beef

The TBARS and WBSF values for beef by diet are displayed in **Figure 11**. Beef from the BLG-SH group showed higher TBARS values compared to the other three groups ($p < 0.001$). Regarding WBSF values, beef from the BLG-SH group displayed lower values compared to beef from G-HAY and G-BLG ($p = 0.017$).

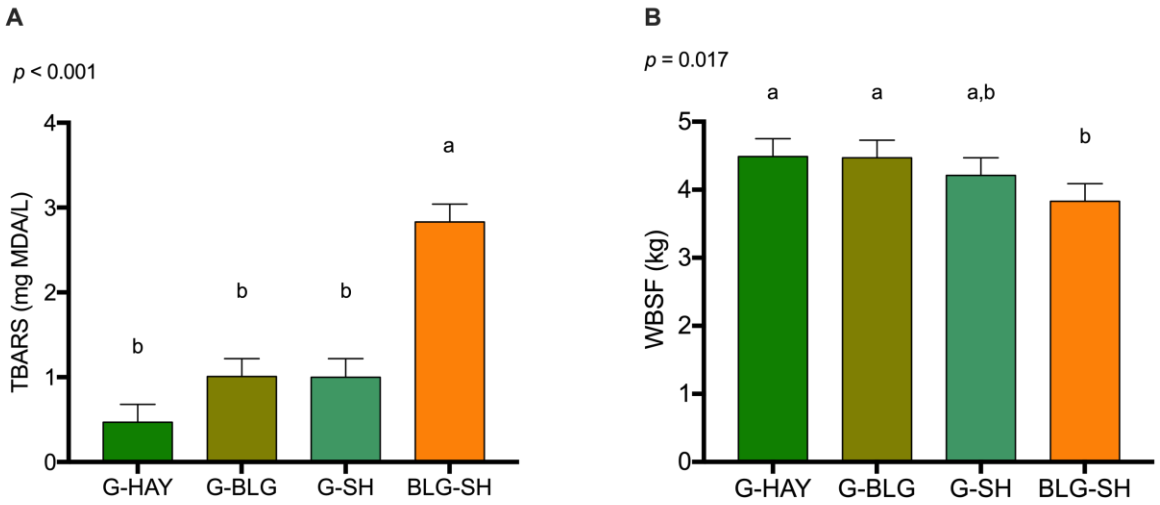

Figure 11. (A) Thiobarbituric acid reactive substances (TBARS) and (B) Warner-Bratzler Shear Force (WBSF) values of beef by diet. Data shown as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment, $n = 117$). G-HAY: grass and hay diet; G-BLG: grass and baleage diet; G-SH: grass and soybean hulls diet; BLG-SH: baleage and soybean hulls diet.

4.5 Discussion

4.5.1 Feeds

4.5.1.1 Proximate composition of the feeds

The proximate analysis of the feeds indicates that SH are higher in fiber than GRASS, HAY, and BLG. This finding is supported by the literature (Poore et al., 2002;Booth et al., 2004). SH are low in lignin and have a high digestibility potential for ruminants (Poore et al., 2002). Therefore, SH provide energy without the management problems associated with high grain diets (Booth et al., 2004). In the present study, SH provided less energy than the other three feed types. However, SH were not consumed by the animals in isolation but as a combination with either GRASS or BLG. Poore et al. (2002) noted that energy levels of SH were variable in various studies, but because of an associative effect on forage digestion, SH appear to have an effective energy value. GRASS contained the most CP and gross energy compared to the other feeds. These

findings confirm previously published results by Krusinski et al. (2022a) showing that pastures contain more CP and gross energy than conserved forages.

4.5.1.2 Fatty acid profile of the feeds

Numerous differences in the FA content of the feeds were observed in the current study. GRASS contained more *n*-3 PUFAs in the form of ALA than all the other feeds. This was expected since grasses contain higher concentrations of ALA (50-75% of total FAs) (Scollan et al., 2014;Bronkema et al., 2019;Krusinski et al., 2022a;Krusinski et al., 2022d). High levels of PUFAs are found in chloroplasts of green plants (i.e., grasses), which may explain the higher concentrations of ALA (Elgersma et al., 2013). Levels of *n*-3 PUFAs drastically decreased in conserved forages (HAY and BLG). While they still contained more PUFAs than SH, conserved forages usually have reduced nutritional quality compared to fresh grasses. This is due to the drying process of HAY and the fermenting process of BLG which result in the oxidation of PUFAs, especially ALA. More specifically, PUFAs are released from the plant membranes and are then oxidized with exposure to air by lipoxygenases (Kalač and Samková, 2010). This process is generally followed by an increase in levels of palmitic acid (C16:0) since SFAs are less prone to oxidation (Van Ranst et al., 2009;Kalač and Samková, 2010). This was confirmed in the present study with HAY and BLG containing more palmitic acid than GRASS. We also reported a higher *n*-6:*n*-3 ratio in SH compared to the other feeds. Bronkema et al. (2019) indicated that SH have a higher LA content, thus increasing the *n*-6:*n*-3 ratio. The results presented in the current study confirm these statements since we found higher levels of *n*-6 PUFAs and lower levels of *n*-3 PUFAs in SH compared to the other feeds, thus increasing the *n*-6:*n*-3 ratio. Interestingly, SH contained more MUFA than the other types of feeds (mainly as oleic acid). O'Callaghan et al. (2019) showed that adding SH to a concentrate diet decreased levels of oleic acid. Ensiled forages

such as BLG have advantages compared to HAY. Ensiling does not greatly impact the FA profile (Kalač and Samková, 2010;Glasser et al., 2013). Ensiling forages protects FAs from oxidation, explaining why oxidation in HAY is generally more prevalent (Kalač, 2011). Our results confirm the more beneficial FA profile of BLG compared to HAY; BLG contained more *n*-3 PUFAs and had a lower *n*-6:*n*-3 ratio compared to HAY. However, it is important to note that the feed composition plays a major role in the nutritional profile of feeds. Different plant species have different effects on the FA profile of feeds (Butler, 2014). A limitation of the current study is the lack of information about the proportion of plant species present in the feeds. Krusinski et al. (2022a) showed that individual plant species affect the FA and antioxidant profiles of pastures.

4.5.1.3 Phytochemical content of the feeds

GRASS and BLG contained the most chlorophyll A, chlorophyll B, and total phenols while SH contained the least of these compounds. There is a strong positive correlation between chlorophyll A and B and ALA in grasses (Khan et al., 2012). Green forages are also known to contain vitamins with antioxidant properties such as vitamin E (Elgersma et al., 2013). The high total phenols levels found in GRASS were expected. It was previously reported that the total phenolic content is higher in grasses than in seeds (Niroula et al., 2019). In a study comparing a complex pasture mixture to a grain diet, the authors reported higher levels of chlorophyll A, chlorophyll B, and total phenols in pasture (Krusinski et al., 2022a). Surprisingly, levels of these antioxidant compounds were not lower in BLG. Drying and fermenting usually decrease concentrations of antioxidants and phenolics (Owens et al., 1997;Butler, 2014;Krusinski et al., 2022d). Tripathi et al. (1995) noted that haymaking may cause more leaf dropping and shattering compared to BLG making (leaves are the most nutritious parts of the plant), which may explain why concentrations of these compounds were reduced in HAY but not in BLG. While SH have

been investigated for their antioxidant potential (Liu et al., 2019), our results indicate that SH have low levels of total phenols when compared to GRASS or BLG. However, it appears that the growth stage of the soybean plant affects its phenolic concentration (Peiretti et al., 2019).

4.5.2 Animal performance and carcass traits

Results in the present study demonstrate that the diet has an impact on animal growth, carcass traits, and meat quality. Initial body weight did not differ between groups, which may be attributed to pre-trial management. The addition of SH to either GRASS or BLG led to higher final body weight, higher total gain, and higher ADG. While the BLG-SH group was expected to be higher than the other groups for these variables, it was interesting to see a similar trend in the G-SH group which was out on pasture. The BLG-SH group was treated as feedlot cattle. Previous studies showed that cattle finished in feedlots have higher final body weight, total weight gain, and ADG compared to pasture-finished cattle (Neel et al., 2007;Maciel et al., 2021). Besides diet, another explanation may be that cattle in feedlots also have less exercise than cattle out on pasture, which reduces their maintenance requirements. The G-SH group was also out on pasture and consumed mostly GRASS, but differences with the BLG-SH group were not significant. Neel et al. (2007) showed that increasing the amount of soybean meal and SH in the cattle's diet led to higher final body weight and ADG. Dennis et al. (2012) reported that animals consuming a diet of only HAY showed higher final body weight and ADG than animals consuming a BLG diet. In the present study, no significant differences were seen between the G-HAY and the G-BLG groups. This might be due to animals consuming HAY and BLG as supplemental feeds while eating mostly GRASS. The plant species used might also differ compared to other studies.

Regarding carcass traits, a similar trend was observed for hot carcass weight with the G-SH and the BLG-SH groups weighing more than the other two groups. This finding aligns with

previous results about weight gain. Maciel et al. (2021) reported that animals in feedlots finished on grain have greater backfat, ribeye size, USDA yield grade, and marbling scores than animals finished on pasture. In the present study, the BLG-SH group showed the same trends, and the same was observed for the G-SH group (which can be attributed to the inclusion of SH). It was previously shown that increasing the amount of soybean meal and SH in the diet increased fat thickness and the USDA yield grade (Neel et al., 2007). Supplementing grass-finished cattle with BLG seems to reduce backfat, USDA yield grade, and marbling score. There is limited evidence in the literature demonstrating how feeding conserved forages affects carcass traits and the nutritional profile of beef (Krusinski et al., 2022d). The present study indicates that including SH in the diet increases weight gain, while HAY and BLG may reduce weight gain, yield grade, and marbling scores.

4.5.3 Beef fatty acids

4.5.3.1 Saturated fatty acid content of beef

No differences in SFAs between groups were seen. Red meat and especially beef are criticized for their high SFA content (Casperson et al., 2020). SFAs increase low-density lipoprotein (LDL) cholesterol, which may increase risks of coronary heart diseases (Billingsley et al., 2018). Based on this, dietary guidelines in the U.S. recommend limiting the intake of SFAs to 10% of daily caloric intake (U.S. Department of Agriculture and U.S. Department of Health and Human Services, 2020). However, not all SFAs have the same health effects. For instance, palmitic acid (C16:0) has a strong LDL cholesterol-raising effect, while stearic acid (C18:0) has a neutral effect on LDL-cholesterol (FAO, 2010;Calder, 2015). While no differences were observed in this study, Baublits et al. (2006) found that supplementing with SH increases C16:0 levels in beef. Based on the feed FA profile, it was expected that HAY and BLG supplementation would increase

the SFA content of beef, especially C16:0. The processing of forages into HAY and BLG generally result in a loss of PUFAs accompanied with an increase in palmitic acid (Van Ranst et al., 2009;Kalač and Samková, 2010). Nevertheless, the lack of significant differences found in the present study might be due to cattle consuming mostly fresh pasture. One limitation of this study is that we did not record the intake of supplemental feeds. Even if the FA profile of the feeds can give us an idea of what to expect in the meat, the gross transfer of dietary SFAs into ruminant products is variable. For example, the transfer of dietary C16:0 into milk fat ranges from 12% to 50% (Loften et al., 2014).

4.5.3.2 Monounsaturated fatty acid content of beef

The MUFA content of beef did not differ between groups. MUFAs make up almost half of beef fat (mostly as oleic acid) (Leheska et al., 2008). Oleic acid consumption has the potential to lower LDL-cholesterol and blood pressure in humans (Calder, 2015). Grain-finished beef usually contains up to 70% more MUFAs than GFB (Duckett et al., 2013;Van Elswyk and McNeill, 2014). In the present study, oleic acid (C18:1 9*c*) was the most abundant FA. The only difference observed was in concentrations of C16:1 9*t* which were lower in beef from the BLG-SH group compared to the other three groups. O'Callaghan et al. (2019) observed that adding SH to a concentrate diet resulted in lower MUFA content in milk. Further, Baublits et al. (2006) found that supplementing cattle diet with SH led to lower levels of C16:1 9*t*. Ruminant *trans*-FAs are produced by the isomerization of MUFAs in the rumen (Vargas-bello-pérez and Garnsworthy, 2013), and grass feeding generally leads to a more favorable rumen pH which allows for more efficient biohydrogenation and isomerization (French et al., 2000;Kraft et al., 2008;Krusinski et al., 2022d). Thus, the higher amount of C16:1 9*t* found in the groups with fresh GRASS was expected. The health effects of ruminant *trans*-FAs remain unclear. Some studies reported the antiatherogenic

and anticarcinogenic effects of ruminant *trans*-FAs (Kalač and Samková, 2010), while others reported potential negative health effects (Gebauer et al., 2015;Verneque et al., 2020).

4.5.3.3 Polyunsaturated fatty acid content of beef

More differences between groups were observed for PUFAs. Overall, beef from the G-HAY group contained more PUFAs than the other three groups. The concentration of *n*-6 PUFAs did not differ between groups, so the variations in PUFA content were due to differences in *n*-3 PUFAs. Both *n*-6 and *n*-3 PUFAs are of interest for human health. Consumption of long-chain *n*-3 PUFAs have anti-inflammatory potential, while *n*-6 PUFAs are generally considered pro-inflammatory (Simopoulos, 2006). This makes the *n*-6:*n*-3 ratio a crucial metric to determine the health effects of a food (Simopoulos, 2002;2006). In the present study, beef from BLG-SH contained less ALA than beef from G-HAY. This was expected since fresh forages contain 50-75% *n*-3 PUFAs, mostly as ALA (Dewhurst et al., 2006). It appears that the addition of SH to the cattle diet reduced the amount of ALA in beef. This finding is supported by results published by Baublits et al. (2006). They reported that the addition of SH to forages resulted in a decrease in *n*-3 PUFAs, especially ALA. Regarding long-chain *n*-3 PUFAs (EPA, DPA, DHA), beef from the G-HAY group contained higher levels of these beneficial FAs than the three other groups. The European Commission considers a food "a source of *n*-3 PUFAs" if 100 g of the food contains at least 40 mg of EPA+DHA or 0.3 g of ALA (Commission Regulation of European Union, 2010). Even if the EPA+DHA content in beef of all four groups were below the limit to qualify as a "source of *n*-3 PUFAs," beef from G-HAY was the closest to meet these standards and can contribute to the intake of these long-chain *n*-3 PUFAs, especially for individuals who have limited access to marine foods (Howe et al., 2006). EPA and DHA are linked to healthier cardiovascular, immune, and cognitive functions (Parolini, 2019;Mendivil, 2021). DPA has been shown to

improve cognitive functions, lower cholesterol, and reduce inflammation (Byelashov et al., 2015). Our results indicate that consuming GFB supplemented with HAY provides higher levels of these long-chain *n*-3 PUFAs compared to the other groups. While lower levels of *n*-3 PUFAs in the groups fed SH were expected, it was surprising to see lower levels of these FAs in the groups fed BLG. Haymaking generally results in the loss of PUFAs because of oxidation and the dropping of leaves compared to BLG (Tripathi et al., 1995;Krusinski et al., 2022d). One explanation might be that animals in the G-BLG group consumed more of their supplemental feed than animals in the G-HAY group. Cattle seem to prefer BLG over HAY (Hunde et al., 2008). If animals consumed more GRASS in the G-HAY group than the G-BLG group, it might explain the differences in long-chain *n*-3 PUFAs.

The Western diet is generally high in *n*-6 PUFAs and low in *n*-3 PUFAs, leading to increased risks of diseases (Simopoulos, 2002). The *n*-6:*n*-3 ratio in the Western diet is estimated to be between 15:1 and 20:1. The optimal *n*-6:*n*-3 ratio to benefit human health is between 1:1 and 4:1 (Simopoulos, 2002;2006). In the present study, the *n*-6:*n*-3 ratio was higher in beef from G-SH and BLG-SH, and lower in beef from G-HAY. Even though we noted significant differences between groups, the *n*-6:*n*-3 ratio was still below 2:1 for all of them. The higher *n*-6:*n*-3 ratio in the groups containing SH was expected due to lower amounts of *n*-3 PUFAs and higher levels of *n*-6 PUFAs in SH compared to the other feeds. Duckett et al. (2009a) found no differences in the *n*-6:*n*-3 ratio when feeding SH to cattle before forage finishing. Baublits et al. (2006), on the other hand, reported a greater *n*-6:*n*-3 ratio when cattle where supplemented with SH. However, the addition of any of the supplemental feeds tested cannot explain the wide variations in GFB found by Bronkema et al. (2019). Increasing the *n*-3 PUFA and CLA content while decreasing the SFA and *n*-6 content are priorities to improve the nutritional quality of beef (Scollan et al., 2006).

4.5.3.4 Biohydrogenation intermediates of beef

Biohydrogenation intermediates including CLA, CLnA, and ADs are formed when LA and ALA undergo biohydrogenation in the rumen (70-95% and 85-100%, respectively) (Lock et al., 2006). No differences in CLnA and AD were observed in this study. The only difference was seen in levels of C18:2 9*c*,11*t*/9*c*,7*t,* with beef from G-HAY containing the most and beef from BLG-SH containing the least. This was expected since GRASS contains more PUFAs than SH. Feeding mostly GRASS to cattle results in a more favorable rumen pH, leading to more efficient biohydrogenation (French et al., 2000;Kraft et al., 2008). However, SH has the potential to increase the CLA content of beef compared to grain-diets, mainly because of their high fiber content resulting in a more optimal rumen pH for biohydrogenation to occur (Kiesling, 2013).

4.5.4 Vitamin E, TBARS, and WBSF

Beef from the three groups fed fresh GRASS contained more vitamin E than beef from BLG-SH. Vitamin E is of interest for human health due to its antioxidant properties (Daley et al., 2010). Duckett et al. (2009a) found no difference in vitamin E levels when pasture was supplemented with SH or not. However, GFB generally contains up to three times more vitamin E than grain-finished beef (Duckett et al., 2009a;Pighin et al., 2016;Logan et al., 2020;Krusinski et al., 2022b). The amount of vitamin E found in GFB is enough to protect the beef from oxidation and extend the shelf-life of meat (De la Fuente et al., 2009;Daley et al., 2010). TBARS is an effective assay to measure lipid oxidation. In the present study, TBARS values were higher in beef from BLG-SH compared to the other three groups. Untrained panelists usually do not detect oxidation flavors until oxidation values reach 2.0 mg MDA/kg of tissue (Greene and Cumuze, 1981). In the current study, only beef from the BLG-SH group exceeded this threshold. Feeding GRASS to cattle usually leads to reduced TBARS values in beef compared to concentrate diets

(Nuernberg et al., 2005). The higher amounts of antioxidants (including vitamin E) present in GFB might explain the better oxidative stability and lower TBARS values (Alothman et al., 2019).

Shear force values were the lowest for beef from BLG-SH compared to beef from G-HAY and G-BLG. Maciel et al. (2021) reported that GFB has higher WBSF values compared to grain-finished beef. These findings indicate that grass-finishing affects the tenderness of beef. Marbling may be a contributing factor to increased meat tenderness (lower WBSF values). Baublits et al. (2006) found no difference in shear force values when including SH to a grass diet. Based on the results of the present study, supplementing the diet of cattle with SH might help with meat tenderness, especially in GFB.

4.6 Conclusions

Based on our findings, we can conclude that SH caused more weight gain in cattle, increased the marbling score of beef, and improved the tenderness of GFB. SH did increase the *n*-6:*n*-3 ratio in beef, but it remained under 2:1. The use of SH as a supplemental feed increased TBARS values as well. Feeding GFB fresh GRASS and HAY resulted in a higher PUFA content, especially higher levels of long-chain *n*-3 PUFAs including EPA, DPA, and DHA. Vitamin E concentrations were also increased in beef on fresh pasture, likely contributing to lower TBARS values (**Figure 12**). In conclusion, none of the supplemental feeds tested in the current study increased the *n*-6:*n*-3 ratio to the values observed previously by Bronkema et al. (2019) in their nutritional survey of commercially available GFB. Future research should investigate other feeds (with different plant species) and determine what ingredients cause large increases in the *n*-6:*n*-3 ratio of GFB. As observed here, the *n*-6:*n*-3 ratio of GFB should remain under 4:1 to benefit human health. GFB has the potential to provide beneficial bioactive compounds for human health including long-chain *n*-3 PUFAs, phenols, and vitamin E.

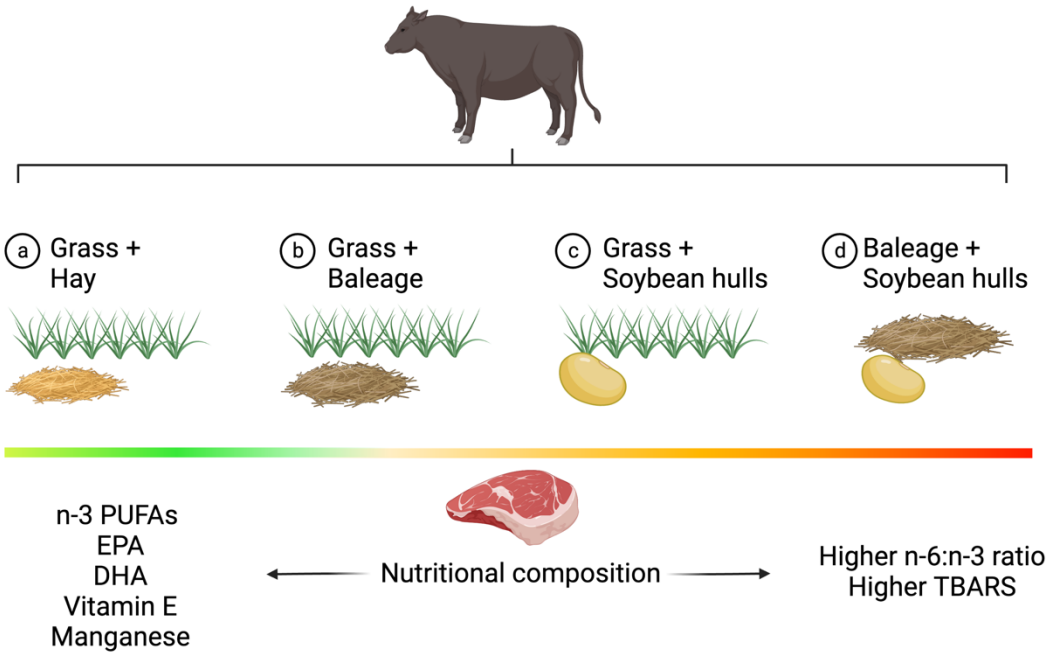

Figure 12. Nutritional composition of grass-finished beef varies by diet. Supplementing grass-finished beef with hay results in higher levels of *n*-3 fatty acids, vitamin E, and manganese. The inclusion of soybean hulls in the diet of grass-finished cattle results in a higher *n*-6:*n*-3 ratio and higher lipid oxidation values. *n*-3 PUFAs: omega-3 polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; TBARS: thiobarbituric acid reactive substances.

CHAPTER 5: MEASURING THE PHYTOCHEMICAL RICHNESS OF MEAT: EFFECTS OF GRASS-, GRAIN-FINISHING SYSTEMS, AND GRAPESEED EXTRACT SUPPLEMENTATION ON THE FATTY ACID AND PHYTOCHEMICAL CONTENT OF BEEF

5.1 Abstract

Grass-finished beef (GFB) has the potential to provide beneficial bioactive compounds for human health including omega-3 polyunsaturated fatty acids (*n*-3 PUFAs), conjugated linoleic acid (CLA), and secondary bioactive compounds such as phytochemicals. The objective of this study was to compare fatty acids (FAs), micronutrients, and phytochemicals of beef fed either a biodiverse pasture (GRASS), a total mixed ration (GRAIN), or a total mixed ration with 5% grapeseed extract (GRAPE). This was a two-year study involving fifty-four Red Angus steers. FAs were analyzed by GC-MS and phytochemicals were analyzed by UPLC-MS/MS. GFB contained higher levels of *n*-3 PUFAs, vitamin E, iron, zinc, stachydrine, hippuric acid, citric acid, and succinic acid than beef from GRAIN and GRAPE ($p < 0.001$ for all). Principal Component Analysis (PCA) identified three clusters most likely corresponding to the three finishing diets. Random Forest analysis showed main phytochemicals capable of predicting cattle diets and Random Forest classification resulted in an error rate of 17.3%. No significant differences were observed in quantified phytochemicals between beef from GRAIN and GRAPE. In conclusion, these results indicate that grass-finishing beef results in higher beneficial bioactive compounds such as *n*-3 PUFAs, micronutrients, and phytochemicals compared to grain-finishing. Additionally, beef phytochemicals can be used to predict cattle finishing diets.

5.2 Introduction

Health organizations recommend reducing red meat consumption for human health and environmental reasons (Willett et al., 2019). Beef is often associated with various metabolic diseases and its production is thought to contribute to climate change (Godfray et al., 2018). While

there are legitimate concerns about the current beef production, putting all beef in the same category may be reductionist (Provenza et al., 2019). Cattle management practices need to be considered when health claims are made. For example, grass-finished beef (GFB) produced in agroecological ways generally aligns with the demands of savvy consumers who are concerned about their health and the environment (Xue et al., 2010). GFB is an important alternative contributing to food sustainability goals, but it remains largely underexplored (Davis et al., 2022). First, GFB contains more omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs), more conjugated linoleic acid (CLA), less omega-6 (*n*-6) PUFAs, and less cholesterol-raising saturated fatty acids (SFAs) than conventional grain-finished beef (Ponnampalam et al., 2006;Alfaia et al., 2009;Van Elswyk and McNeill, 2014;Krusinski et al., 2022b). Second, other health-enhancing phytochemicals (such as polyphenolic compounds) are also thought to be more abundant in GFB compared to grain-finished beef (van Vliet et al., 2021b).

Differences in the nutritional profile between grass- and grain-finished beef have been extensively studied (Garcia et al., 2008;Leheska et al., 2008;Alfaia et al., 2009;Duckett et al., 2009a;Daley et al., 2010;Van Elswyk and McNeill, 2014;Krusinski et al., 2022b). However, these studies mostly focused on fatty acids (FAs), minerals, and vitamins of beef. Phytochemicals are secondary compounds produced by plants which may have health-enhancing properties such as antioxidant and anti-inflammatory effects (Serra et al., 2021;van Vliet et al., 2021b). Phenolic compounds are secondary metabolites derived from plants which contain at least one aryl ring with at least one hydroxyl group attached (O'Connell and Fox, 2001). There are more than 8000 different phenolic compounds, and they can be classified as either non-flavonoids (phenolic acids), flavonoids, or tannins (Cianciosi et al., 2018;Pogorzelska-Nowicka et al., 2018;Serra et al., 2021;Krusinski et al., 2022d). While they are non-essential for major biological mechanisms, they

still offer health benefits especially when they act as antioxidants and work as chain breakers or radical scavengers (Kumar et al., 2015;Cianciosi et al., 2018). Polyphenolic compounds may protect cells against oxidative damage leading to the protection against various metabolic diseases caused by oxidative stress (Scalbert et al., 2005). Common food sources that contain significant amounts of polyphenolic compounds include fruits, vegetables, herbs, nuts, cocoa, and tea (Serra et al., 2021). For example, fruits such as apples or grapes contain up to 200-300 mg of polyphenols per 100 g of food (Scalbert et al., 2005). Dietary intake of polyphenols varies by country and eating habits (Del Bo et al., 2019). In the U.S., it was found that the polyphenol intake reflects the low consumption of fruits and vegetables (Huang et al., 2020). Based on these findings, it becomes crucial to identify foods and production practices that can be sources of polyphenols.

Ruminant products may contribute to the dietary intake of polyphenols. It was previously reported that phenolic compounds from the diet accumulate in the milk and meat of ruminants (O'Connell and Fox, 2001;Serra et al., 2021;van Vliet et al., 2021a;van Vliet et al., 2021b). Polyphenols founds in milk and meat mostly come from the plants animals forage on; the polyphenolic profile of milk and meat varies depending on the plant species present in the animal's diet (van Vliet et al., 2021b). Numerous studies reported higher total phenolic content and antioxidant activity in milk and meat from ruminants foraging on pastures compared to concentrate or mixed diets (Gatellier et al., 2004;Lopez-Andres et al., 2014;Chen et al., 2015;Luo et al., 2019). Diverse pastures are usually higher in chlorophyll, carotenoids, and phenols than concentrate grain diets (Krusinski et al., 2022a). More specifically, individual plant species found in diverse pastures have different effects on bioactive compounds. In one study, alfalfa was positively correlated with phenolics, orchard grass was positively correlated with chlorophyll B but negatively correlated with carotenoids, and meadow fescue was negatively correlated with chlorophyll B (Krusinski et

al., 2022a). Different kinds of pastures with differing plant species also display varying polyphenolic profiles (Reynaud et al., 2010). These results indicate that different plant species present in ruminant diets may result in varying transfer rates of bioactive compounds from plants to meat.

There has been interest in using plant byproducts and waste from the food industry to enhance the nutritional profile of meat (Brenes et al., 2008;Muñoz-González et al., 2019;Niderkorn and Jayanegara, 2021;Krusinski et al., 2022c). In wine-producing regions, like Michigan, great quantities of waste and byproducts are generated causing economic and ecological issues (Brenes et al., 2008). Grape byproducts such as grapeseed extracts (GSE) are rich in polyphenols including anthocyanins, proanthocyanins, and flavanols, and can be fed to animals to increase the polyphenolic content and the oxidative stability of FAs in meat (Brenes et al., 2008;Arola-Arnal et al., 2013;Muñoz-González et al., 2019). Feeding GSE directly to animals instead of adding them during the processing stages of meat allows bioactive compounds to remain bioavailable and to be metabolized by the animal (Antonini et al., 2020). A previous study found a dose-dependent increase in muscle polyphenols when GSE were fed to rats (Serra et al., 2013). Further, it was reported that the polyphenols found in grape byproducts were absorbed in sufficient amounts to modulate antioxidant activities in chicken muscles (Brenes et al., 2008). An additional study in chickens found that GSE polyphenols were absorbed and remained bioactive in chicken meat (Muñoz-González et al., 2019). These findings indicate that feeding GSE to animals might result in meat with extended shelf-life, higher levels of PUFAs, and higher concentrations of polyphenols (Serra et al., 2013).

While there were attempts to identify and quantify polyphenolic compounds in goat milk and cheese (Delgadillo-Puga et al., 2019;Delgadillo-Puga and Cuchillo-Hilario, 2021), chicken

meat (Brenes et al., 2008;Muñoz-González et al., 2019), and cow milk (Besle et al., 2010), only a few studies focused on phytochemicals found in beef. A recent study compared the nutritional profiles of GFB and a plant-based alternative (including phenolic compounds) and found a 90% difference between both products (van Vliet et al., 2021a). Additionally, another recent study reported higher levels of polyphenolic compounds in pasture-finished bison compared to grain-finished bison (van Vliet et al., 2023). These studies confirmed that phytochemical compounds from the animal's diet accumulate in their milk and meat, and that it is possible to identify and quantify these bioactive compounds. However, only a few studies focused on differences in phytochemicals between GFB and grain-finished beef. Therefore, the objective of the current study was to compare the vitamins, minerals, FAs, and phytochemicals in beef finished either on a diverse pasture, on grain in feedlot, or on grain in feedlot and supplemented with GSE.

5.3 Materials and methods

This research protocol for animal use and procedures has been approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC #201800155).

5.3.1 Experimental design, animals, and diets

This two-year study was conducted in 2019 and 2020 at the Michigan State University Upper Peninsula Research and Extension Center (UPREC) located in Chatham, MI (latitude: 46°20' N, longitude: 86°55' W; elevation: 271 m). Three treatments were tested: 1) a complex pasture mixture (GRASS), 2) a total mixed feedlot ration (GRAIN), and 3) a total mixed feedlot ration supplemented with 5% (dry matter - DM) GSE for the last 30 days (GRAPE). The experimental design for the GRASS and GRAIN groups was previously described (Maciel et al., 2021;Krusinski et al., 2022b). Seventy (n = 70) and 54 steers (n = 54) (14-20 months of age) were randomly allocated to the diets in 2019 and 2020, respectively. Sixty steers in 2019 and 44 steers

in 2020 were randomly stratified and allocated to one of three pens for each of the GRASS and GRAIN treatments (for each diet and each year, n = 10 animals per pen, three pens). This design was followed in 2019, but due to lower male births in 2020, only two groups with seven steers each were formed for the GRAIN diet. For GRASS and GRAIN, two breeds were initially used: Red Angus and Red Angus x Akaushi crossbreed. For GRAPE, ten Red Angus steers each year were kept in one same feedlot pen.

For this manuscript, only Red Angus beef samples were analyzed. For each year, nine samples per diet were randomly chosen. For GRASS and GRAIN in 2019, three samples per pen per year were randomly picked. The same design was followed for GRASS in 2020. Since only two pens for GRAIN were formed in 2020 due to lower male births, all seven samples were picked with an additional two random samples from 2019. Since GRAPE samples all came from the same pen, nine samples for each year were randomly chosen. The total number of beef samples for this study is 54 (n = 54).

The botanical composition of the diets was described previously (Krusinski et al., 2022a;Krusinski et al., 2022b). Briefly, the plant species found in GRASS were meadow fescue (*Schedonorus pratensis* (Huds.) P. Beauv.), red clover (*Trifolium pratense* L.), timothy grass (*Phleum pratense*), alfalfa (*Medicago sativa*), white clover (*Trifolium repens* L.), birdsfoot trefoil (*Lotus corniculatus*), chicory (*Cichorium intybus*), orchardgrass (*Dactylis glomerata* L.), and dandelion (*Taraxacum oficinale* L.). The GRAIN diet was composed of orchardgrass hay, dry corn, high moisture corn, and pellets (36% crude protein). The nutritional profiles of the GRASS and GRAIN diets were previously described in detail by Krusinski et al. (2022a). For GRAPE, 5% of GSE (DM basis) was added to GRAIN during the last 30 days of the finishing period. GSE was obtained from Pioneer Enterprises (Lewiston, ID, USA).

5.3.2 Sample collection

Each year, animals were slaughtered on the same day in a United States Department of Agriculture (USDA) regulated facility (16-18 months old for GRAIN and GRAPE and 24-26 months old for GRASS). Ribeye samples were collected between the 11^{th} and 13^{th} rib on the left side of the carcass. Beef samples were then further processed at the Michigan State University Meat Laboratory; steaks were cut in 1 × 1 cm cubes and flash frozen with liquid nitrogen. Beef samples were stored at -80 °C until further analysis.

Feed samples were collected every two weeks. GRASS samples were collected by randomly clipping three 0.25 m^2 quadrats to a 5 cm stubble in pre-grazing areas. GRAIN and GRAPE samples were collected every two weeks from the mixers and composited by month for each diet as described previously (Krusinski et al., 2022a). For FA analysis, the number of samples was 21 GRASS, 15 GRAIN, and 7 GRAPE in 2019 and 24 GRASS, 10 GRAIN, and 5 GRAPE.

5.3.3 Proximate analysis

Feed samples underwent proximate analysis based on protocols described by Maciel et al. (2021) and Krusinski et al. (2022a). Briefly, samples were dried in a forced-air oven at 105 °C for 8 h. Ash content was determined by oxidizing feed samples at 500 °C in a muffle furnace. Protocols by Mertens (2002) were followed to determine neutral detergent fiber (NDF) with the addition of amylase and sodium sulfite. Acid detergent fiber (ADF) was analyzed following protocols described in AOAC (2000). Crude protein (CP) was measured as previously described (Hach et al., 1987). Finally, gross energy was determined using a bomb calorimeter.

5.3.4 Fatty acid analysis

The protocol was described previously (Sergin et al., 2021;Krusinski et al., 2022a;Krusinski et al., 2022b;Krusinski et al., 2022c). Chemicals for FA analysis were purchased

from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. For extraction, a microwave-assisted method was used as described by Bronkema et al. (2019). Lyophilized and ground feed samples and minced beef samples were mixed with 8 mL 4:1 ethyl acetate:methanol (*v/v*) and underwent extraction in a CEM Mars 6 microwave digestion system equipped with a 24-vessel rotor and GlassChem tubes (CEM Corp., Matthews, NC, USA). Samples were then filtered using Whatman filter paper grade 597 into another set of test tubes containing 3.5 mL HPLC water and centrifuged to separate the top organic layer. The top layer was transferred to another set of tubes and evaporated under nitrogen gas. Samples were reconstituted with 4:1 dichloromethane:methanol (*v/v*) to reach a concentration of 20 mg of oil/mL.

A modified protocol from Jenkins (2010) was used for the creation of FA methyl esters (FAMEs). Briefly, 100 μL containing 2 mg of oil was resuspended in toluene with 20 μg of internal standard (methyl 12-tridecenoate, U-35M, Nu-Chek Prep, Elysian, MN, USA). After resuspending, 2 mL of 0.5 N anhydrous potassium methoxide was added before being heated at 50 °C for 10 min. Then, 3 mL of methanolic HCl (5%) was added before heating the samples at 80 °C for 10 min. After cooling down, 2 mL of HPLC water and 2 mL of hexane were added, and samples were centrifuged at 2,500 RPM for 5 min. The top layer was removed and dried under nitrogen gas. Next, FAMEs were resuspended in 1 mL of isooctane (final concentration was 2 mg/mL). Samples were then transferred to gas chromatography-mass spectrometry (GC-MS) vials with glass inserts.

For the quantification of FAMEs, a PerkinElmer (Waltham, MA, USA) 680/600S GC-MS in electron impact mode (70 eV) was used. The GC-MS was equipped with a HP-88 column (100 m, 0.25 mm ID, 0.2 μM film thickness) from Agilent Technologies (Santa Clara, CA, USA). One μL of feed samples was injected (GC temperature: 250 °C). One μL of beef samples was injected

twice (20:1 split) (GC temperatures: 175 °C and 150 °C). For detailed temperature settings, please see Krusinski et al. (2022c). A third injection in splitless mode followed for beef samples. This GC-MS method was adapted from Kramer et al. (2008). The carrier gas was helium (1 mL/min), and MS data were recorded in full scan mode (m/z 70-400 amu).

Identification of FAMEs was performed using MassLynx V4.1 SCN 714 (Water Corp., Milford, MA, USA). Retention time and EI mass fragmentation were compared to a reference standard containing Supelco 37 Component FAME Mix with mead acid, docosatetraenoic acid, *n*-3 DPA, *n*-6 DPA, and palmitelaidic acid (Cayman Chemical, Ann Arbor, MI, USA). The CLA standard UC-59M from Nu-Chek Prep (Elysian, MN, USA) was used for the identification of CLA isomers. For FAs not included in the standards, elution order and EI mass fragmentation were used to identify them. FAMEs were quantified by using a standard curve including reference and internal standards.

5.3.5 Vitamin E and mineral analysis of beef

The protocols for vitamin E and mineral analysis of beef were previously reported (Bronkema et al., 2019;Krusinski et al., 2022c). For vitamin E, the protocol was adapted from Rettenmaier and Schüep (1992). In brief, 1 g of beef was homogenized with 5 mL of water. Proteins were precipitated with ethanol, and fat-soluble vitamins were extracted using hexane. Samples were centrifuged and the top layer was evaporated. Evaporated samples were resuspended in the chromatographic mobile phase and placed in vials. A six-point curve was made by serial diluting a vitamin E solution (Sigma-Aldrich, St. Louis, MO, USA) in ethanol (50 μg/mL to 0.2 μg/mL). A Waters Acquity system equipped with a Symmetry C18, 1.7 μm, 2.1 × 50 mm analytical column and Water Empower Pro Chromatography Manager software (Water Corp., Milford, MA, USA) were used for chromatography analysis. The mobile phase was acetonitrile:methylene

chloride:methanol (70:20:10, *v/v/v*) and the flow rate was 0.5 mL/min. Detection was performed at UV absorption 292 nm.

The mineral analysis protocol was adapted from Wahlen et al. (2005). Beef samples were dried and digested overnight in an oven at 95 °C using ten times the dry tissue mass of nitric acid. Samples were then diluted with water to 100 times the dried tissue mass. The Agilent 7900 Inductivity Coupled Plasma-Mass Spectrometer (ICP-MS) (Agilent Technologies, Inc., Santa Clara, CA, USA) was used. A six-point calibration curve and bovine liver and mussel standards (National Institute of Standards and Technology, Gaithersburg, MD, USA) were used as controls.

5.3.6 Polyphenolic profiling

In preparation for analysis, feed samples were freeze-dried in a Harvest Right Home Freeze Dryer (Harvest Right, North Salt Lake, UT, USA) for 18.5 h and ground through a 1 mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA) with dry ice as reported by Krusinski et al. (2022a). Beef samples were minced and pulverized on dry ice using a mortar and pestle. UHP-LC-MS-grade acetonitrile, methanol, DMSO, formic acid and water (Supelco LiChrosolv®) were ordered from Sigma-Aldrich (St. Louis, MO, USA). A QReSS™ internal standards kit, containing [U-$^{13}C_6$] phenylalanine, was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Purified standards of compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA) and/or Cayman Chemical (Ann Arbor, MI).

The protocol by van Vliet et al. (2023) was followed for this analysis. Briefly, 200 mg of pulverized beef and 50 mg of feed samples were mixed with 1000 μL and 500 μL, respectively of methanol. At this time, 10 μL of QReSS internal standard (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) were added to the samples. Proteins were then precipitated under vigorous shaking for 10 min in a QIAGEN TissueLyser II with two 5 mm glass beads (QIAGEN Sciences,

Germantown, MD, USA). Samples were then centrifuged at 23,000 × g for 10 min at 4 °C. Supernatants were removed and transferred to a new set of tubes. For beef samples, 2 mL of water with 1% formic acid was added. For feed samples, 1 mL was added. Strata C18-E cartridges (Phenomenex, Torrance, CA, USA) were used for solid phase extraction (SPE). Cartridges were activated with 1 mL of methanol with 1% formic acid before being washed with 1 mL of water with 1% formic acid. Samples were then loaded onto the cartridges. After passing the samples through the cartridges, beef samples were washed with 2 mL of water with 1% formic acid while feed samples were washed with 1.2 mL of water with 1% formic acid. Beef and feed samples were eluted with 1.2 mL of methanol in 0.1% formic acid. Samples were then evaporated under a gentle stream of nitrogen gas before being reconstituted with 100 and 200 μL of methanol in 1.5 mL LC-MS amber vials (Agilent, Santa Clara, CA, USA) with 250 μL glass inserts.

Compounds were simultaneously detected as precursor ion/product ion pair using multiple reaction monitoring (MRM) using ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The platform utilized an SCIEX Hybrid Triple Quad 7500 (Framingham, MA, USA) with a front-end Shimadzu Nexera 40 Series (Kyoto, Japan) liquid chromatography system. The sample extracts were kept at 10 °C in an auto-sampler and compounds were separated at 30 °C using a reverse phase Kinetex F5 100A column (2.1 mm × 150 mm, 2.6 μM) column from Phenomenex (Torrance, CA, USA) with binary mobile phases of water (A) and acetonitrile (B), both containing 0.1% formic acid (*v/v*). Samples were run in both negative and positive electrospray ionization mode. The following source parameters were used in negative mode: 1,600 V for the ionspray voltage, 550 °C for the temperature, 40 psi for the curtain gas, 40 psi for the nebulizer gas (GS1), 60 psi for the heating gas (GS2). In the negative mode, the linear gradient consisted of an initial composition of 5% B for 2.1 min with a flow rate of 0.2

mL/min, which was ramped up gradually to 95% B and a maximum flow rate of 0.46 mL/min over 14 min to keep a constant pressure, prior to being switched to 5% B for the final 4 minutes with a minimum flow rate of 0.175 mL/min.

The following source parameters were used in positive mode: 2,000 V for the ionspray voltage, 550 °C for the temperature, 40 psi for the curtain gas, 40 psi for the nebulizer gas (GS1), 60 psi for the heating gas (GS2). In the positive mode, the linear gradient consisted of an initial composition of 5% B for 2.1 min with a flow rate of 0.2 mL/min, which was ramped up gradually to 95% B and a maximum flow rate of 0.46 mL/min over 14 min to keep a constant pressure, prior to being switched to 5% B for the final 4 minutes with a minimum flow rate of 0.175 mL/min. For both modes, a pooled matrix sample (sample generated by taking a small volume from samples from different experimental conditions), a double blank (100% methanol), and mixture of purified standards of target compounds was injected using an unscheduled method to determine presence of compounds in the matrix sample and their retention times for the scheduled method. In both modes, the cycling time in the scheduled method was set to 1000 msec and the dwell time ranged from 3 to 250 msec depending on the number of MRMs triggered. Double blank (100% methanol) and blank internal standard samples (methanol spiked with [U-$^{13}C_6$] phenylalanine) were ran every 15 samples for quality control purposes.

Analyst 3.1 software (AB Sciex, Framingham, MA, USA) was used to acquire and analyze the chromatographic data. Peaks were integrated using area-under-the-curve and normalization was performed using [U-$^{13}C_6$] phenylalanine as the internal standard to account for any loss of material during sample preparation. Unlabeled external standard mixes were run in parallel to the samples with known concentrations of the different metabolites to allow for quantitation (in mg/100 g) of various compounds with relevant nutritive value and for which a standard was

available. For compounds with no relevant nutritive value or for which no standard was ran concurrently, the data is expressed as arbitrary units (AU).

5.3.7 Statistical analysis

The statistical analysis was performed using RStudio (R Core Team, Vienna, Austria). A linear regression model was used to test the effect of diet on fatty acids, micronutrients, and quantified phytochemicals in beef. Diet, year, and pen were considered fixed effects. The experimental unit was each animal. Tukey's HSD was used for post hoc comparison and results were considered significant at $p < 0.05$. Values below the limit of detection were treated as zeroes for all analyses. Results were reported as mean ± standard error from the mean (SEM). Principal Component Analysis (PCA) and Random Forest (RF) analysis were conducted using MetaboAnalyst 5.0 (metaboanalyst.ca) as described previously (van Vliet et al., 2021a). For these, phytochemical compounds were first normalized to mass and then log transformed. The goal was to visualize data sets and identify the top metabolites that discriminate between groups using mean decrease accuracy.

5.4 Results

5.4.1 Nutritional composition of the diets

The proximate composition and the FA profile of the diets are displayed in **Table 16**. Overall, significant differences were seen between GRASS and the other two diets. GRAIN and GRAPE did not significantly differ. The two diets containing TMR, GRAIN and GRAPE, contained significantly more DM than GRASS ($p < 0.001$). On the other hand, GRASS contained significantly more ash, CP, NDF, ADF, and gross energy compared to the other two diets ($p < 0.001$). Regarding FAs, GRASS contained significantly more SFAs compared to GRAIN and GRAPE ($p < 0.001$). More specifically, differences were observed for C12:0 through C15:0 and

C20:0 through C24:0. GRAIN and GRAPE contained almost five times more MUFAs than GRASS ($p < 0.001$), even though GRASS displayed more palmitoleic acid (C16:1). GRAIN and GRAPE contained seven times more oleic acid than GRASS ($p < 0.001$). Significant differences were also observed for PUFAs with GRASS containing more total PUFAs than GRAIN and GRAPE ($p < 0.001$). More specifically, GRASS contained fifteen times more *n*-3 PUFAs compared to the other two diets ($p < 0.001$), while GRAIN and GRAPE contained significantly higher levels of *n*-6 PUFAs compared to GRASS ($p < 0.001$). This was reflected in the *n*-6:*n*-3 ratio of the diets with GRAIN and GRAPE having a ratio 63 and 75 times higher than GRASS, respectively ($p < 0.001$).

Table 16. Nutritional composition of the diets

	GRASS[1]	GRAIN[2]	GRAPE[3]	*p*-value
C10:0	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.216
C12:0	0.21 ± 0.02 [a]	0.03 ± 0.00 [b]	0.03 ± 0.01 [b]	< 0.001
C14:0	0.66 ± 0.05 [a]	0.07 ± 0.00 [b]	0.07 ± 0.00 [b]	< 0.001
C15:0	0.13 ± 0.01 [a]	0.03 ± 0.00 [b]	0.03 ± 0.00 [b]	< 0.001
C16:0	14.13 ± 0.22	13.82 ± 0.20	13.35 ± 0.17	0.156
C16:1 *c*9	0.18 ± 0.02 [a]	0.09 ± 0.01 [b]	0.09 ± 0.01 [b]	< 0.010
C16:1 *c*7	1.06 ± 0.04 [a]	0.14 ± 0.01 [b]	0.12 ± 0.02 [b]	< 0.001
C17:0	0.20 ± 0.01 [a]	0.06 ± 0.00 [b]	0.07 ± 0.00 [b]	< 0.001
C18:0	1.73 ± 0.07	1.71 ± 0.05	1.67 ± 0.05	0.898
C18:1 *c*9	3.17 ± 0.26 [b]	22.27 ± 0.14 [a]	22.15 ± 0.31 [a]	< 0.001
C18:1 *c*11	0.52 ± 0.05	0.52 ± 0.01	0.48 ± 0.01	0.896
C18:2 *n*-6 (LA)[4]	14.09 ± 0.42 [b]	56.11 ± 0.57 [a]	57.03 ± 0.64 [a]	< 0.001
C18:3 *n*-3 (ALA)[5]	61.60 ± 0.93 [a]	4.29 ± 0.30 [b]	3.95 ± 0.66 [b]	< 0.001
C20:0	0.62 ± 0.03 [a]	0.42 ± 0.02 [b]	0.41 ± 0.04 [b]	< 0.001
C22:0	0.75 ± 0.04 [a]	0.17 ± 0.01 [b]	0.17 ± 0.01 [b]	< 0.001
C24:0	0.74 ± 0.03 [a]	0.23 ± 0.01 [b]	0.22 ± 0.01 [b]	< 0.001
∑ SFA[6]	19.36 ± 0.38 [a]	16.57 ± 0.27 [b]	16.03 ± 0.24 [b]	< 0.001
∑ OCFA[7]	0.33 ± 0.01 [a]	0.09 ± 0.00 [b]	0.10 ± 0.00 [b]	< 0.001
∑ MUFA[8]	4.94 ± 0.25 [b]	23.02 ± 0.13 [a]	22.98 ± 0.30 [a]	< 0.001
∑ PUFA[9]	75.71 ± 0.57 [a]	60.41 ± 0.32 [b]	60.99 ± 0.34 [b]	< 0.001
∑ *n*-6[10]	14.09 ± 0.42 [b]	56.12 ± 0.57 [a]	57.04 ± 0.63 [a]	< 0.001
∑ *n*-3[11]	61.62 ± 0.93 [a]	4.29 ± 0.30 [b]	3.95 ± 0.66 [b]	< 0.001
n-6:*n*-3 ratio[12]	0.24 ± 0.01 [b]	15.12 ± 1.28 [a]	18.42 ± 2.34 [a]	< 0.001

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$. Fatty acids reported as % of total. [1]GRASS: diverse pasture; [2]GRAIN: total mixed ration (TMR); [3]GRAPE: TMR + 5% DM grapeseed extract; [4]LA: linoleic acid; [5]ALA: α-linolenic acid; [6]∑ SFA = all saturated FAs (10:0-24:0); [7]∑ OCFA = all odd chain FAs (13:0, 15:0, 17:0); [8]MUFA = all monounsaturated FAs (16:1, 18:1); [9]∑ PUFA = LA + ALA + C20:3 *n*-3; [10]∑ *n*-6 = LA; [11]∑ *n*-3 = ALA + C20:3 *n*-3; [12]*n*-6:*n*-3 ratio = ∑ *n*-6/∑ *n*-3; [*]reported in %DM, [13]DM: dry matter (%); [14]CP: crude protein; [15]NDF: neutral detergent fiber; [16]ADF: acid detergent fiber; [17]Energy (cal/g).

Table 16. (cont'd)

	GRASS[1]	GRAIN[2]	GRAPE[3]	*p*-value
DM[13]	21.38 ± 0.63 [b]	79.80 ± 0.97 [a]	82.36 ± 1.32 [a]	< 0.001
Ash[*]	6.60 ± 0.17 [a]	4.02 ± 0.20 [b]	3.79 ± 0.34 [b]	< 0.001
CP[14*]	13.40 ± 0.47 [a]	9.65 ± 0.18 [b]	9.29 ± 0.32 [b]	< 0.001
NDF[15*]	51.82 ± 0.92 [a]	21.02 ± 0.39 [b]	20.59 ± 0.62 [b]	< 0.001
ADF[16*]	33.38 ± 0.72 [a]	10.09 ± 0.26 [b]	9.94 ± 0.39 [b]	< 0.001
Energy[17]	4465.49 ± 11.98 [a]	4265.90 ± 15.98 [b]	4274.76 ± 28.55 [b]	< 0.001

5.4.2 Fatty acid and micronutrient content of beef

5.4.2.1 Fatty acids

The FA profile of beef by diet is displayed in **Table 17**. No significant differences were observed for total SFAs and individual SFAs ($p > 0.05$). Regarding MUFAs, no significant differences were reported for total MUFAs, but beef from the GRAIN group contained more C14:1 9*c* than beef from GRASS, with beef from GRAPE not containing significantly different concentrations of this MUFA compared to the other two groups ($p < 0.05$). Additionally, significant differences were observed for C16:1 9*t* and C18:1 11*t* with beef from the GRASS group having higher concentrations of these FAs compared to beef from the other two groups ($p < 0.001$). Most differences were seen for *n*-3 and *n*-6 PUFAs. Beef from GRASS contained ~4.5 times and ~6 times more total *n*-3 PUFAs than beef from GRAIN and GRAPE, respectively ($p < 0.001$). More specifically, beef from GRASS contained higher concentrations of ALA, EPA, and DPA than beef from the other two groups ($p < 0.001$). For DHA, significant differences were observed between beef from GRASS and beef from GRAIN, but not for beef from GRAPE ($p < 0.05$). For total *n*-6 PUFAs ($p < 0.05$) and some individual *n*-6 FAs, significant differences were observed with beef from GRAIN containing more of this class of PUFAs than GRASS, while GRAPE did not significantly differ from the other two groups. These differences were reflected in the *n*-6:*n*-3

ratio with beef from GRASS having the lowest ratio (1.65:1) and beef from GRAIN (8.39:1) and GRAPE (9.82:1) having the highest ($p < 0.001$). Finally, no significant differences were observed for CLA, AD, and total FA ($p > 0.05$).

Table 17. Fatty acid profile of beef by diet (mg per 100 g beef)

		GRASS[1]	GRAIN[2]	GRAPE[3]	*p*-value
∑ SFA[4]		767.56 ± 188.89	996.16 ± 194.19	868.22 ± 266.53	0.700
	C12:0	1.04 ± 0.25	1.09 ± 0.26	0.63 ± 0.35	0.527
	C14:0	31.29 ± 10.15	53.81 ± 10.43	49.95 ± 14.32	0.287
	C15:0	8.16 ± 2.23	3.97 ± 2.29	3.34 ± 3.15	0.330
	C16:0	424.06 ± 107.99	681.64 ± 111.01	564.21 ± 152.37	0.261
	C17:0	26.17 ± 8.62	10.18 ± 8.86	23.16 ± 12.16	0.403
	C18:0	272.22 ± 66.47	242.23 ± 68.33	224.09 ± 93.79	0.907
∑ MUFA[5]		748.55 ± 194.06	944.75 ± 188.04	1076.92 ± 258.41	0.572
	C14:1 9*c*	5.57 ± 3.30 [b]	19.10 ± 3.39 [a]	13.67 ± 4.65 [a,b]	< 0.050
	C16:1 9*c*	60.47 ± 54.83	117.74 ± 56.37	240.76 ± 77.37	0.174
	C16:1 9*t*	3.51 ± 0.33 [a]	1.08 ± 0.20 [b]	1.05 ± 0.27 [b]	< 0.001
	C18:1 9*c*	565.28 ± 148.34	733.76 ± 152.50	732.59 ± 209.30	0.694
	C18:1 9*t*	2.24 ± 0.95	1.44 ± 0.27	1.75 ± 0.36	0.578
	C18:1 11*t*	36.69 ± 11.11 [a]	1.54 ± 2.02 [b]	1.70 ± 2.70 [b]	< 0.001
∑ PUFA[6]		113.84 ± 12.60	111.65 ± 12.95	95.29 ± 17.78	0.669

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$. [1]GRASS: beef fed a diverse pasture; [2]GRAIN: beef fed a total mixed ration (TMR); [3]GRAPE: beef fed TMR + 5% DM grapeseed extract; [4]∑ SFA = all saturated FAs (10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 22:0); 5∑ MUFA = all monounsaturated FAs (14:1, 16:1, 17:1, 18:1, 20:1); [6]∑ PUFA = LA + ALA + GLA + Eicosadienoic + Eicosatrienoic + DGLA + Mead + Arachidonic + EPA + DTA + DPA *n*-3 + DHA; [7]∑ *n*-3 = ALA + EPA + DHA + DPA *n*-3 + Eicosatrienoic; [8]ALA: α-linolenic acid; [9]EPA: eicosapentaenoic acid; [10]DPA: docosapentaenoic acid; [11]DHA: docosahexaenoic acid; [12]∑ *n*-6 = LA + GLA + Eicosadienoic + DGLA + Arachidonic + DTA; [13]LA: linoleic acid; [14]*n*-6:*n*-3 ratio = ∑ *n*-6/∑ *n*-3; [15]∑ CLA = sum of conjugated linoleic acid isomers (*c*9, *t*11/*t*7, *c*9 18:2 + *t*11, *c*13 18:2 + *t*11, *t*13 18:2 + *t,t* 18:2); [16]∑ AD (Atypical Dienes) = sum of non-conjugated linoleic acid isomers (*t*11, *t*15 18:2 + *t*9, *t*12 18:2 + *c*9, *t*14/*c*9, *t*13 18:2 + *t*11, *c*15 18:2 + *c*9, *t*16 18:2 + *c*9, *c*15 18:2 + *c*12, *c*15 18:2); [17]∑ CLnA = sum of conjugated linolenic acid isomers (*c*9, *t*11, *t*15 18:3 + *c*9, *t*11, *c*15 18:3); [18]∑ FA = sum all of FAs.

Table 17. (cont'd)

	GRASS[1]	GRAIN[2]	GRAPE[3]	*p*-value
∑ *n*-3[7]	46.03 ± 4.79 [a]	10.13 ± 4.92 [b]	7.73 ± 6.76 [b]	< 0.001
C18:3 *n*-3 (ALA)[8]	24.32 ± 3.70 [a]	2.32 ± 3.81 [b]	2.22 ± 5.23 [b]	< 0.001
C20:3 *n*-3	0.30 ± 0.07 [a]	0.03 ± 0.07 [b]	0.09 ± 0.09 [a,b]	< 0.050
C20:5 *n*-3 (EPA)[9]	7.41 ± 0.38 [a]	1.80 ± 0.39 [b]	1.10 ± 0.53 [b]	< 0.001
C22:5 *n*-3 (DPA)[10]	13.39 ± 0.96 [a]	5.67 ± 0.99 [b]	3.96 ± 1.36 [b]	< 0.001
C22:6 *n*-3 (DHA)[11]	0.61 ± 0.08 [a]	0.32 ± 0.08 [b]	0.35 ± 0.11 [a,b]	< 0.050
∑ *n*-6[12]	67.07 ± 9.04 [b]	100.32 ± 9.29 [a]	86.61 ± 12.73 [a,b]	< 0.050
C18:2 *n*-6 (LA)[13]	46.95 ± 7.07	65.61 ± 7.27	57.47 ± 9.97	0.195
C18:3 *n*-6	0.29 ± 0.05	0.40 ± 0.05	0.42 ± 0.07	0.243
C20:2 *n*-6	0.33 ± 0.09	0.57 ± 0.10	0.55 ± 0.13	0.186
C20:3 *n*-6	3.34 ± 0.49 [b]	5.87 ± 0.50 [a]	4.61 ± 0.69 [a,b]	< 0.010
C20:4 *n*-6	14.61 ± 1.51 [b]	22.53 ± 1.56 [a]	19.21 ± 2.14 [a,b]	< 0.010
C22:4 *n*-6	1.56 ± 0.49 [b]	5.34 ± 0.51 [a]	4.36 ± 0.70 [a]	< 0.001
n-6:*n*-3 ratio[14]	1.65 ± 0.44 [b]	8.39 ± 0.45 [a]	9.82 ± 0.62 [a]	< 0.001
∑ CLA[15]	5.14 ± 1.20	2.28 ± 1.23	2.86 ± 1.69	0.243
C18:2 9*c*,11*t*/9*c*,7*t*	4.01 ± 1.05	1.63 ± 1.08	1.96 ± 1.48	0.269
∑ AD[16]	18.07 ± 5.07	9.29 ± 5.21	17.01 ± 7.16	0.438
∑ CLnA[17]	0.36 ± 0.05	0.25 ± 0.05	0.25 ± 0.07	0.292
C18:3 9*c*,11*t*,15*t*	0.12 ± 0.03 [a]	0.02 ± 0.03 [b]	0.05 ± 0.04 [a,b]	< 0.050
C18:3 9*c*,11*t*,15*c*	0.24 ± 0.03	0.23 ± 0.03	0.20 ± 0.04	0.758
∑ FA[18]	1686.08 ± 400.01	2080.23 ± 399.93	2090.96 ± 549.44	0.495

5.4.2.2 Micronutrients

Main micronutrients concentrations in beef by diet are shown in **Figure 13**. Beef from GRASS contained significantly more vitamin E, iron, and zinc compared to beef from the other two groups ($p < 0.001$). No significant differences were observed between beef from GRAIN and beef from GRAPE ($p > 0.05$).

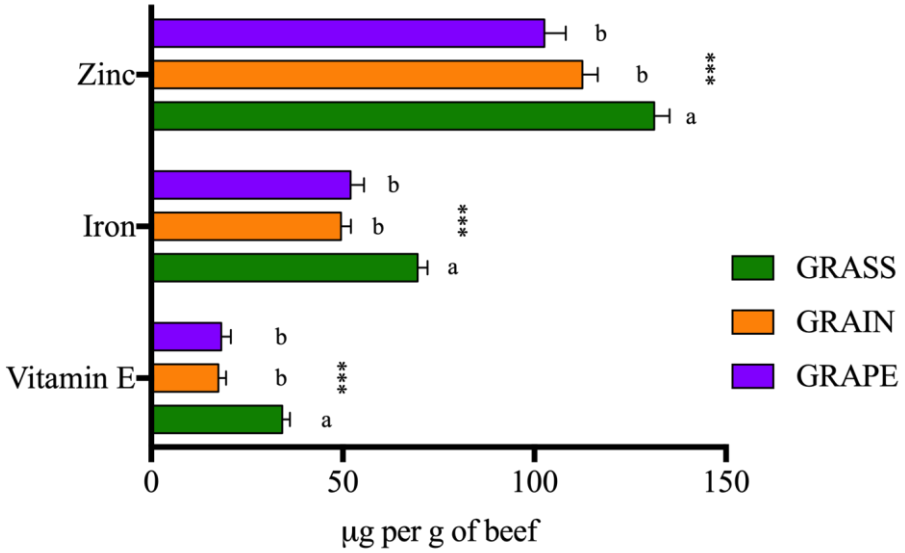

Figure 13. Main micronutrients concentrations in beef by diet (µg per g of beef). Values are reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$ ('***' $p < 0.001$). GRASS: beef fed a diverse pasture; GRAIN: beef fed a total mixed ration (TMR); GRAPE: beef fed TMR + 5% DM grapeseed extract.

5.4.3 Phytochemical profile of beef

5.4.3.1 Data visualization and identification of top discriminating compounds

Results from the PCA and RF analysis of the phytochemical data are displayed in **Figure 14**. PCA (**A**) displayed slight separation between beef from GRASS, GRAIN, and GRAPE as shown by the 30% variation along principal component 1. Three clusters corresponding to the three finishing diets can be observed on the PCA plot, but with some overlaps. The RF plot (**B**) showed distinctions in specific phytochemicals in beef by diets. Stachydrine and succinic acid

were the two most discriminating phytochemicals, followed by citric acid, 4-hydrobenzoic acid, allantoin, and vanillic acid. RF classification also showed good prediction of group with an overall out of bag (OOB) error rate of 17.3%, but with some difficulty in predicting the GRAIN group (37.5% class error) (**Table 18**).

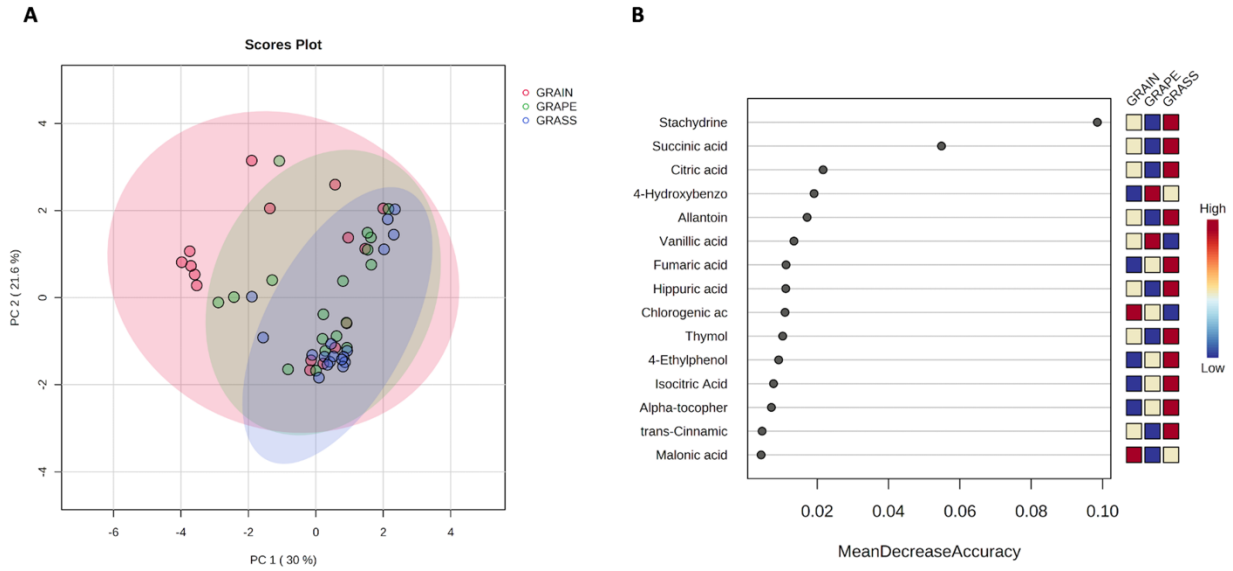

Figure 14. Principal Component Analysis (PCA) and Random Forest (RF) plots. (**A**) PCA plot showing separation and clusters based on finishing diet with some overlaps. (**B**) RF variable importance plot showing main phytochemicals capable of discriminating beef based on finishing diet. The y-axis represents phytochemicals in order of importance for group classification (from top to bottom). The x-axis shows mean decrease accuracy, with a higher value indicating the importance of that phytochemical in predicting groups.

Table 18. Random Forest Classification of beef by diet

	GRASS[1]	GRAIN[2]	GRAPE[3]	Class Error	OOB[4]
GRASS	17	1	0	5.6%	
GRAIN	0	10	6	37.5%	17.3%
GRAPE	0	2	16	11.1%	

[1]GRASS: beef fed a diverse pasture; [2]GRAIN: beef fed a total mixed ration (TMR); [3]GRAPE: beef fed TMR + 5% DM grapeseed extract; [4]OOB: Overall out of bag error rate.

5.4.3.2 Differences in quantified phytochemicals in beef

Quantified phytochemicals in beef by diet (in mg per 100 g of beef) are displayed in **Table 19**. Beef from GRASS contained significantly higher levels of stachydrine, hippuric acid, citric acid, succinic acid, and fumaric acid compared to beef from GRAIN and GRAPE ($p < 0.001$). These results complete the findings from the RF analysis reported above. Beef from GRAIN and GRAPE contained higher concentrations of p-coumaric acid than beef from GRASS ($p < 0.05$). No significant difference by diet were observed for the rest of the quantified phytochemicals ($p > 0.05$).

Table 19. Quantified phytochemicals in beef by diet (mg per 100 g beef)

	GRASS[1]	**GRAIN[2]**	**GRAPE[3]**	***p*-value**
Stachydrine	0.65 ± 0.04 [a]	0.26 ± 0.05 [b]	0.28 ± 0.06 [b]	< 0.001
4-Ethylphenol	3.92 ± 0.44	3.00 ± 0.45	3.83 ± 0.60	0.287
Hippuric acid	13.37 ± 1.20 [a]	7.77 ± 1.24 [b]	5.52 ± 1.66 [b]	< 0.001
Citric acid	379.20 ± 35.20 [a]	79.10 ± 36.30 [b]	51.40 ± 48.70 [b]	< 0.001
Succinic acid	36.80 ± 2.66 [a]	17.80 ± 2.74 [b]	13.70 ± 3.67 [b]	< 0.001
Fumaric acid	2.68 ± 0.34 [a]	0.90 ± 0.35 [b]	0.62 ± 0.47 [b]	< 0.001
Caffeic acid	0.04 ± 0.02	0.04 ± 0.02	0.01 ± 0.03	0.570
p-Coumaric acid	1.56 ± 0.25 [b]	2.47 ± 0.25 [a]	2.53 ± 0.34 [a]	< 0.050
4-Hydroxybenzoic acid	0.38 ± 0.22	0.02 ± 0.23	0.85 ± 0.30	0.058
Gallic acid	0.01 ± 0.02	0.00 ± 0.02	0.05 ± 0.03	0.370
Ethyl gallate	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.871
Vanillic acid	0.18 ± 0.16	0.28 ± 0.16	0.38 ± 0.22	0.733
D-Tartaric acid	2.29 ± 0.52	2.34 ± 0.53	1.71 ± 0.71	0.734
Pyrocatechol sulfate	0.52 ± 0.09	0.55 ± 0.10	0.70 ± 0.13	0.501
Coixol	1.07 ± 0.36	1.16 ± 0.37	1.73 ± 0.49	0.510

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$. [1]GRASS: beef fed a diverse pasture; [2]GRAIN: beef fed a total mixed ration (TMR); [3]GRAPE: beef fed TMR + 5% DM grapeseed extract.

5.5 Discussion

5.5.1 Nutritional composition of the diets

In the present study, differences were noted only between GRASS and the two other diets. No significant differences were observed for FAs or proximate composition between GRAIN and GRAPE. Differences in nutritional composition between pasture and TMR were reported in detail in Krusinski et al. (2022a). Grasses usually contain higher levels of SFAs and PUFAs (especially *n*-3) when compared to grains (Glasser et al., 2013;Klopatek et al., 2022). Higher concentrations of *n*-3 PUFAs in grasses are due to the accumulation of such FAs in leaf tissue of fresh pasture, with levels depending on the leaf-to-stem ratio (Khan et al., 2012;Butler, 2014;Alothman et al., 2019). Forages usually contain 50-75% of *n*-3 PUFAs as part of their FA composition (Dewhurst et al., 2006). Findings in the present study align with these numbers, with GRASS containing ~61% of *n*-3 PUFAs. Grains are usually higher in MUFAs and *n*-6 PUFAs when compared to grasses. This is mainly due to the growth of ears and the accumulation of these FAs in those ears (Khan et al., 2012). Here, more than 50% of FAs in GRAIN and GRAPE were *n*-6 PUFAs. The concentrations of *n*-3 and *n*-6 PUFAs in the diets were ultimately reflected in the *n*-6:*n*-3 ratio which was significantly lower in GRASS compared to the other two TMR diets. While such differences were anticipated between grasses and TMR, more differences were expected between GRAIN and GRAPE as grapeseed oil is composed of ~75% *n*-6 PUFAs (Garavaglia et al., 2016). However, since only 5% (DM basis) were added to the TMR for the GRAPE diet, it is possible that such amounts were too low to reflect a difference in the nutritional profile of the diets. Vinyard et al. (2021) included either 15% or 30% (DM basis) of grape pomace to a TMR diet. They found that ADF and NDF increased with the concentration of grape pomace in the diet compared to TMR

alone. However, Nudda et al. (2019) reported similar proximate composition values between TMR and TMR with grape pomace, aligning with results presented in the current study.

5.5.2 Beef fatty acids and micronutrients

5.5.2.1 Fatty acids

Differences in the FA profile of beef from grass and grain finishing systems were widely reported in the literature (Realini et al., 2004;Garcia et al., 2008;Alfaia et al., 2009;Duckett et al., 2013;Krusinski et al., 2022b). The absence of significant differences between groups regarding SFAs aligns with what others described (Duckett et al., 2013;Krusinski et al., 2022b). While some reported that concentrations of SFAs in GFB are higher than grain-finished beef, this is mainly because FAs were reported as percent of total FAs (Duckett et al., 2009b;Van Elswyk and McNeill, 2014). GFB is generally leaner, resulting in no significant differences compared to concentrations of SFAs in grain-finished beef when reported as mg per 100 g of beef (Krusinski et al., 2022d). Manso et al. (2016) reported a decrease in some SFAs in the milk of ewes supplemented with 10% (DM basis) of grape pomace compared to the milk of ewes fed a simple TMR/forage concentrate diet. However, this decrease was not observed when ewes were supplemented with only 5% (DM basis) of grape pomace, indicating a dose-dependent response. Moate et al. (2014) reported similar findings in dairy cows and attributed this decrease to the presence of grape residues containing lignin which are not fermentable in the rumen. Since no decrease in SFAs was observed in the current study, it was most likely due to the lower dose of GSE added to the cattle diet.

Surprisingly, no differences were seen in total MUFA concentrations in the present study. This was unexpected since grain-finished beef generally contains 30-70% more MUFAs than GFB (Duckett et al., 2013;Van Elswyk and McNeill, 2014;Krusinski et al., 2022d). Interestingly, differences were noted for a couple of individual MUFAs with grain-finished beef containing more

tetradecenoic acid (C14:1 9*c*) than GFB, and GFB containing more palmitelaidic acid (C16:1 9*t*) and vaccenic acid (C18:1 11*t*) than grain-finished beef and beef supplemented with GSE. Krusinski et al. (2022b) reported similar results regarding individual MUFAs with GFB having higher levels of specific *trans*-MUFAs and grain-finished beef having higher concentrations of specific *cis*-MUFAs. When high levels of *trans*-MUFAs are reported in GFB, it is generally due to higher concentrations of beneficial vaccenic acid (Mapiye et al., 2015;Klopatek et al., 2022;Krusinski et al., 2022b). In general, MUFAs are of interest for their low-density lipoprotein (LDL) cholesterol-lowering potential (Calder, 2015) and for their contribution to the overall palatability of beef (Chung et al., 2006;Smith et al., 2006).

In the present study, most differences were seen for *n*-3 and *n*-6 PUFAs. As expected, GFB contained more *n*-3 PUFAs (including ALA, EPA, DPA, and DHA) than grain-finished beef. These long-chain PUFAs are associated with healthier cardiovascular and cognitive functions (Parolini, 2019;Mendivil, 2021). On the other hand, grain-finished beef contained more *n*-6 PUFAs than GFB. This class of PUFAs may be pro-inflammatory compared to their *n*-3 counterpart which may be anti-inflammatory (Simopoulos, 2006). Surprisingly, beef fed grain and supplemented with GSE was not significantly different from grain-finished beef and GFB regarding *n*-6 PUFAs. Ianni et al. (2019) noted that the inclusion of grape pomace in the diet of cattle usually results in higher proportions of LA in beef, mainly because grape byproducts contain great concentrations of this *n*-6 FA. However, Manso et al. (2016) noted that the increase in LA in milk from ewes fed grape byproducts is dose-dependent and significant changes are seen when at least 10% (DM basis) of grape supplementation is added to the diet. The *n*-6:*n*-3 ratio is generally used for health claims associated with GFB (Daley et al., 2010;Krusinski et al., 2022d). An ideal ratio for human health is hypothesized to be around 1:1-4:1 (Simopoulos, 2002;2006). Here, GFB

had a more optimal *n*-6:*n*-3 ratio for human health (1.65:1) compared to the other two groups that had a ratio closer to 10:1 (a value sometimes associated with adverse health effects (Simopoulos, 2002)).

5.5.2.2 Vitamin E, zinc, and iron

Higher levels of vitamin E, iron, and zinc are expected for GFB compared to grain-finished beef (Horcada et al., 2020;Krusinski et al., 2022b). Higher concentrations of vitamin E in GFB are generally enough to protect meat from oxidation, leading to extended shelf-life (De la Fuente et al., 2009). The antioxidant potential of vitamin E also protects cells against free radicals, which can benefit human health (Daley et al., 2010;Horcada et al., 2020). Untea et al. (2022) showed the oxidative stability-influencing parameters of grape pomace and noted that it contains significant amounts of vitamin E and zinc. Vitamin E is a free radical scavenger and breaks the chain of lipid peroxidation, but zinc can also protect cells from iron-initiated lipid oxidation (Untea et al., 2022). Based on this, it could be assumed that the inclusion of GSE in the cattle diet would increase zinc and vitamin E concentrations compared to TMR alone. However, no such differences were noted in the present study. There is most likely a dose-dependent effects for these compounds and the levels of GSE added were probably too low to observe significant differences.

5.5.3. Phytochemical profile of beef

The PCA and RF plots displayed separations between beef from different finishing systems. Beef samples tested in this study all came from similar genetics steers, indicating that differences observed were most likely due to differences in finishing diets (GRASS vs. GRAIN vs. GRAPE). One limitation from the current study is that the phytochemical profile of the diets was not reported, so the extent of transfer of phytochemicals from plants to the meat cannot be established with certainty. O'Connell and Fox (2001) stated that most polyphenolic compounds

found in dairy products are derived from feeds, even though some of them may be the products of amino acid catabolism.

Grasses are generally high in antioxidants including vitamin E, chlorophyll, carotenoids, and phenols (Krusinski et al., 2022a). In the present study, beef from GRASS contained higher levels of numerous phytochemicals including stachydrine, hippuric acid, citric acid, and succinic acid compared to beef from GRAIN and GRAPE. These specific phytochemicals were also identified in the RF analysis as compounds capable of predicting diets. Stachydrine and hippuric acid were also identified as cattle diet-discriminating compounds by others (Carrillo et al., 2016;van Vliet et al., 2023), even though van Vliet et al. (2023) reported higher levels of stachydrine in pen-finished bison compared to pasture-finished bison. This phytochemical is found in high concentrations in chestnuts, alfalfa, and Chinese medicinal herbs, and exhibits bioactivities for the treatment of fibrosis, cardiovascular diseases, cancers, brain diseases, and inflammation in humans (Cheng et al., 2020). Since stachydrine is found in alfalfa, it was expected to find higher concentrations of this bioactive compound in beef from GRASS since the complex diverse pasture fed to these animals was made of ~10% alfalfa (Krusinski et al., 2022a;Krusinski et al., 2022b). Besle et al. (2010) identified hippuric acid as a major compound capable of indicating cattle finishing diets (with higher levels found in the milk from animals kept on grasslands). Higher concentrations of this phytochemical in the milk and meat of grass-finished animals can probably be attributed to the presence of phenolic acids in pasture-based diets (Rocchetti et al., 2022). Citric acid was the most abundant phytochemical quantified in this study (with beef from GRASS containing 379.20 mg of citric acid per 100 g of beef). Citric acid is mostly found in fruits, especially citrus fruits and has several health benefits including increasing the bioavailability and absorption of minerals and reducing risks of kidney stone formation (Nii et al., 2006;Mahato et

al., 2018). Supplee and Bellis (1921) noted that pasture feeding may increase concentrations of citric acid in milk in some instances. For comparison, fresh apricots contain 30-50 mg of citric acid per 100 g (Gurrieri et al., 2001). In the present study, 100 g of GFB contained 7-12 times more citric acid than 100 g of apricots. Succinic acid was also abundant in GFB. Gatmaitan et al. (2021) reported a decrease in relative abundance of succinic acid in grain-finished beef and indicated that this compound can be used for the authentication of GFB. Beef from GRAIN and GRAPE (fed mainly a TMR) contained higher concentrations of p-coumaric acid than beef from GRASS. This phenolic acid is one of the main phenolic compound reported in corn-based diets (Rocchetti et al., 2022).

While clear differences were observed between beef from GRASS compared to the other two groups, distinctions were not as obvious between beef from GRAIN and beef from GRAPE. The PCA plot showed overlaps between clusters with the GRAPE group overlapping with GRAIN and GRASS, which may indicate a transfer of phytochemicals from the diet to the meat. The quantified phytochemicals presented in this study are not exclusive to GSE, which may explain why no significant differences were observed between beef from GRAPE and beef from the other two diets. Based on the RF biochemical importance plot, it appears that vanillic acid and 4-hydrobenzoic acid have the potential to discriminate beef from cattle supplemented with GSE even though no significant differences were noted when these compounds were quantified. Vanillic acid is one of the most significant hydrobenzoic acid found in grapes (Nudda et al., 2019;Sabra et al., 2021). Whether supplementing cattle diets with GSE increases phytochemicals in beef remains uncertain, even though higher plasma polyphenols have been reported in cattle supplemented with grape byproducts (Beslo et al., 2022). Another important point is that only 5% (DM basis) of GSE were added to the TMR for the GRAPE group, which may not be enough to observe significant

changes. It appears that the effects of GSE on the beef nutritional profile are dose-dependent (Serra et al., 2013;Manso et al., 2016).

Overall, the differences in phytochemicals between grass- and grain-finished beef noted in this study were in agreement with what was previously reported on products from grazing animals compared to animals fed a conventional grain diet (Besle et al., 2010;Hilario et al., 2010;Carrillo et al., 2016;Delgadillo-Puga et al., 2019;van Vliet et al., 2021b;van Vliet et al., 2023).

5.6 Conclusions

In this study we evaluated the FA and phytochemical profiles of beef samples from grass- and grain-finishing systems, with a third group supplemented with GSE used as "positive control" to confirm the transfer of biochemicals from plants to the meat. Overall, beef from the grass-finishing system (GRASS) displayed the most beneficial nutritional profile for human health with a lower *n*-6:*n*-3 ratio and higher levels of long-chain *n*-3 PUFAs, vitamin E, zinc, iron, and most phytochemicals including stachydrine, hippuric acid, citric acid, succinic acid, and fumaric acid compared to grain-finished beef (GRAIN) and grain-finished beef supplemented with GSE (GRAPE) (**Figure 15**). Interestingly, beef from GRAPE was somewhere in the middle between GRASS and GRAIN for a few specific FAs such as total *n*-6 PUFAs, C20:3 *n*-3, and DHA. The same observation was made with PCA where some overlap was seen, especially between GRASS and GRAPE. Random Forest classification allowed us to identify the most important phytochemicals for group separation, with stachydrine, succinic acid, and citric acid being the top three compounds capable of separating beef from different diets.

While higher levels of phytochemicals were expected with GSE supplementation, the response is generally dose-dependent and the 5% (DM basis) added to the cattle diet in this study were most likely not enough to raise levels of bioactive compounds in beef. The main limitation

of this study was the lack of phytochemical profiling of the diets, making the assumption of transfer of phytochemicals from plants to the meat more difficult. We also did not quantify phytochemicals specific to grapes, which does not allow us to determine the deposition rate of such compounds in beef supplemented with GSE. However, this study displayed extensive nutritional profiles of beef from two common finishing systems (GRASS and GRAIN), and a third finishing diet worth exploring in more detail using a byproduct from the wine industry (GRAPE). We were also able to quantify phytochemicals and report them in amounts relevant for human health (mg per 100 g of beef). Future studies should investigate the addition of phytochemical-rich byproducts to cattle diets (and in varying amounts) and their effects on the nutritional profile of beef. Additionally, quantifying these bioactive compounds and reporting results that can be used by consumers is crucial. More research is needed to link livestock production systems, nutritional profiles of animal products, and human health. Such findings could also be used for the authentication of GFB.

In conclusion, the *n*-6:*n*-3 ratio, total *n*-3 PUFAs, micronutrients, and phytochemicals are compounds that can be used to determine the finishing diet of cattle. GFB with higher amounts of beneficial bioactive compounds may favor human health.

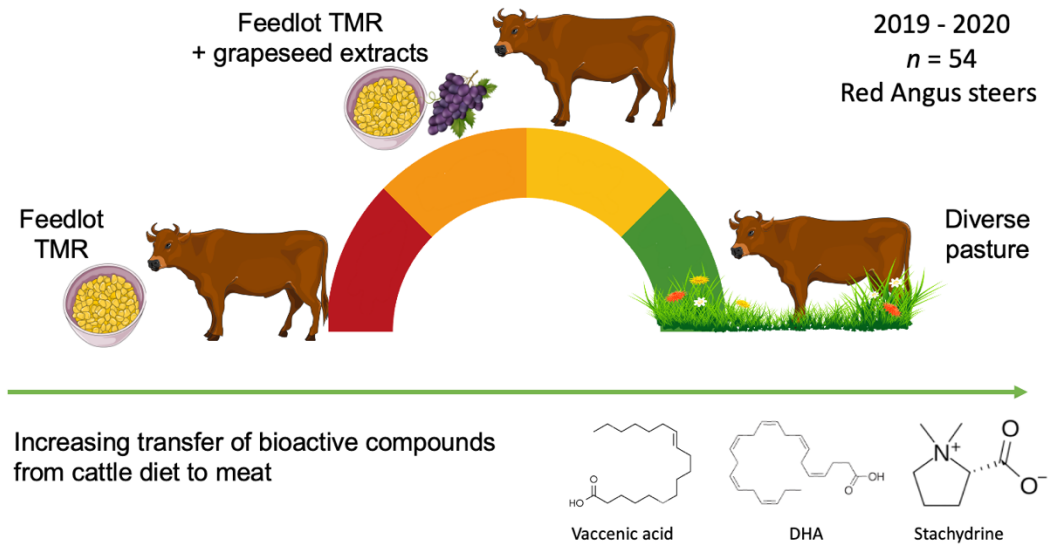

Figure 15. Transfer of bioactive compounds from diets to meat. Grass-finished beef contained higher levels of bioactive compounds such as *n*-3 PUFAs, vaccenic acid, and phytochemicals (such as stachydrine) compared to grain-finished beef and grain-finished beef supplemented with grapeseed extracts (GSE). TMR: total mixed ration.

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

This dissertation investigated the effects of finishing diets and breeds on the nutritional profile of GFB to better understand the large variations in fatty acids (FAs) and micronutrients observed previously (Bronkema et al., 2019). To do so, the following topics were covered in each chapter:

Chapter 2 focused on FAs and antioxidants found in commonly used feedstuffs including a complex pasture mixture and a traditional grain-based diet. Variations of these bioactive compounds over the grazing season were assessed, and correlations were reported to better understand the relationship between individual plant species, weather conditions, and bioactive compounds. This study was relevant because demand for grass-finished products keeps increasing and global climate change makes it harder to predict variations in the nutritional profile of grasslands.

Chapter 3 investigated the effects of diet and breed on the nutritional profile of beef. Since diet and breed are the two most influential factors capable of modifying the nutritional profile of beef, it was important to determine the specific effects of each factor and their interaction to better comprehend and predict nutritional variations in beef. In this chapter, two breeds and two diets were tested: Red Angus and Red Angus x Akaushi steers finished either on a diverse pasture mixture or a traditional grain diet.

Chapter 4 examined the impacts of supplemental feeds (hay, baleage, and soybean hulls) on the nutritional profile of GFB. Depending on the region and the climate, fresh forages may not always be available, and some producers may need to rely on conserved forages. It was hypothesized that soybean hulls high in *n*-6 PUFAs and conserved forages with reduced nutritional quality would cause variations in the nutritional profile of beef.

Chapter 5 assessed the effects of cattle diets (complex pasture mixture, grain diet, and grain diet supplemented with grapeseed extracts) on beef FAs, micronutrients, and phytochemicals. Differences in FA profile and micronutrients between grass- and grain-finished beef have been extensively studied, but less is known about secondary plant compounds that are transferred from feeds to the meat. This study profiled a wide array of phytochemicals in beef (including polyphenols) and determined the impacts of cattle diet on the phytochemical richness of beef.

This dissertation highlighted the importance of measuring variations in the nutritional quality of feedstuffs and how it affects the nutritional profile of beef. The complex pasture mixture contained higher concentrations of *n*-3 PUFAs and antioxidants compared to the grain diet. Additionally, trends in specific FAs were identified with *n*-3 FAs being more abundant in early and late grazing season and *n*-6 FAs following an opposite trend. Correlations also gave good indications about the effects of individual plant species and weather conditions on bioactive compounds (**chapter 2**). Further, this research suggests that finishing cattle on a diverse pastures (containing a wide array of different plant species) improves levels of beneficial bioactive compounds in beef compared to finishing cattle on grain. This includes long-chain *n*-3 PUFAs, the *n*-6:*n*-3 ratio, CLA, vaccenic acid, iron, zinc, and vitamin E. Here, the diet effect was more dominant than the breed effect and the diet × breed interaction, indicating that finishing diets have a high potential for modifying the nutritional composition of beef (**chapter 3**). Regarding supplemental feeds, supplementing GFB with hay made from drying a diverse pasture resulted in higher concentrations of long-chain *n*-3 PUFAs (including EPA, DPA, and DHA) and a lower *n*-6:*n*-3 ratio in beef compared to beef supplemented with baleage or soybean hulls (**chapter 4**). GFB also contained higher concentrations of phytochemicals including stachydrine, hippuric acid, citric

acid, succinic acid, and fumaric acid compared to grain-finished beef and beef supplemented with grapeseed extracts. Principal Component Analysis (PCA) and Random Forest Analysis showed differences in the phytochemical profiles of beef by diet and identified stachydrine, succinic acid, and citric acid as the top three compounds capable of separating beef from different diets (**chapter 5**). Nevertheless, results reported in this dissertation could not explain the large nutritional variations reported in the previously published nutritional survey of commercially available GFB (Bronkema et al., 2019).

6.2 Future directions

6.2.1 Different feedstuffs

The supplemental feeds tested as part of this dissertation (hay, baleage, soybean hulls) could not explain the large nutritional variations in GFB observed previously (Bronkema et al., 2019). However, it was concluded that cattle diets have a significant effect on the nutritional profile of beef, suggesting a need to investigate this route further. While guidelines prohibit the use of grain and grain byproducts for GFB production, it cannot be ruled out that some producers may supplement their animals with such feeds. This could explain higher *n*-6:*n*-3 ratios in beef. One example is distiller's grains. Various studies reported that the inclusion of distiller's grains to cattle diets increased the *n*-6:*n*-3 ratio in beef in a dose-dependent manner compared to controls (Gill et al., 2008;Mello et al., 2012;de Nazare Santos Torres et al., 2022). A review by Merayo et al. (2020) stated that "the values of the *n*-6:*n*-3 ratio have been shown to be at levels expected for beef from intensive systems." Future studies should supplement GFB with distiller's grains (or other grain-based diets) in the finishing phase to test whether the inclusion of such ingredients is responsible for nutritional variations.

6.2.2 Establish stronger labeling regulations and authentication methods for grass-finished beef

Pasture-raised dairy and meat products carry premium values and nutritional attributes of interest for health-savvy consumers, indicating an urgent need for better authentication of products from grassland origins (Prache et al., 2020). GFB products can be authenticated by 1) FAs, 2) phytochemicals, 3) vitamin E, and 4) gene expression (Monahan et al., 2018). There is currently no federal standard for "grass-fed" and "grass-finished" labels in the U.S., but producers still need to follow the USDA Food Safety Inspection Service (FSIS) guidelines to use these claims (this includes sending written documentation about the animal's diet, but no regular inspections are conducted). This led independent organizations such as the American Grassfed Association to start their own third-party certifications (American Grassfed Association, 2022). Their control procedure involves sending inspectors to farms to ensure that guidelines are followed. However, no empirical analysis on nutrient density and/or transcriptomics is conducted on beef samples to guarantee "grass-fed" and "grass-finished" standards. Based on this, future research should focus on implementing better testing/authentication methods using nutrient density data and transcriptomics to certify that "grass-fed" and "grass-finished" expectations are met.

6.2.3 Effects of beef consumption from different production systems on human health

While various epidemiological studies have linked the consumption of red meat to metabolic diseases, the impact of different beef production systems has not been considered and putting all beef under the same umbrella might be reductionist. As indicated in this dissertation, numerous studies have shown differences in the nutritional profile of beef coming from different production systems, with GFB generally having a nutrient profile favoring human health. However, the effects of consuming GFB vs. grain-finished beef on human health still need further

investigation. Arya et al. (2010) showed that the consumption of meat from animals foraging on diverse pastures lowered the postprandial levels of inflammatory markers in humans compared to grain-finished beef. Another study on pecorino cheese made from sheep foraging on diverse pastures decreased levels of pro-inflammatory cytokines in consumers (Sofi et al., 2010). It was also reported that consuming beef finished on monocultured pastures or on grain did not have the same beneficial effects on inflammatory markers than meat from animals foraging on diverse pastures (Gilmore et al., 2011). More data are needed to determine the effects of beef consumption on human health, especially beef raised using different production methods. This indicates a critical need for future research to create a direct link between livestock production systems and human health.

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APPENDIX A: SUPPLEMENTAL INFORMATION AND DATA

Table A1. GC-MS supporting information for fatty acid methyl esters (FAMEs)

Fatty acid	Injection[1]	Retention time (min)	Quantitative ion[2]	Major fragmentation ions from feed FAMEs[3]
C8:0	30:1 split	13.88	74	74 (100), 87 (29), 101 (11), 143 (5), 158 (3)
C12:0	30:1 split	18.12	74	74 (100), 75 (17), 83 (7), 87 (66), 129 (8), 143 (6), 171 (7)
C14:0	30:1 split	20.82	74	74 (100), 75 (15), 83 (7), 87 (87), 129 (8), 143 (26), 157 (7), 199 (13), 242 (14)
C16:0	30:1 split	24.83	74	74 (100), 75 (20), 83 (10), 87 (80), 143 (20), 227 (11), 270 (16)
C16:1 *n*-7	30:1 split	26.62	74	74 (100), 83 (90), 87 (77), 96 (82), 98 (68), 110 (38), 123 (30), 152 (35), 194 (31), 236 (35), 268 (11)
C16:1 *n*-9	30:1 split	26.30	74	74 (97), 84 (79), 87 (62), 96 (100), 98 (84), 110 (46), 152 (40), 194 (33), 236 (43), 268 (8)
C18:0	30:1 split	31.33	74	74 (100), 75 (24), 87 (72), 143 (21), 199 (10), 255 (10), 298 (18)
C18:1 *n*-7	30:1 split	34.27	74	74 (91), 83 (100), 96 (81), 97 (92), 111 (39), 123 (27), 180 (23), 222 (22), 264 (57), 296 (10)
C18:1 *n*-9	30:1 split	33.88	74	74 (82), 83 (100), 96 (81), 97 (92), 110 (39), 123 (27), 180 (23), 222 (22), 264 (57), 296 (10)
C18:2 *n*-6	30:1 split	38.42	81	79 (43), 81 (100), 95 (69), 96 (48), 110 (32), 121 (16), 150 (14), 263 (8), 294 (14)
C18:3 *n*-3	30:1 split	43.06	79	79 (100), 93 (62), 95 (84), 108 (48), 121 (21), 136 (13), 149 (13), 292 (7)
C20:0	30:1 split	41.49	74	74 (100), 87 (90), 143 (15), 326 (11)
C22:0	30:1 split	48.68	143	74 (50), 87 (100), 143 (6), 199 (2), 354 (1)
C24:0	30:1 split	54.95	74	74 (100), 87 (54), 97 (20), 143 (15), 382 (10)
C15:0	30:1 split	22.60	74	74 (100), 75 (19), 87 (79), 143 (22), 213 (10), 256 (9)
C17:0	30:1 split	27.67	74	74 (97), 75 (31), 87 (100), 143 (20), 185 (10), 241 (10), 284 (19)

[1]Injection used for analysis; [2]Ion used for extracted ion chromatogram and used for quantification; [3] 70 eV, mass range of m/z 70–400 amu.

Table A2. Mean concentrations of saturated and monounsaturated fatty acids in beef by diet, breed, and diet x breed interaction (% of total fatty acids)[1]

Fatty acid	Diet (D)		Breed (B)		Significance of ANOVA[4]		
	GRASS	GRAIN	RA[2]	AK[3]	D	B	D x B
∑ SFA[5]	43.86 ± 2.84	45.68 ± 2.86	45.12 ± 2.84	44.41 ± 2.84	NS	NS	NS
C10:0	0.11 ± 0.03	0.08 ± 0.03	0.10 ± 0.03	0.09 ± 0.03	NS	NS	NS
C12:0	0.06 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	NS	***	NS
C13:0	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	*	NS	NS
C14:0	1.86 ± 0.08	2.52 ± 0.09	2.02 ± 0.08	2.36 ± 0.08	***	***	NS
C15:0	0.38 ± 0.06	0.18 ± 0.06	0.26 ± 0.06	0.29 ± 0.06	***	NS	NS
C16:0	25.87 ± 1.94	31.24 ± 1.98	28.67 ± 1.89	28.44 ± 1.89	**	NS	NS
C17:0	1.06 ± 0.23	0.52 ± 0.23	0.79 ± 0.22	0.80 ± 0.22	***	NS	NS
C18:0	14.36 ± 0.70	10.92 ± 0.71	13.06 ± 0.70	12.21 ± 0.70	***	*	NS
C19:0	0.05 ± 0.02	0.02 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	***	NS	NS
C20:0	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	***	NS	NS
C22:0	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	NS	NS	NS
∑ BCFA[6]	1.53 ± 0.05	0.75 ± 0.06	1.08 ± 0.05	1.19 ± 0.05	***	NS	NS
C14:0 *iso*	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	***	NS	NS
C15:0 *iso*	0.14 ± 0.04	0.08 ± 0.04	0.10 ± 0.04	0.11 ± 0.04	NS	NS	NS
C15:0 *anteiso*	0.10 ± 0.02	0.03 ± 0.02	0.06 ± 0.02	0.07 ± 0.02	***	NS	NS
C16:0 *iso*	0.11 ± 0.04	0.08 ± 0.04	0.09 ± 0.04	0.09 ± 0.04	**	NS	NS
C17:0 *iso*	0.54 ± 0.07	0.19 ± 0.07	0.35 ± 0.07	0.38 ± 0.07	***	NS	NS
C17:0 *anteiso*	0.54 ± 0.04	0.30 ± 0.04	0.40 ± 0.04	0.45 ± 0.04	***	NS	NS
C18:0 *iso*	0.07 ± 0.03	0.07 ± 0.03	0.07 ± 0.03	0.07 ± 0.03	NS	NS	NS

[1]Values reported as means ± SEM (standard error of mean). [2]RA: Red Angus; [3]AK: Red Angus x Akaushi; [4]NS: not significant; $p > 0.05$; $*p < 0.05$; $**p < 0.01$ $***p < 0.001$.
[5]∑ SFA = all saturated FAs (10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 22:0); [6]∑ BCFA = sum of all branched chain FAs (*iso*14:0, *iso*15:0, *anteiso*15:0, *iso*16:0, *iso*17:0, *anteiso*17:0, *iso*18:0); [7]∑ MUFA = all monounsaturated FAs (14:1, 16:1, 17:1, 18:1, 20:1); [8]∑ *c*MUFA = 14:1, 17:1, sum of *c*16:1, *c*18:1, and *c*20:1; [9]∑ *t*MUFA = sum of *t*16:1 and *t*18:1.

Table A2. (cont'd)

Fatty acid	Diet (D)		Breed (B)		Significance of ANOVA		
	GRASS	GRAIN	RA	AK	D	B	D x B
∑ MUFA[7]	44.59 ± 1.21	46.17 ± 1.28	44.62 ± 1.14	46.14 ± 1.14	NS	*	NS
∑ *c*MUFA[8]	41.21 ± 0.89	45.07 ± 0.98	42.39 ± 0.79	43.90 ± 0.79	*	*	NS
C14:1 9*c*	0.35 ± 0.08	0.75 ± 0.08	0.50 ± 0.08	0.60 ± 0.08	***	*	NS
C16:1 9*c*	4.42 ± 1.28	6.62 ± 1.28	5.26 ± 1.28	5.78 ± 1.28	***	*	NS
C16:1 10*c*	0.24 ± 0.10	0.25 ± 0.10	0.23 ± 0.10	0.26 ± 0.10	NS	*	NS
C16:1 11*c*	0.10 ± 0.04	0.17 ± 0.04	0.12 ± 0.04	0.14 ± 0.04	***	**	NS
C17:1 9*c*	0.53 ± 0.01	0.44 ± 0.02	0.47 ± 0.01	0.50 ± 0.01	**	NS	NS
C18:1 9*c*	33.46 ± 1.06	34.72 ± 1.11	33.72 ± 1.02	34.47 ± 1.02	NS	NS	NS
C18:1 11*c*	1.24 ± 0.09	1.38 ± 0.10	1.30 ± 0.09	1.32 ± 0.09	NS	NS	NS
C18:1 12*c*	0.17 ± 0.03	0.15 ± 0.03	0.16 ± 0.03	0.16 ± 0.03	NS	NS	NS
C18:1 13*c*	0.22 ± 0.06	0.27 ± 0.06	0.23 ± 0.06	0.26 ± 0.06	**	NS	NS
C18:1 14*c*	0.04 ± 0.02	0.02 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	***	NS	NS
C18:1 15*c*	0.07 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	**	NS	*
C20:1 9*c*	0.11 ± 0.04	0.10 ± 0.04	0.10 ± 0.04	0.11 ± 0.04	NS	NS	*
C20:1 11*c*	0.26 ± 0.15	0.29 ± 0.15	0.27 ± 0.15	0.28 ± 0.15	NS	NS	NS
∑ *t*MUFA[9]	3.37 ± 0.36	1.04 ± 0.37	2.20 ± 0.37	2.22 ± 0.37	***	NS	NS
C16:1 9*t*	0.30 ± 0.10	0.09 ± 0.10	0.20 ± 0.10	0.19 ± 0.10	**	NS	NS
C16:1 10-12*t*	0.50 ± 0.08	0.26 ± 0.08	0.35 ± 0.08	0.37 ± 0.08	***	NS	NS
C18:1 6-8*t*	0.10 ± 0.03	0.05 ± 0.03	0.08 ± 0.03	0.07 ± 0.03	***	NS	NS
C18:1 9*t*	0.13 ± 0.04	0.10 ± 0.04	0.12 ± 0.04	0.12 ± 0.04	**	NS	NS
C18:1 10*t*	0.12 ± 0.03	0.07 ± 0.03	0.10 ± 0.03	0.09 ± 0.03	**	NS	NS
C18:1 11*t*	1.35 ± 0.07	0.15 ± 0.08	0.74 ± 0.08	0.76 ± 0.08	***	NS	NS
C18:1 12*t*	0.19 ± 0.02	0.08 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	***	NS	NS
C18:1 13,14*t*	0.38 ± 0.05	0.10 ± 0.05	0.24 ± 0.05	0.25 ± 0.05	***	NS	NS
C18:1 15*t*	0.17 ± 0.05	0.09 ± 0.06	0.14 ± 0.06	0.13 ± 0.06	*	NS	NS
C18:1 16*t*	0.17 ± 0.01	0.05 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	***	NS	NS

Table A3. Mean concentrations of polyunsaturated fatty acids in beef by diet, breed, and diet x breed interaction (% of total fatty acids)[1]

Fatty acid	Diet (D)		Breed (B)		Significance of ANOVA[4]		
	GRASS	GRAIN	RA[2]	AK[3]	D	B	D x B
∑ PUFA[5]	8.62 ± 1.69	6.56 ± 1.71	8.06 ± 1.69	7.12 ± 1.69	*	NS	NS
∑ *n*-3[6]	3.24 ± 0.35	0.74 ± 0.36	2.11 ± 0.35	1.88 ± 0.35	***	NS	NS
C18:3 *n*-3 (ALA)[7]	1.58 ± 0.10	0.23 ± 0.11	0.93 ± 0.10	0.88 ± 0.10	***	NS	NS
C20:3 *n*-3	0.02 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	***	NS	NS
C20:5 n-3 (EPA)[8]	0.59 ± 0.08	0.12 ± 0.09	0.39 ± 0.08	0.32 ± 0.08	***	NS	NS
C22:5 n-3 (DPA)[9]	0.99 ± 0.14	0.36 ± 0.14	0.73 ± 0.14	0.62 ± 0.14	***	NS	NS
C22:6 n-3 (DHA)[10]	0.06 ± 0.03	0.02 ± 0.03	0.04 ± 0.03	0.04 ± 0.03	*	NS	NS
∑ *n*-6[11]	5.31 ± 1.32	5.74 ± 1.34	5.88 ± 1.33	5.17 ± 1.33	NS	NS	NS
C18:2 n-6 (LA)[12]	3.62 ± 0.77	3.76 ± 0.78	3.88 ± 0.80	3.51 ± 0.80	NS	NS	NS
C18:3 *n*-6	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	NS	NS	NS
C20:2 *n*-6	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	NS	NS	NS
C20:3 *n*-6	0.26 ± 0.06	0.31 ± 0.06	0.31 ± 0.06	0.26 ± 0.06	NS	NS	NS
C20:4 *n*-6	1.24 ± 0.38	1.33 ± 0.38	1.40 ± 0.38	1.16 ± 0.38	NS	NS	NS
C22:4 *n*-6	0.14 ± 0.11	0.29 ± 0.11	0.23 ± 0.11	0.19 ± 0.10	**	NS	NS
n-6:*n*-3 ratio[13]	1.61 ± 0.39	8.36 ± 0.41	5.04 ± 0.39	4.92 ± 0.39	***	NS	NS
C20:3 *n*-9	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.06 ± 0.02	NS	NS	NS

[1]Values reported as means ± SEM (standard error of mean). [2]RA: Red Angus; [3]AK: Red Angus x Akaushi; [4]NS: not significant; $p > 0.05$; $*p < 0.05$; $**p < 0.01$ $***p < 0.001$.
[5]∑ PUFA = LA + ALA + GLA + Eicosadienoic + Eicosatrienoic + DGLA + Mead + Arachidonic + EPA + DTA + DPA *n*-3 + DHA; [6]∑ *n*-3 = ALA + EPA + DHA + DPA *n*-3 + Eicosatrienoic; [7]ALA: α-linolenic acid, [8]EPA: eicosapentaenoic acid: [9]DPA: *n*-3 docosapentaenoic acid, [10]DHA: docosahexaenoic acid; [11]∑ *n*-6 = LA + GLA + Eicosadienoic + DGLA + Arachidonic + DTA; [12]LA: linoleic acid; [13]*n*-6:*n*-3 ratio = ∑ *n*-6/∑ *n*-3; [14]∑ CLnA = sum of conjugated linolenic acid isomers (*c*9, *t*11, *t*15 18:3 + *c*9, *t*11, *c*15 18:3); [15]∑ Atypical Dienes (AD) = sum of non-conjugated linoleic acid isomers (*t*11, *t*15 18:2 + *t*9, *t*12 18:2 + *c*9, *t*14/c9, *t*13 18:2 + *t*11, *c*15 18:2 + *c*9, *t*16 18:2 + *c*9, *c*15 18:2 + *c*12, *c*15 18:2); [16]∑ CLA = sum of conjugated linoleic acid isomers (*c*9, *t*11/*t*7, *c*9 18:2 + *t*11, *c*13 18:2 + *t*11, *t*13 18:2 + *t,t* 18:2).

Table A3. (cont'd)

Fatty acid	Diet (D)		Breed (B)		Significance of ANOVA		
	GRASS	GRAIN	RA	AK	D	B	D x B
∑ CLnA[14]	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	**	NS	NS
C18:3 9*c*,11*t*,15*t*	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	**	NS	NS
C18:3 9*c*,11*t*,15*c*	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	NS	NS	NS
∑ AD[15]	0.92 ± 0.04	0.53 ± 0.05	0.72 ± 0.04	0.72 ± 0.04	***	NS	NS
C18:2 11*t*,15*t*	0.21 ± 0.01	0.08 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	***	NS	NS
C18:2 9*t*,12*t*	0.04 ± 0.02	0.01 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	***	NS	NS
C18:2 9*c*,14*t*/9*c*,13*t*	0.13 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	***	NS	NS
C18:2 11*t*,15*c*	0.22 ± 0.01	0.04 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	***	NS	NS
C18:2 9*c*,16*t*	0.23 ± 0.08	0.19 ± 0.08	0.23 ± 0.08	0.20 ± 0.08	NS	NS	NS
C18:2 9*c*,15*c*	0.07 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	***	NS	NS
C18:2 12*c*,15*c*	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	**	NS	NS
∑ CLA[16]	0.34 ± 0.14	0.16 ± 0.14	0.25 ± 0.14	0.26 ± 0.14	***	NS	NS
C18:2 9*c*,11*t*/9*c*,7*t*	0.24 ± 0.08	0.11 ± 0.08	0.17 ± 0.08	0.18 ± 0.08	***	NS	NS
C18:2 11*t*,13*c*	0.04 ± 0.02	0.02 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	**	NS	NS
C18:2 11*t*,13*t*	0.03 ± 0.02	0.02 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	**	NS	NS
C18:2 *t*,*t*	0.03 ± 0.02	0.02 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	NS	NS	NS

Table A4. Mean concentrations of saturated and monounsaturated fatty acids by diet (% total fatty acids)

	G-HAY[1]	G-BLG[2]	G-SH[3]	BLG-SH[4]	*p*-value
∑ SFA[5]	35.94 ± 1.63 [b]	37.97 ± 1.63 [a,b]	38.21 ± 1.64 [a,b]	41.10 ± 1.63 [a]	0.003
C10:0	0.26 ± 0.22	0.35 ± 0.22	0.34 ± 0.22	0.38 ± 0.22	0.111
C12:0	0.08 ± 0.04	0.08 ± 0.04	0.08 ± 0.04	0.08 ± 0.04	0.994
C13:0	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.113
C14:0	1.46 ± 0.11	1.55 ± 0.11	1.58 ± 0.11	1.69 ± 0.11	0.489
C15:0	0.25 ± 0.01 [a,b]	0.28 ± 0.01 [a]	0.22 ± 0.01 [b]	0.22 ± 0.01 [b]	0.020
C16:0	21.36 ± 1.03 [b]	22.60 ± 1.04 [b]	23.36 ± 1.04 [a,b]	25.46 ± 1.03 [a]	< 0.001
C17:0	0.58 ± 0.03 [b]	0.68 ± 0.03 [a]	0.61 ± 0.03 [a,b]	0.69 ± 0.03 [a]	0.034
C18:0	11.34 ± 1.16	11.73 ± 1.17	11.47 ± 1.17	11.95 ± 1.16	0.522
C19:0	0.28 ± 0.23 [b]	0.44 ± 0.23 [a]	0.32 ± 0.24 [a,b]	0.40 ± 0.23 [a,b]	0.018
C20:0	0.12 ± 0.05	0.10 ± 0.05	0.10 ± 0.05	0.10 ± 0.05	0.148
C22:0	0.20 ± 0.06 [a]	0.11 ± 0.06 [b]	0.11 ± 0.06 [b]	0.12 ± 0.06 [b]	0.003
∑ BCFA[6]	1.47 ± 0.17	1.59 ± 0.17	1.48 ± 0.17	1.39 ± 0.17	0.333
C14:0 *iso*	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.093
C15:0 *iso*	0.09 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.656
C15:0 *anteiso*	0.10 ± 0.02	0.10 ± 0.02	0.08 ± 0.02	0.07 ± 0.02	0.088
C16:0 *iso*	0.10 ± 0.04	0.11 ± 0.04	0.09 ± 0.04	0.10 ± 0.04	0.182
C17:0 *iso*	0.57 ± 0.03	0.60 ± 0.03	0.59 ± 0.03	0.50 ± 0.03	0.069
C17:0 *anteiso*	0.52 ± 0.04	0.58 ± 0.04	0.54 ± 0.04	0.55 ± 0.04	0.541
C18:0 *iso*	0.08 ± 0.03	0.07 ± 0.03	0.07 ± 0.03	0.07 ± 0.03	0.336

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment, $n = 117$). [1]G-HAY: grass and hay diet; [2]G-BLG: grass and baleage diet; [3]G-SH: grass and soybean hulls diet; [4]BLG-SH: baleage and soybean hulls diet; [5]∑ SFA = total saturated FAs; [6]∑ BCFA = total branched chain FAs; [7]∑ MUFA = total monounsaturated FAs; [8]∑ *c*MUFA = total *cis*-monounsaturated FAs; [9]∑ *t*MUFA = total *trans*-monounsaturated FAs; [10]∑ FA = all FAs in mg/100 g of meat.

Table A4. (cont'd)

	G-HAY	G-BLG	G-SH	BLG-SH	*p*-value
∑ MUFA[7]	41.96 ± 0.69 [b]	44.27 ± 0.70 [a,b]	45.06 ± 0.70 [a]	43.57 ± 0.69 [a,b]	0.021
∑ *c*MUFA[8]	37.04 ± 2.00 [b]	38.23 ± 2.00 [a,b]	40.45 ± 2.01 [a]	39.77 ± 2.00 [a,b]	0.018
C14:1 9*c*	0.35 ± 0.06	0.38 ± 0.06	0.37 ± 0.06	0.33 ± 0.06	0.484
C16:1 9*c*	4.88 ± 0.29	4.85 ± 0.30	5.32 ± 0.30	4.90 ± 0.29	0.488
C16:1 10*c*	0.62 ± 0.21 [a,b]	0.75 ± 0.21 [a]	0.55 ± 0.21 [b]	0.53 ± 0.21 [b]	0.005
C16:1 11*c*	0.29 ± 0.21	0.39 ± 0.21	0.32 ± 0.21	0.35 ± 0.21	0.115
C17:1 9*c*	0.53 ± 0.06	0.59 ± 0.06	0.54 ± 0.06	0.56 ± 0.06	0.509
C18:1 9*c*	26.31 ± 3.36 [b]	27.43 ± 3.37 [a,b]	29.64 ± 3.37 [a]	29.56 ± 3.36 [a]	0.007
C18:1 11*c*	1.73 ± 0.16 [a]	1.46 ± 0.16 [b]	1.60 ± 0.16 [a,b]	1.14 ± 0.16 [b]	0.001
C18:1 12*c*	0.32 ± 0.08	0.33 ± 0.08	0.31 ± 0.08	0.32 ± 0.08	0.743
C18:1 13*c*	0.35 ± 0.23 [b]	0.48 ± 0.23 [a]	0.43 ± 0.23 [a,b]	0.46 ± 0.23 [a]	0.021
C18:1 14*c*	0.14 ± 0.04	0.13 ± 0.04	0.11 ± 0.04	0.12 ± 0.04	0.087
C18:1 15*c*	0.20 ± 0.12	0.29 ± 0.12	0.24 ± 0.12	0.26 ± 0.12	0.111
C20:1 9*c*	0.43 ± 0.26	0.43 ± 0.26	0.39 ± 0.26	0.39 ± 0.26	0.511
C20:1 11*c*	0.89 ± 0.22 [a]	0.72 ± 0.22 [b]	0.65 ± 0.22 [b,c]	0.57 ± 0.22 [c]	< 0.001
∑ *t*MUFA [9]	4.92 ± 1.51 [a,b]	6.04 ± 1.51 [a]	4.60 ± 1.51 [b]	3.80 ± 1.51 [b]	< 0.001
C16:1 9*t*	0.93 ± 0.23 [a]	1.04 ± 0.23 [a]	0.85 ± 0.23 [a]	0.52 ± 0.23 [b]	< 0.001
C16:1 10,11,12*t*	0.80 ± 0.37 [b]	1.09 ± 0.37 [a]	0.82 ± 0.37 [b]	0.82 ± 0.37 [b]	0.013
C18:1 6-8*t*	0.25 ± 0.15 [a,b]	0.34 ± 0.15 [a]	0.23 ± 0.15 [b]	0.29 ± 0.15 [a,b]	0.037
C18:1 9*t*	0.25 ± 0.17 [b]	0.38 ± 0.17 [a]	0.30 ± 0.17 [a,b]	0.32 ± 0.17 [a,b]	0.026
C18:1 10*t*	0.21 ± 0.17 [b]	0.37 ± 0.17 [a]	0.25 ± 0.17 [a,b]	0.24 ± 0.17 [b]	0.005
C18:1 11*t*	1.48 ± 0.14 [a]	1.60 ± 0.14 [a]	1.19 ± 0.14 [a,b]	0.65 ± 0.14 [b]	< 0.001
C18:1 12*t*	0.19 ± 0.07 [a]	0.21 ± 0.07 [a]	0.17 ± 0.07 [a,b]	0.15 ± 0.07 [b]	0.007
C18:1 13,14*t*	0.41 ± 0.03 [a]	0.41 ± 0.03 [a]	0.34 ± 0.03 [a,b]	0.31 ± 0.03 [b]	0.002
C18:1 15*t*	0.21 ± 0.27	0.42 ± 0.27	0.30 ± 0.27	0.36 ± 0.27	0.126
C18:1 16*t*	0.18 ± 0.05 [a]	0.19 ± 0.05 [a]	0.15 ± 0.05 [b]	0.14 ± 0.05 [b]	0.030
∑ FA[10]	729.92 ± 83.69	698.46 ± 84.92	833.36 ± 86.21	840.46 ± 83.69	0.550

Table A5. Mean concentrations of polyunsaturated fatty acids by diet (% total fatty acids)

	GHAY[1]	GBLG[2]	GSH[3]	BLGSH[4]	*p*-value
∑ PUFA[5]	16.04 ± 1.20 [a]	11.79 ± 1.21 [a,b]	11.56 ± 1.22 [a,b]	10.44 ± 1.20 [b]	0.020
∑ *n*-3[6]	7.68 ± 0.56 [a]	4.61 ± 0.56 [b]	3.92 ± 0.56 [b]	3.78 ± 0.56 [b]	< 0.001
C18:3 n-3 (ALA)[7]	1.64 ± 0.22 [a]	1.64 ± 0.22 [a]	1.29 ± 0.22 [a,b]	1.14 ± 0.22 [b]	0.015
C20:3 *n*-3	0.14 ± 0.06 [a]	0.11 ± 0.06 [a,b]	0.09 ± 0.06 [b]	0.10 ± 0.06 [b]	0.005
C20:5 *n*-3 (EPA)[8]	1.55 ± 0.09 [a]	0.91 ± 0.10 [b]	0.77 ± 0.10 [b]	0.71 ± 0.09 [b]	< 0.001
C22:5 *n*-3 (DPA)[9]	3.96 ± 0.49 [a]	1.76 ± 0.49 [b]	1.57 ± 0.49 [b]	1.61 ± 0.49 [b]	< 0.001
C22:6 *n*-3 (DHA)[10]	0.39 ± 0.07 [a]	0.20 ± 0.07 [b]	0.20 ± 0.07 [b]	0.22 ± 0.07 [b]	< 0.001
∑ *n*-6[11]	7.80 ± 0.89	6.88 ± 0.90	7.41 ± 0.90	6.39 ± 0.89	0.573
C18:2 *n*-6 (LA)[12]	4.57 ± 0.82	4.18 ± 0.82	4.63 ± 0.82	3.91 ± 0.82	0.668
C18:3 *n*-6	0.12 ± 0.07	0.12 ± 0.07	0.10 ± 0.07	0.11 ± 0.07	0.416
C20:2 *n*-6	0.16 ± 0.03 [a]	0.10 ± 0.03 [b]	0.11 ± 0.03 [b]	0.10 ± 0.03 [b]	0.034
C20:3 *n*-6	0.37 ± 0.04	0.36 ± 0.04	0.39 ± 0.04	0.36 ± 0.04	0.927
C20:4 *n*-6	1.76 ± 0.30	1.68 ± 0.30	1.73 ± 0.30	1.46 ± 0.30	0.725
C22:4 *n*-6	0.83 ± 0.21 [a]	0.43 ± 0.21 [b]	0.46 ± 0.21 [b]	0.46 ± 0.21 [b]	< 0.001
n-6:*n*-3 ratio[13]	1.03 ± 0.23 [c]	1.49 ± 0.23 [b]	1.89 ± 0.23 [a]	1.70 ± 0.23 [a,b]	< 0.001
C20:3 *n*-9	0.56 ± 0.14 [a]	0.31 ± 0.14 [b]	0.23 ± 0.14 [b]	0.26 ± 0.14 [b]	0.001

Values reported as means ± standard error. Different letters denote statistical significance at $p < 0.05$ (mixed model analysis, post-hoc comparison performed using Tukey's adjustment, $n = 117$). [1]G-HAY: grass and hay diet; [2]G-BLG: grass and baleage diet; [3]G-SH: grass and soybean hulls diet; [4]BLG-SH: baleage and soybean hulls diet; [5]∑ PUFA: total polyunsaturated FAs; [6]∑ *n*-3: total *n*-3 FAs; [7]ALA: α-linolenic acid; [8]EPA: eicosapentaenoic acid; [9]DPA: docosapentaenoic acid; [10]DHA: docosahexaenoic acid; [11]∑ *n*-6: total *n*-6 FAs; [12]LA: linoleic acid; [13]*n*-6:*n*-3 ratio: ∑ *n*-6/∑ *n*-3; [14]∑ CLnA: total conjugated linolenic acid isomers; [15]∑ Atypical Dienes: total non-conjugated linoleic acid isomers; [16]∑ CLA: total conjugated linoleic acid isomers.

Table A5. (cont'd)

	GHAY	GBLG	GSH	BLGSH	*p*-value
∑ CLnA[14]	0.26 ± 0.17	0.26 ± 0.17	0.22 ± 0.17	0.23 ± 0.17	0.422
C18:3 9*c*,11*t*,15*t*	0.13 ± 0.09	0.13 ± 0.09	0.12 ± 0.09	0.12 ± 0.09	0.502
C18:3 9*c*,11*t*,15*c*	0.13 ± 0.09	0.12 ± 0.09	0.11 ± 0.09	0.11 ± 0.09	0.287
∑ AD[15]	2.61 ± 1.03 [a]	2.64 ± 1.03 [a]	2.14 ± 1.03 [a,b]	2.11 ± 1.03 [b]	0.008
C18:2 11*t*,15*t*	0.55 ± 0.13 [a]	0.47 ± 0.13 [a,b]	0.37 ± 0.13 [b,c]	0.34 ± 0.13 [c]	< 0.001
C18:2 9*t*,12*t*	0.28 ± 0.16	0.36 ± 0.16	0.30 ± 0.16	0.30 ± 0.16	0.074
C18:2 9*c*,14*t*/9*c*,13*t*	0.39 ± 0.19	0.43 ± 0.19	0.34 ± 0.19	0.34 ± 0.19	0.156
C18:2 11*t*,15*c*	0.54 ± 0.13 [a]	0.49 ± 0.13 [a]	0.35 ± 0.13 [b]	0.32 ± 0.13 [b]	< 0.001
C18:2 9*c*,16*t*	0.30 ± 0.13	0.30 ± 0.13	0.27 ± 0.13	0.27 ± 0.13	0.489
C18:2 9*c*,15*c*	0.32 ± 0.18	0.37 ± 0.18	0.31 ± 0.18	0.32 ± 0.18	0.237
C18:2 12*c*,15*c*	0.23 ± 0.12	0.22 ± 0.12	0.20 ± 0.12	0.21 ± 0.12	0.662
∑ CLA[16]	1.50 ± 0.52 [a]	1.25 ± 0.52 [b]	1.10 ± 0.52 [b,c]	0.93 ± 0.52 1	< 0.001
C18:2 9*c*,11*t*/9*c*,7*t*	0.83 ± 0.14 [a]	0.61 ± 0.14 [b]	0.52 ± 0.15 [b]	0.37 ± 0.14 [c]	< 0.001
C18:2 11*t*,13*c*	0.27 ± 0.13 [a]	0.24 ± 0.13 [a,b]	0.21 ± 0.13 [a,b]	0.21 ± 0.13 [b]	0.029
C18:2 11*t*,13*t*	0.21 ± 0.12	0.21 ± 0.12	0.19 ± 0.12	0.19 ± 0.12	0.554
C18:2 *t*,*t*	0.19 ± 0.12	0.19 ± 0.12	0.16 ± 0.12	0.17 ± 0.12	0.322

APPENDIX B: INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) FORMS

MICHIGAN STATE
UNIVERSITY

APPROVAL OF SUBMISSION

December 18, 2018

Dear Jason Edward Rowntree:

This is to notify you that your application to use vertebrate animals in research, testing or instruction has been approved by the Institutional Animal Care and Use Committee (IACUC).

The protocol is approved from 12/18/2018 to 12/19/2021.

Investigator:	Jason Edward Rowntree
Type of Review:	New Protocol Application
IACUC ID:	PROTO201800155
Title of Protocol:	Enhancing healthfulness and demand of upper mid-western, locally produced beef
Funding:	Enhancing healthfulness and demand of Upper Midwestern, locally-produced beef (National Inst of Food & Agriculture)

According to regulations and Michigan State University policies, no significant changes may be made to your research without submitting an amendment to the IACUC for review and approval before any changes can be implemented.

All principal investigators should conduct their animal activities in accordance with the following regulations and requirements: USDA regulations (9 CFR Parts 1, 2, & 3), the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy), the *Guide for the Care and Use of Laboratory Animals*, 8th Edition (the Guide), and the *Guide for the Care and Use of Agricultural Animals in Research and Teaching*, 3rd Edition (Ag Guide).

MSU is registered with the United States Department of Agriculture (34-R-0017) and has an approved Animal Welfare Assurance (A3955-01) from the NIH Office of Laboratory Animal Welfare (OLAW). In addition, all components of the University are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC Unit #1047).

NOTE: If this research is DOD funded please remember that you must submit necessary approval notification to the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

Sincerely,

Susan M. Barman

Animal Care Program

Institutional Animal Care and Use Committee
Michigan State University

4000 Collins Road, Room 145
Lansing, MI 48910

517-432-8103
Fax: 517-432-8105
iacuc@msu.edu
animalcare.msu.edu

MICHIGAN STATE
UNIVERSITY

APPROVAL OF SUBMISSION

March 18, 2020

Dear Jason Edward Rowntree:

This is to notify you that your application to use vertebrate animals in research, testing or instruction has been approved by the Institutional Animal Care and Use Committee (IACUC).

The protocol is approved from 3/18/2020 to 3/19/2023.

Investigator:	Jason Edward Rowntree
Type of Review:	New Protocol Application
IACUC ID:	PROTO202000054
Title of Protocol:	Impact of supplementation on grass-fed beef nutritive value
Funding:	Green Acres Grass-fed Beef Research (GreenAcres Foundation) Michigan State University

According to regulations and Michigan State University policies, no significant changes may be made to your research without submitting an amendment to the IACUC for review and approval before any changes can be implemented.

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