In vitro screening of sorghum parental lines for digestibility as a step toward development of superior sorghum hybrids for cattle feeding

by

Firman Nasiu

B.S., Hasanuddin University, 1999
M.S., Gadjah Mada University, 2013

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

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Abstract

Fifty-one cultivars of sorghum parental lines were used in a series of *in vitro* assays to assess fermentation by mixed ruminal microorganisms as a step toward the development of superior sorghum hybrids. Sorghum grains were milled to pass a 1-mm screen of a cyclone mill, and subsequently incubated for 30 hours with a mixture of artificial saliva and strained ruminal contents from fistulated cattle. The study was designed as an incomplete randomized block design due to the large number of sorghum cultivars tested. Maximum cumulative gas production (*K*) and time required to reach half the maximum gas production ($t_{½}$) were different (P<0.01) across sorghum cultivars. Similarly, terminal pH of cultures and *in vitro* dry matter disappearance (IVDMD) were also different among sorghum parental lines (P<0.01). Production of volatile fatty acids (VFA) also was assessed, and substantial differences among cultivars were noted for concentrations of propionate, iso-butyrate, isovalerate, valerate, and acetate:propionate ratio (P<0.01), but no differences were observed for concentrations of acetate, butyrate, and total VFA production (P>0.05). Furthermore, forty-eight sorghum cultivars of the parental lines from *in vitro* experiment were investigated to measure the gelatinization temperatures using differential scanning calorimetry (DSC). Results showed that onset temperature (T_o) ranged from 67.54 to 83.90ºC, peak temperature (T_p) ranged from 74.94 to 98.35ºC, and conclusion temperature (T_c) ranged from 74.53 to 105.32ºC. In addition, gelatinization enthalpies (ΔH_{gel}) were ranged from 1.06 to 6.49 J/g. Lower peak temperature and gelatinization enthalpies could indicate higher digestibility of sorghum grain tested. Variations in results demonstrated from both *in vitro* assay and DSC analysis suggest there is potential for development of sorghum cultivars that are more suitable than current cultivars as feeds for ruminants.

Keywords: Sorghum, *in vitro*, gas production, gelatinization

Abbreviations: *in vitro* dry matter disappearance, IVDMD; volatile fatty acid, VFA; acetate: propionate, AP; differential scanning calorimetry, DSC; onset temperature, T_o; peak temperature, T_p; conclusion temperature, T_c.

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Chapter 1 - Literature Review

1.1. Introduction

Historically, the evolutionary improvement of sorghum grain was due to its drought tolerance (Quinby & Martin, 1954; Smith & Frederiksen, 2000) and suitability for growing under arid and semi-arid condition (Haussmann et al., 1998; Verma et al., 2018; McCuistion et al., 2019). The water scarcity resistance has made sorghum more desirable to grow in under-irrigated fields than corn (Smith & Frederiksen, 2000), the number one grain produced in the world (Bean et al., 2019). Areas such as Texas and Kansas have favorable conditions for growing sorghum and are the major producers of sorghum in the Unites States (Shahbandeh, 2024).

Sorghum grain has been used in ruminant diets for many years due to its energy and protein content. In the livestock industry, the nutritive value of grain sorghum is comparable with other conventional grain feed such as maize, wheat, and barley (Spicer et al., 1986; Hancock et al., 1992). Sorghum exhibits an average feeding value of 92% that of corn (Zinn, 1991) and a greater apparent metabolizable energy value compared to corn or wheat (Hulan & Proudfoot, 1982; Black et al., 2005). Compared to corn, sorghum also contains more crude protein, ash (Hulan & Proudfoot, 1982), and the amino acids leucine, alanine, and phenylalanine (Breuer & Dohm, 1972). Moreover, high concentrations of phenolic compounds within sorghum have drawn significant attention because these secondary metabolites possess anti-inflammatory and antioxidant properties that not only benefit humans (Awika & Rooney, 2004) but also have the potential to improve animal health (Lefter et al., 2019).

Grain sorghum is valued in ruminant diets for its starch content, which provides high energy density to support production (Humer & Zebeli, 2017; Ronda et al., 2019). Starch digestion and energy utilization efficiency from sorghum can be variable, and digestibilities of

protein and amino acids often are inferior to those of other cereal grains (Liu et al., 2013). The digestibility of sorghum starch in ruminant diets is variable due to a range of factors such as grain type, processing method, duration for starch degradation in the small intestine, feed surface exposed for enzymatic hydrolysis (Owens et al., 1986), and biochemical and structural features of grain endosperm (Wong et al., 2009). Environmental and genetic factors primarily affect the grain's biochemical composition and starch properties (Beta & Corke, 2001).

Attempts to improve sorghum grain digestibility have focused on various strategies, including genetic modification (Wong et al., 2010), grain processing methods (Theurer, 1986), and the use of reducing agents (Hamaker et al., 1987). These efforts have shown significant improvement in sorghum digestibility through studies using *in vitro* and *in vivo* methods. Additional work is needed to explore the digestibility of different varieties of sorghum with various characteristics. This study investigated attributes of sorghum parental lines using *in vitro* techniques as a step toward developing superior sorghum hybrid for use in cattle production.

1.2. Sorghum Introduction and Improvement

1.2.1. Sorghum introduction

Sorghum is an ancient crop cultivated for thousands of years (Hagerty, 1941; De Wet & Harlan, 1971). Archeological and botanical evidence suggest that cultivation and pre-domestication of sorghum took place in the eastern Sudanese savannah near the lower Blue Nile and White Nile rivers in the fourth millennium B.C. (Winchell et al., 2017; Stemler et al., 1975; Murdock, 1960; Venkateswaran et al., 2019; Martin, 1970). From its origin and domestication place in northeastern Africa, sorghum spread across the African continent (Ananda et al., 2020). Sorghum distribution continued from Western Ethiopia to West Africa around 1500-2000 B.C. and was moved across the Sudan to the region of the Upper Niger River by the Cushite people

(Doggett, 1970). Furthermore, due to human migration, sorghum distribution reached eastern and southern Africa (Venkateswaran et al., 2019).

The geographical distribution of Sorghum from Africa to Asia was brought along with movement of people (Kimber, 2000) through dhow traffic on the trading route between East Africa and India via Arabia around 1500 B.C. (Doggett, 1970; Kimber, 2000; Winchell et al., 2018). When brought to India, sorghum taxa had probably been domesticated (Kimber, 2000), and most were *bicolor* types (Boivin & Fuller, 2009). Some authors suggest that sorghums shipped to India were East African sorghums due to the close relation between Indian sorghums and north-east African sorghums (Doggett, 1970). The trading route continued from India to China along the coast of Asia and from Indochina to China through the Mekong River (Venkateswaran et al., 2019; Doggett, 1970). Sorghum distribution in China occurred during the Hubilie Khan era from 1260 to 1295 following its introduction to China by Genghis Khan between AD 1206 and 1228 (Hagerty, 1941). Furthermore, studies estimate that sorghum reached the Middle East and the Mediterranean region from India or Africa through Arabia by 700 B.C. (Dogget, 1970).

Sorghum's introduction to the United States began in the middle of the nineteenth century when guinea corn and chicken corn entered the country through the slave trade (Venkateswaran et al., 2019; Doggett, 1970). However, the first sorghum officially introduced was broomcorn, which Benjamin Franklin transported back from Europe in 1725 (Duncan et al., 1991). The first grain sorghums successfully introduced and that contributed significantly to crop improvement in the United States were White and Brown Durras transported to California from Egypt in 1874 (Smith & Frederiksen, 2000; Quinby & Martin, 1954). White Durras were more desirable than Brown Durras due to the bitterness of Brown Durra seed. White Durra reached the Great Plains

quickly and became popular in Kansas during the early1890s (Smith & Frederiksen, 2000). Other varieties from North Africa introduced in the United States following the durra varieties were Milo in about 1880, Feterita in 1906, and Hegari in 1908 (Doggett, 1970). In addition, kafir varieties moved from South Africa to the United States in 1876 and Shallu from India in 1890 (Doggett, 1970). In the early 1910s, dwarf yellow Milo, due to farmers' selection, became an essential sorghum grain in the Southern Great Plains (Quinby & Martin, 1954).

Meanwhile, sorghum distribution reached Argentina, Paraguay, and Australia in the early years of settlement. Sorghum became popular in those countries after its introduction from the United States (Martin, 1970). Recently, sorghum has been spread globally and has become one of the top five grains produced (Bean et al., 2019). The United States, Nigeria, Brazil, India, and Mexico are the top five countries for sorghum production in the world (USDA, 2024).

1.2.2. Sorghum improvement

Because grain sorghum has been an essential crop across the globe, national and international programs become crucial to facilitate the development of superior cultivars to improve sorghum production. The United States Department of Agriculture (USDA), the Food and Agriculture Organization (FAO) of the U.N., and the United Nations Development Program (UNDP) are the entities that are intensively promoting the research on sorghum cultivars (Aruna & Cheruku, 2019). The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) also has played a significant role in these efforts (Reddy et al., 2010). Furthermore, the Sorghum Conversion Program initiated by Texas A&M-USDA has contributed to the development of elite sorghum hybrids by converting tall and late-maturing tropical sorghum to short and early-maturing sorghum (Dahlberg et al., 1996). This conversion program is essential

to the sorghum improvement program in the United States (Rooney, 2004) and continues to provide superior germplasm for many breeders worldwide (Tari et al., 2013).

Grain sorghum improvement and development have been a focus of research and breeding programs worldwide. These efforts have primarily aimed to enhance yield, adaptation, quality, and disease resistance while also addressing biotic and abiotic stress (Hoffman & Rooney, 2014; Aruna & Cheruku, 2019; Reddy et al., 2008). Integrating traditional and new technologies, including molecular genetic tools, has further enhanced sorghum improvement (Rooney, 2004). Population improvement methods, such as recurrent selection and hybrid development, have also been employed to exploit heterosis (Reddy & Kumar, 2008).

Various factors influence the genetic improvement and development program of grain sorghum worldwide. These include the need to enhance yield and adaptation, the availability of diverse germplasm, and the use of gene families to improve agronomic traits (Aruna & Cheruku, 2019; Hailemariam, 2023). Furthermore, transgenic technology can enhance resistance to pests, pathogens, and environmental stress and improve nutritional quality (Visarada, 2008). The discovery of cytoplasmic and genetic male sterility has enabled easy cross-pollination in sorghum, leading to the development of pure-line varieties and hybrids (Reddy et al., 2008). Moreover, molecular breeding approaches, such as those focused on drought tolerance and quality improvement, are essential for enhancing sorghum's resilience and nutritional value (Bejiga et al., 2021; Mofokeng et al., 2017).

The development of sorghum hybrids has focused on five races of *Sorghum bicolor*, including durra, kafir, guinea, caudate, and bicolor races (Aruna & Cheruku, 2019). Each race is developed based on specific characteristics important for sorghum hybridization. The kafir race has made a significant contribution to the development of sorghum hybrids, particularly in

China, where it has played a vital role in the development of R-lines (Li & Li, 1998), which is in line with the genetic diversity and potential for hybridization found in kafir race (Ganesamurthy et al., 2010). The primary sorghum race in Ethiopia, durra, has significantly contributed to the development of sorghum hybrids. It is widely grown and adaptively differentiated into various races, ecotypes, and varieties, explicitly adapting to different environments (Mola & Ejeta, 2021). The genetic diversity among these landraces has been high, with specific genotypes being divergent and suitable for hybridization programs (Ganesamurthy et al., 2010). The role of the durra race in the development of sorghum hybrids corresponds with its association with morphological traits and yield components, which are essential factors in hybrid performance (Tesso et al., 2011; Haussmann et al., 1998).

The contribution of the caudatum race to the development of sorghum hybrids has to be considered, particularly in terms of its genetic diversity and potential for improving drought tolerance. The caudatum race, which Chari-Nile-speaking peoples in Africa likely developed, is known for its hardness and high grain production (Stemler et al., 1975). This race has been critical in developing hybrid parents, particularly in the African Great Lakes region (Reddy et al., 2006; Mutava et al., 2011). The genetic diversity of cultivated sorghum, including the caudatum race, has been studied, with the caudatum genotype showing stability in grain yield across different environments (Deu et al., 2006; Mutava et al., 2011). Another sorghum landrace that is important in the development of sorghum hybrid is guinea. Rattunde et al. (2013) found that Mali's first sorghum hybrid based on Guinea-race germplasm showed significant yield advantages.

Major sorghum producing countries such as China, the United States, Australia, Brazil, and Mexico have participated in developing sorghum hybrids (Aruna & Cheruku, 2019).

Hybridization of sorghum in the United States began in 1914 through introduction of Dakota Amber Sorgo, Gooseneck Sorgo, Honey Sorgo, Sumac Sorgo, Red Amber Sorgo, Black-hull Kafir, Dwarf Milo, and Feterita (Vinall & Cron, 1921; Huskins & Smith, 1932; Hume & Franzke, 1934; Marcy, 1937; Norgaard, 1942; Duncan et al., 1991). Vinall and Cron of USDA introduced Chiltex and Premo in 1923, and these were the first results of artificial hybridization to grow (Martin, 1937; Duncan et al.,1991; Hoffman & Rooney, 2014; Rooney, 2004). In the following years, other elite cultivars were introduced throughout the United States, including Finney, Texas Milo, Wheatland, Martin, and many other cultivars grown in different climate conditions (Quinby & Martin, 1954; Duncan et al., 1991).

Like other sorghum producing countries, the United States breeding programs have focused on enhancing yield potential per plant, heterosis, test weight, panicle size, and grain number per panicle (Pfeiffer et al., 2019). About 60% of total yield gains in U.S. sorghum production have resulted from sorghum breeding programs (Pfeiffer et al., 2019). Moreover, the development of hetero-waxy and waxy sorghum hybrids has also been of interest in sorghum development programs due to their potential to improve the nutritive value of sorghum grain (Wong et al., 2009; Rooney & Pflugfelder, 1986). Hybrid development in the United States continues the sorghum hybridization program by focusing on the exploitation of kafir and durra race (Duncan et al., 1991). Recent technologies such as doubled haploids, high-throughput phenotyping, and genomic selection have been applied in sorghum breeding programs, which are expected to accelerate the invention of superior sorghum hybrids (Pfeiffer et al., 2019). Hybridization programs have also contributed to a remarkable increase in sorghum production in Australia (Stephens et al., 2012) and Argentina (Gizzi & Gambin, 2016), as these countries have significantly increased sorghum yield and production in the past few decades.

1.3. Characteristics of Sorghum Grain

1.3.1. Structure of sorghum grain

The structure and composition of sorghum grain are complex and vary between different varieties (Bean et al., 2019), which are highly influenced by genetics and environmental conditions (Serna-SaldÍvar & Espinosa-Ramírez, 2019). Morphologically, sorghum kernels consist of three components: pericarp (outer layer), endosperm (storage tissue), and germ (Waniska, 2000). Composition of sorghum kernels has been observed to be 6% pericarp, 84% endosperm, and 10% germ (Rooney & Miller, 1982; Rooney & Serna-Saldivar, 2000). The pericarp is crucial in determining grain quality parameters (Guindo et al., 2016). The outer layer of the pericarp comprises the epicarp, mesocarp, and endocarp (Earp & Rooney, 1982). The endocarp component of the pericarp consists of cross and tube cell layers to transport water throughout the kernel (Guindo et al., 2016). The pericarp is characterized by irregular air spaces, round starch grain in soft or opaque endosperm varieties, and tightly packed structure with polygonal starch grain in hard or translucent endosperm varieties (Hoseney et al., 1974). The pericarp thickness is influenced by the quantity of starch in the mesocarp cells, with thick pericarp varieties containing more starch (Earp et al., 2004).

The endosperm of sorghum grain serves as a storage component for nutrients, mainly starch and protein (Hoseney et al., 1974; Rooney & Miller, 1982). It also plays a role in the physical characteristics of the grain, such as its dimension and weight (Rooney et al., 2005; Anglani, 1998). Endosperm tissue comprises an aleurone layer, peripheral, corneous area, and floury area (Earp & Rooney, 1982). The aleurone layer contains protein, ash, and oil, and the peripheral area contains protein and starch (Waniska, 2000). Peripheral endosperm tissue is very dense, hard, and resistant to moisture penetration and enzyme degradation (Rooney &

Pflugfelder, 1986). In sorghum kernels, peripheral endosperm proportion is greater than that of corn (Watson, 1984), which may explain the lower digestibility of grain sorghum compared to corn (Rooney & Pflugfelder, 1986)

The structure of sorghum grain embryo is a complex system that undergoes significant changes during germination and seedling growth. The scutellum, an essential tissue in the embryo, plays a crucial role in mobilizing reserve food materials and can produce α-amylase, an enzyme that breaks down starch (Aisien & Palmer, 1983). The endosperm, which surrounds the embryo, also undergoes structural changes, with different varieties possessing distinct characteristics (Hoseney et al., 1974; Zheng & Wang, 2010).

1.3.2. Chemical composition and characteristics of sorghum starch

Starch is synthesized in amyloplasts (Morrison & Karkalas, 1990) and is comprised of two significant molecules: amylose and amylopectin (French, 1973; Martin & Smith, 1995). In addition to amylose and amylopectin, a trace amount of branched amylose may also be present (Rooney & Pflugfelder, 1986). Amylose is synthesized by granule-bound starch synthase (CBSS; Ball et al., 1998; Denyer et al., 1999; Seung, 2020) and characterized as a linear polymer of α-1,4 linked D-glucose units (Tester et al., 2004) and a few α-1,6 branch points (Bean et al., 2019). The proportion of amylose in starch is affected by the plant species and the genetic variation of the species (Rooney & Pflugfelder, 1986). Amylose content in most sorghum starch ranges from 20 to 30%, which is typical for regular sorghum varieties (Zhu, 2014). In a study comparing ten sorghum varieties grown under the same condition, Beta et al. (2000) found that the amylose content of sorghum starch varied from 21.5 to 29.9%. Similarly, Cagampang and Kirleis (1985) reported that the amylose content of starch from fifteen pearled sorghum cultivars ranged from 24.8% to 25.9%. The ratio of amylose:amylopectin affects characteristics of starch (Bean et al.,

2019). Normal sorghum, which contains greater amylose content compared to hetero-waxy and waxy sorghum, is less digestible (Zhu, 2014). Hibberd et al. (1982) compared the *in vitro* digestibility of isolated starch from nine varieties of grain sorghum and four varieties of corn and reported higher IVDMD and IVGP from varieties of waxy sorghum. A similar result was highlighted from an *in vitro* and *in situ* study to investigate the nutritional properties of different sorghum genotypes: non-waxy (WxWxWx), intermediate waxy (WxWxwx and Wxwxwx), and waxy (wxwxwx) (Lichtenwalner et al., 1978). They revealed a significant increase in starch digestibility as the waxy gene increased as measured by nutrient disappearance and gas production.

Amylopectin is much larger than amylose and is the major component of starch (Rooney & Pflugfelder, 1986; Bean et al., 2019). It is a highly branched polymer of α-1,4-linked D-glucose units with α-1,6 branch points every 20 to 25 glucose residues (Tester et al., 2004; Watson, 1984; Rooney & Pflugfelder, 1986). Amylopectin comprises 70 to 85 % of the starch component (Bean et al., 2019). The concentration of amylopectin affects the gelatinization property (Zhu, 2014), eventually affecting the starch's digestibility. Gelatinization is an irreversible denaturation status of starch after applying sufficient energy and moisture to break hydrogen bonds in the crystalline area (Zobel, 1984). Once the starch is gelatinized, it becomes more susceptible to enzymatic degradation (Theurer, 1986). Beta et al. (2000) reported the amount of energy used to disrupt the intramolecular hydrogen bonds of starch granules in some varieties of sorghum was less for starches containing greater amylopectin content. They further explained that the low energy required for gelatinization is associated with greater digestibility of starch within the grains. In addition to amylose and amylopectin, some starches also contain intermediate fractions recognized as less branched amylopectin and amylose (Whistler et al.,

1984). However, due to the difficulties of the extraction process, those intermediate fractions are poorly investigated (Rooney & Pflugfelder, 1986).

Starch-protein interaction is a significant factor affecting the starch digestibility of grain sorghum, which is most likely due to the presence of kafirins (Wong et al., 2009). In some varieties of sorghum, the protein composition of endosperm is unique because prolamin, which is the major storage protein in grain sorghum, develops intermolecular cross-links by sulfide bonds called cross-linked kafirins (El Nour et al., 1998). The presence of extensive cross-linking in kafirin limits digestibility of protein and the starch granules enmeshed therein (Hamaker & Bugusu, 2003; Wang et al., 2009). In the cooking state, when heat is applied to the sorghum flour, kafirin is indigestible even with the reduction of sulfide bonds, indicating that another factor such as molecular weight of individual kafirins may affect the cross-linking of sorghum protein (Belton et al., 2006). The cross-linked fraction appears to be higher in sorghum than in other grains (Wong et al., 2009), which may explain corn's higher digestibility than grain sorghum (Liu et al., 2013). Peripheral endosperm in non-waxy sorghum types contains dense and resistant protein that protect the endosperm's inner components (Bean et al., 2019). This region is persistent in mechanical and enzymatic degradation (Rooney & Pflugfelder, 1986). However, corneous endosperm in waxy-type sorghum lacks protein, making it susceptible to mechanical and enzymatic breakdown (Waniska, 1989). The development of waxy and hetero-waxy types of sorghum is promising to improve grain sorghum's starch digestibility because those sorghum types exhibit less protein in the peripheral endosperm.

1.3.3. Phytochemical compounds of grain sorghum

One factor affecting grain sorghum's starch digestibility is the presence of anti-nutritional compounds, particularly tannin (Rooney & Pflugfelder, 1986). This phenolic compound has been

found to decrease starch digestibility by interacting specifically with amylose and linear fragments of amylopectin (Barros et al., 2012). Research has shown that the tannin content in grain sorghum can significantly affect the starch digestibility. In a study comparing the effect of tannin extract on amylase activity before and after the addition of amylopectin, Mkandawire et al. (2013) observed a reduced amylase activity when tannin was allowed to interact with the amylase enzyme before the addition of amylopectin. However, a significant increase of amylase activity was observed when amylase was added after tannin and amylopectin were mixed. Another study by Awika (2016) investigated the effect of tannin on the characteristics of partially gelatinized starch. They demonstrated that when complexed with starch, tannins can increase crystallinity, pasting temperature, and slow digesting starch, as well as double-resistant starch. In addition, the effect of tannin on starch digestibility is concentration dependent (Barros et al., 2012).

Tannin inhibition on starch and protein, leading to the lower digestibility of feed ingredients including sorghum grain, can be proposed through two mechanisms: 1) direct interaction of tannins and microbial cell wall and secreted enzymes and 2) complex of tannins with nutrients such as protein, carbohydrate, and mineral (McSweeney et al., 2001). Even though tannins have a solid affinity for binding protein relative to other substrates (Waghorn, 2008), tannins have been proven to inhibit starch digestion (Mkandawire et al., 2013). Tanner et al. (1994) studied the inhibition of protein hydrolysis by proanthocyanidins. They suggested that tannins may alter the rumen's protein metabolism by forming a tannin-protein complex and interfering with the activities of the susceptible sites of proteases. Similarly, Min et al. (2000) investigated the degradation of ribulose-1,5-*bi*phosphate carboxylase/oxygenase (*EC* 4.1.1.39;

Rubisco) by rumen microorganisms in the presence of tannins. They concluded that condensed tannins may depress protein digestion by protecting it from ruminal degradation.

Considering the negative effect that tannins may possess on digestion of starch and other nutrients, some studies have been conducted to alter tannin inhibition. Babikir and El Tinay (1993) reported an increase *in vitro* protein digestibility and reduced tannin concentration by soaking the whole grain in water or sodium carbonate in a study using two cultivars of sorghum grain. Another method to decrease tannin was presented by Hassan and El Tinay (1995), who concluded that fermentation can decrease tannin concentration in sorghum grain. Ruminal microorganisms can hydrolyze tannins (Field, 1987; Bhat et al., 1998), making them have less interaction with nutrients. However, degradation of hydrolysable tannins in the ruminal environment produces toxic compounds (Doss et al., 2009) that can adversely affect fibrolytic microorganisms *Ruminococcus flavefaciens* and anaerobic fungi populations (Jayanegara et al., 2015). The effect of tannins on bacteria is species-specific as condensed tannins from *Lotus corniculatus* suppress some proteolytic bacteria, but total ruminal microbial protein remained unchanged (Min et al., 2002)

Regardless of the negative effects of tannin, the presence of tannin still has potential benefits for animal health and the environment. The role of tannins in improving health status is due to the capacity of tannins to act as a biological antioxidant that protects cells from damage by free radicals (Hagerman et al., 1998). Studies have also shown the use of tannins to reduce methane emissions produced by ruminants (Jayanegara et al., 2015; Rira et al., 2019; Aboagye et al., 2019).

Another anti-nutritional compound present in sorghum that may affect starch digestibility is phytic acid (Thompson & Yoon, 1984). Like tannins, phytic acid may reduce starch

digestibility by binding to amylase, interacting with the protein embedded in the starch molecule, or directly binding to the starch component (Yoon et al., 1983). However, phytic acid may not adversely affect animal performance at low concentrations, as Bowman et al. (2005) observed. By investigating the use of low phytic acid-P mutant barley in a finishing beef cattle diet, they found that performance of animal fed different levels of phytic acid-P in the diet were similar, as were the nutrient digestibilities among treatments. The absence of a negative effect on animal performance, as reported by Bowman et al. (2005), may be due to the capability of ruminal microbes to hydrolyze phytic acid (Zebeli & Humer, 2016). Discussion on the effect of phytic acid on starch digestibility through either *in vitro* or *in vivo* studies is limited. However, it is convincing that phytic acid may play a significant role in starch digestion.

Carotene is another relatively abundant phytochemical in grain sorghum (Kean et al., 2007). The presence of carotene is beneficial as it serves as a precursor for synthesis of vitamin A, which is linked to cardiovascular disease in humans (Kritchevsky, 1999) and infectious disease in ruminants (LeBlanc et al., 2004). The excellent properties of carotene in increasing health status may be attributed to its antioxidant capacity and impact on immune function (Chew, 1993). Because vitamin A is essential for both humans and ruminants, development of new sorghum hybrids has focused yellow endosperm sorghum, as it contains higher carotene content (Rooney & Pflugfelder, 1986; Kean et al., 2007; Waniska, 2000). The digestibility of yellow-endosperm sorghum is mainly affected by genetics, as Streeter et al. (1991) reported that the hetero-yellow hybrid has higher digestibility than pure yellow endosperm. They also highlighted uniformity in observation parameters in pure yellow sorghum hybrids due to the lower genetic modification in parental varieties. The presence of phenolic compounds, such as tannin, may reduce the biological activity of carotene due to the tannin inhibition of enzymatic activity during

digestion and absorption of the protein complex. Marques et al. (2021) suggested that polyphenols may inactivate the digestion enzyme through complex formation with the enzyme's active site. Thus, developing a low-tannin sorghum hybrid may increase the availability of carotene in sorghum grain.

1.4. Digestibility of Grain Sorghum

1.4.1. Effect of structure and chemical composition of the starch

The extent of starch digestion and utilization represents grain quality, including sorghum, when fed to animals. Grain with higher digestibility is more likely to improve animal performance (Theurer, 1986; Streeter et al., 1989). Starch's structure and chemical composition are major factors affecting sorghum grain's digestibility (Rooney & Pflugfelder, 1986). Compared to corn, sorghum endosperm contains a thicker peripheral layer, which is rich in protein (Watson, 1984). Sorghum grain digestibility is inferior to that of corn (Liu et al., 2013). However, with proper processing methods such as steam-flaking, the digestibility of the sorghum grain can be increased to a level similar to corn (Zinn, 1991).

The structure and chemical composition of sorghum grain are primarily affected by genetics and environment (Beta & Corke, 2001; Huntington, 1997). Lichtenwalner et al. (1978) observed a marked difference in the digestibility between non-waxy (WxWxWx), intermediate waxy (WxWxwx and Wxwxwx), and waxy (wxwxwx), representing different genotypes of grain sorghum. By comparing two different cultivars of waxy and non-waxy Kafir and Redlan through *in vitro* and *in vivo* studies, they reported increases in starch digestibility with presence of the waxy gene. They further highlighted that protein solubility is greater in homozygous waxy grain than in non-waxy grain. A similar study on two different types of endosperms has been done by Streeter et al. (1990) by comparing three pure-line sorghum varieties, including Dorset (normal

endosperm), Dwarf Redlan (waxy endosperm), and 1133 (waxy endosperm). They found no significant differences in ruminal or total tract starch digestibility among varieties. However, starch digestion before the caecum was greater in the waxy sorghum type. Another comparison of digestibility of different endosperm types of sorghum has been reported by Wester et al. (1992) by comparing waxy, hetero-waxy, and non-waxy hybrids of sorghum. They reported the fastest *in vitro* starch disappearance in the waxy type compared to the other types. In another study, Pedersen et al. (2007) investigated the effect of waxy allele wx^a and wx^b on the thermal properties of sorghum starch. They reported a higher gelatinization temperature of the waxy type than the wild type. The higher gelatinization temperature in the waxy type might be due to the formation of a complex between the amylose-forming enzyme, granule-bound starch synthase, and amylopectin content, creating a solid covalent bond that is resistant to enzymatic hydrolysis (van de Wal et al., 1998). Greater digestibility of the waxy hybrid of sorghum was expected because the waxy hybrid contains >90% amylopectin, which is more susceptible to enzymatic degradation (Rooney & Pflugfelder, 1986; Tovar et al., 1977).

Environmental variation significantly impacts sorghum starch composition, which will further affect starch properties. Beta and Corke (2001) investigated eight local Zimbabwean landrace varieties, one improved cultivar (SV2), and one hybrid (DC-75). They reported significant variability in amylose content from the same sorghum grain varieties grown under different environmental conditions. Further investigation by Beta and Corke (2001) confirmed the significant effect of genetic and environmental conditions on sorghum grain's pasting, textural, and thermal properties. Similarly, Kaufman et al. (2017) investigated two normal-endosperm sorghum hybrids, Seneca and TX631*TX436, grown under different growing seasons in 2008 and 2009. They reported a slightly higher amylose content of the hybrids grown in 2008

as the temperature was higher than in 2009. They further suggested that the higher amylose content was associated with reduced crystallinity and lamellar periodicity peak intensity as the plant matured. The significant effect of growing conditions on starch composition was also reported by Li et al. (2013) through a study on sorghum starch biosynthesis as affected by different growing temperatures. They reported a significant increase in the ratio of long to short amylopectin branches and a lower degree of branching in the inbred lines grown at temperatures up to 38/21 °C (day/night) compared to 32/22 °C.

1.4.2. Effect of protein-starch interaction

Starch digestibility of grain sorghum is of significant interest in ruminant studies, and can be impacted by protein-starch interactions. Strong sulfide covalent bonds between protein molecules (El Nour et al., 1998), where starch granules are attached (Hamaker & Bugusu, 2003; Wang et al., 2009), limits the access of enzymes to the starch, resulting in lower starch digestibility (Rooney & Pflugfelder, 1986). Compared to corn, storage protein in sorghum, which is called kafirin, contains a greater cross-linked fraction and is more hydrophobic, resulting in formation of undegradable intermolecular disulfide-bonds with other proteins and with other molecule such as polyphenols and lipid, which are stronger than those in corn (Belton et al., 2006; Hamaker & Bugusu, 2003).

Based on solubility, molecular weight, and structure, kafirins are classified into α-, β-, and γ- kafirin (Shull, 1991), with the α-kafirin being the predominant kafirin class (~80%) followed by γ-kafirin (~15%), and β-kafirin (~5%) (Wong et al., 2009). Because kafirin comprises 70-80% of the protein in sorghum flour (Hamaker et al., 1995), the presence of kafirin molecules has a significant impact on starch digestibility (Wang et al., 2009). In addition, high concentrations of kafirin in polymeric forms linked by disulfide bonds increased its propensity to

limit enzyme penetration into protein molecules (Duodu et al., 2003; El Nour et al., 1998; Hamaker et al., 1987). The distribution of disulfide bonds might have a greater negative influence on digestibility than the presence of kafirin itself. In a study to investigate the digestibility of protein and starch from two sorghum lines, KS48 and KS51, which are identical pedigree but different digestibility, Wong et al. (2009) reported a lower digestibility in KS51 than KS48 even though KS48 contains a higher amount of kafirins. They further explained that the low digestibility of the KS51 line was due to the relative abundance of intermolecular disulfide bonds observed in cross-linked glutelin. Thus, even though the presence of kafirin could be a crucial factor (El Nour et al., 1998), the concentration of intermolecular disulfide bonds seems to be more effective in reducing starch digestibility of sorghum grain (Wong et al., 2009).

Starch digestibility is highly associated with the breakdown of the protein matrix and protein body in grain sorghum (Wong et al., 2009; Gómez et al., 2016). Starch molecules attached to protein are more accessible for digestion after protein degradation (Chandrashekar & Kirleis, 1988; Gomez et al., 2016). Because sorghum proteins predominantly consist of kafirins (Hamaker et al., 1995), various methods have been applied to reduce the effect of kafirins on protein digestibility. Hamaker et al. (1987) investigated the effect of reducing agents on uncooked and cooked cereals of sorghum, maize, barley, rice, and wheat. Their report on protein digestibility indicated the involvement of kafirin in reducing protein digestibility of cooked flours, with the greatest reduction in sorghum. They further highlighted that reducing agents such as dithiothreitol, bisulfite, 2-mercaptoethanol, and L-cysteine up to 100 mM effectively increase protein digestibility with sorghum, which was the most affected flour. Konigsberg (1972) reported that 2-mercaptoethanol had been extensively utilized for the complete reduction of

disulfide bridges, which is associated with increased protein digestibility (Oria et al., 1995) and is more effective in sorghum compared to other cereals (Hamaker et al., 1987).

1.4.3. Effect of phytochemical compounds

The uniqueness of sorghum grain is not only limited to the cross-linked disulfide kafirins that affect starch digestibility (Rooney & Pflugfelder, 1986), but also to the presence of phytochemical compounds such as the polymeric polyphenols called tannins (Waniska, 2000). Some sorghum cultivars contain higher tannin concentrations than others, making them less digestible than low-tannin cultivars (Barros et al., 2012; Amoako & Awika, 2016; Mkandawire et al., 2013). Adverse effects of tannins on starch digestion might be due to their ability to form complexes with other components in the sorghum endosperm, such as polysaccharides, proteins, and minerals (Amoako & Awika, 2016; Waghorn, 2008; McSweeney et al., 2001). Barros et al. (2012) investigated effects of tannin content on starch digestibility *in vitro*. They observed that the presence of tannins reduced starch digestibility of corn and that tannins have greater affinity for amylose than for the amylum component. A similar result was noted by Rocchetti et al. (2020) in a study to investigate pigmented sorghum polyphenols as inhibitors of starch digestibility. They concluded starch digestibility decreased in the presence of tannins as a result of formation of insoluble complexes between starch and tannins that were resistant to hydrolysis. Another inhibition mechanism of starch digestibility by tannins is binding the active site of the starch-degrading enzyme, leading to the reduction of enzyme activity (Barros et al., 2012; Waghorn et al., 2008).

Some methods have been applied to reduce tannin concentration because tannin adversely impacts digestibility of sorghum starch and protein (Barros et al., 2012; Babikir & El Tinay, 1993). Sodium carbonate in an *in vitro* experiment increased protein digestibility of

sorghum flour after being soaked at 100°C for 20 minutes (Babikir & El Tinay, 1993). Adetunji et al. (2015) used 0.4% NaOH solution to inactivate tannins in sorghum grain. By steeping the milled sorghum grain in NaOH solution, they reported a 60-80% reduction of α-amylase inhibition and increased free amino nitrogen. Reducing α-amylase inhibitors is crucial to increasing α-amylase activity (Rooney & Pflugfelder, 1986); thus, an increase in sorghum starch digestibility could be expected (Amoako & Awika, 2016).

1.4.4. Effect of ruminal fermentation

Ruminal fermentation significantly affects starch digestibility (Ørskov, 1986), with various factors influencing this process. The presence of starch-digesting bacteria, and to a lesser extent fungi and protozoa, and their activities become a crucial factor in determining starch digestibility and in maintaining energy generated from ruminal fermentation (Gómez et al., 2016; Mendoza et al., 1993). However, starch digestion and absorption in the rumen may be energetically less efficient than in the small intestine (Owens et al., 1986; Huntington et al., 2006; Reynolds, 2006) because the loss of energy amounting to 12 to 20% for heat and methane during ruminal fermentation may occur (Ørskov, 1986).

Ruminal starch digestion is initiated by attachment and colonization of the feed particles by ruminal bacteria, with *Streptococcus bovis*, *Butyrivibrio fibrisolvens*, *Bacteriodes ruminocola*, and *Selenomonas ruminantium* as the predominant starch-digesting bacteria (Huntington, 1997). Amylases produced by the amylolytic bacteria then hydrolyze the α-1,4 and α-1,6 glycosidic bonds of amylose and amylopectin, resulting in dextrins and oligosaccharide constituents (Cerrilla & Martínez, 2003; Gómez et al., 2016) with maltose and glucose as final end products (Cotta, 1988). Rate of enzymatic starch degradation is determined by the accessibility of enzymes to starch molecules (Theurer et al., 1999). However, presence of starch-protein (El

Nour et al., 1998) and protein-tannin complexes (Barros et al., 2012) may limit enzyme penetration, allowing starch granules to remain intact and undigested (Huntington, 1997). The employment of grain processing methods with a combination of heat, moisture, time, and mechanical interaction increases the feed particle surface and provides access for enzyme penetration (Zinn et al., 2008). Thus, starch digestion might be expected to be higher (Rooney & Pflugfelder, 1986).

Improvement in starch digestibility in response to grain processing increases energy availability for ruminal fermentation and animal growth (Corona et al., 2005). Excessive rapid digestion of starch may harm ruminal fermentation and animal performance (Ørskov, 1986). Rapid fermentation of starch increases organic acid production, leading to a decline in ruminal pH (Lee et al., 2003; Mould et al., 1983). At low ruminal pH, growth and activity of fiber-digesting bacteria are inhibited because they favor an optimum ruminal pH between 6.2 and 6.6 (Shriver et al., 1986). In a study using ruminally cannulated cows, Lechartier & Peyraud (2011) reported that a diet containing 41% of DM as starch and 28% of DM as rapidly degradable carbohydrate decreased fibrinolytic activity, VFA production, and acetate:propionate as ruminal pH dropped below 6. Moreover, reducing ruminal pH to <5.6 alters rumen metabolism and function, animal performance, and health (Nagaraja & Titgemeyer, 2007). Further adverse consequence is that a constantly low ruminal pH stimulates ruminal acidosis (Devries et al., 2014; Nagaraja & Titgemeyer, 2007).

Nutritive value of sorghum grain is equivalent to other conventional starch source such as barley, wheat, and corn, even after grain processing (Spicer et al., 1986; Hancock et al., 1992). Thus, sorghum has the potential to provide energy for animal production while maintaining the optimum ruminal function (Humer et al., 2018). Because starch of sorghum grain is less

degradable in the rumen, more starch is available for intestinal digestion, which is more efficient for energy production (Owens et al., 1986; Huntington et al., 2006; Reynolds, 2006). However, intestinal starch digestion by cattle is limited due to the limited production of key carbohydrase enzymes (Harmon et al., 2004).

1.4.5. Effect of intestinal digestion

An appreciable amount of starch escapes ruminal digestion as the consequence of several factors such as starch structure, starch interaction with other nutrients, anti-nutritional compounds, and grain processing (Owens et al., 1986; Rooney & Pflugfelder, 1986). About 30 to 40% of intake starch could escape the ruminal degradation and then flow to the duodenum, where intestinal digestion occurs (Ørskov, 1986). The capacity of starch digestion in the intestine ranges from 45 to 85% of starch entering the duodenum (Huntington, 1997). Starch digestion in the small intestine of ruminants involves the secretion of pancreatic amylase (Gilbert et al., 2015), which hydrolyzes amylose and amylopectin into limit dextrins and oligosaccharides (Gray, 1992: Harmon, 1993). By the action of maltase and iso-maltase, starch digestion is completed by producing free glucose (Harmon, 1992). However, intestinal starch digestion by cattle may be limited by insufficient pancreatic amylase production (Harmon et al., 2004).

The amount of starch intake degraded by intestinal digestion ranges from 5 to 20%, with the predominant digestion occurring in the small intestine (Huntington, 1997). In a study involving six sorghum grain hybrids, Streeter et al. (1991) reported that total tract starch digestibility ranged from 78.9 to 82.3%, with the small intestine digestibility averaging from 6.4 to 8.6% of intake starch and from 20.55 to 26.8% of entry starch to the duodenum. They suggested that the low starch digestibility in the small intestine could be due to the chemical structure of the sorghum starch and the lack of pancreatic amylase activity. In addition, the low

digestibility of starch may not reflect the total capacity of starch digestion in the small intestine because much of the rapidly degraded fraction may have undergone ruminal digestion (Owens et al., 1986). Different results were reported by Zinn (1991) from a study using four cannulated steers to compare the feeding value of steam-flaked corn and sorghum. They highlighted that the total tract digestibility of SFC and SFS were 99.6% and 99.1%, respectively, whereas digestibilities within the small intestine were 89.3% and 84.8% of small intestinal digestion, respectively. Starch digestibility reported by Zinn (1991) is greater than that reported by Streeter et al. (1991), suggesting that application of heat, moisture, and mechanical treatment during hydrothermal processing enhances starch digestibility (Theurer et al., 1999; Zinn et al. 2002).

Feed fractions can escape digestion in the small intestine and flow to the large intestine where they can be fermented, producing VFA and microbial protein (Owens et al., 1986). Of the small instestinally undigested fraction, 35 to 50% is digested in the large intestine (Harmon et al., 2004), where digestive efficiency is poorest (Owens et al., 1986; Harmon & McLeod, 2001). Karr et al. (1966) reported that a significant amount of starch escaped digestion in the small intestine when steers were fed high levels of starch up to 63.5% of the diet DM. They further highlighted that inadequate amylase production in the small intestine and rapid passage rate due to digestive disturbances with high-starch diets affect the amount of starch that reaches the large intestine. Streeter et al. (1989) compared digestibility of high-moisture sorghum grain and dry-rolled corn in beef heifers. They presented a linear decrease in starch digestibility in the large intestine by adding high-moisture sorghum to the diet. They further suggested that the low digestibility of high-moisture sorghum is due to extensive ruminal degradation of sorghum starch, reducing starch flow to the large intestine. Starch digestion in the large intestine is more beneficial for low-processed sorghum than well-processed grain because incomplete ruminal and

small intestinal degradation may allow for subsequent digestion in the large intestine (Hibberd et al., 1985).

1.4.6. Effect of grain processing

Various methods are available to effectively process grain sorghum to make it a valuable energy source for feedlot cattle. These include steam flaking, reconstitution, and micronization, which have been found to improve starch digestibility, cattle performance, and carcass characteristics (Theurer, 1986; Schake et al., 1972). Reconstitution is one of the most favorable processing alternatives for grain sorghum regarding net value and energy cost (Schake et al., 1981). This method involves grinding the grain, adding water, and ensiling the mixture, which can enhance calf performance (Abdelgadir & Morrill, 1994). In a study to compare effects of dry-rolling or reconstitution on starch and protein digestibility of hetero-yellow, red, and brown sorghum, Hibberd et al. (1985) reported a significant increase in total tract digestion of starch in reconstituted grains of red sorghum from 86.9% of the intake to 98.4% and a modest increase in brown sorghum. They further explained that the poorer digestibility of reconstituted brown than red sorghum might be due to the presence of tannin that was not degraded during the reconstitution process. A similar result was presented by Buchanan-Smith et al. (1968), who observed that the reconstitution process significantly increased starch and reduced sugar digestibility compared to the coarse-grind method. Rooney and Pflugfelder (1986) highlighted the role of starch-protein interaction and the physical form of the granule in starch digestibility, suggesting that reconstitution can alter these factors. However, the effect of reconstitution on fermentation characteristics of ensiled grain and growth performance of feedlot heifers was found to be variable (Huck et al., 1999).

Steam flaking of sorghum grain has been observed to have several positive effects (Owens et al., 1997). The steam-flaking process method involves the application of moisture and heat, followed by mechanical disruption of starch granules by compressing the kernels through rolls to form thin flakes (Rooney & Pflugfelder, 1986). Zinn et al. (2008) found that it increases energy value of grains, enhances intestinal nitrogen digestion, and improves feed efficiency. The improved nutritive value of steam-flaked grain is due, in part, to the disruption of the protein matrix encapsulating starch granules, which enhances ruminal starch fermentation (Rooney & Pflugfelder, 1986). In addition, mechanical interaction during the steam-flaking process increases surface area, exposing the grain for easier enzymatic degradation (Gømez et al., 2016; Xiong et al., 1990). Theurer et al. (1999) compared steam-flaking and dry-roll methods in sorghum processing and reported a greater ruminal (82 vs. 67%) and total tract (98.9 vs. 96.5%) starch digestibility for steers fed steam-flaked sorghum than dry-rolled sorghum. Another comparison of feeding value between steam-flaked and dry-rolled sorghum was presented by Theurer et al. (2002). They reported that steam-flaked sorghum increased whole-body N retention by around 15% and transfer of blood urea N to the gut by around 40%, which could increase the potential absorption of microbial protein.

Flake density is one factor to consider when using steam-flaked method in grain processing because it affects the nutritive value of steam-flaked grain (Xiong et al., 1991). Swingle et al. (1999) fed different bulk densities of steam-flaked sorghum to growing and finishing steers. They observed that the optimal flake density for steam-flaked sorghum to improve efficiency, diet net energy, and starch and protein digestibility is around 360 g/L. Swingle et al. (1999) further reported that decreasing flake density below 360 g/L did not improve performance or carcass merit in feedlot cattle. Chen et al. (1994) and Moore et al.

(1992) reported that steam flaking increases milk yield and utilization in dairy cows, with the latter study noting that a flake density of 360 g/L (28 lb/bu) is optimal. Similarly, Reinhardt et al. (1997) summarized that optimum bulk density for steam-flaked sorghum grain to improve feedlot steer performance and mill efficiency is 360 g/L (58.7% starch gelatinization). A study conducted by Zinn et al. (2008) reported that the use of tempering agents to incorporate additional moisture to the grain during the steam flaking process may not further improve feeding value of sorghum. However, the moisture level and flake density of steam-flaked corn, a similar grain, can influence its feeding value (Sindt et al., 2006).

Another method that is applicable to increase the starch digestibility of sorghum grain is dry-roll processing. However, compared to steam flaking, which significantly increased starch digestibility (Oliveira et al., 1995), the dry rolling method can reduce ruminal digestion of organic matter and starch, as well as total tract digestion of organic matter, nitrogen, and starch (Zinn et al., 2008). The extent of barley rolling can also impact starch digestibility, with coarsely rolled barley resulting in lower digestibility of organic matter and starch in the rumen and total tract compared to medium, medium-flat, and flat rolled barley (Beauchemin et al., 2001). Similarly, dry-rolled corn has been found to have lower ruminal and total tract starch digestion compared to steam-flaked corn (Corona et al., 2005).

Micronization is another grain processing method that uses gas-generated infra-red to heat the grain (Douglas et al., 1991). Micronization of grain has been found to increase the digestibility of starch, inactivate enzymes, and reduce anti-nutritional factors (Deepa & Hebbar, 2016). Most importantly, the micronization process may disrupt the protein matrix of the grain kernel, increasing its susceptibility to enzymatic degradation (Douglas et al., 1991). Temperature is a critical factor in the micronization process because temperature may affect protein solubility

in micronized grain, eventually affecting product quality (Shiau, 1982). Some studies have reported benefits of the micronization method for improving sorghum grain's feeding value and animal performance. Croka & Wagner (1975) compared micronized and dry-rolled sorghum grain and reported that even though a difference in total ruminal VFA concentration was not noted, a greater molar concentration of propionic acid and reduction in AP ratio were observed in cattle fed micronized sorghum. They further explained that cattle fed micronized sorghum grain performed better than cattle fed dry-rolled sorghum, which was shown by lower feed consumption and improved feed efficiency. Similarly, Hinman and Johnson (1974) evaluated the feeding value of micronized sorghum with densities of 412, 322, and 232 g/ compared to dry-rolled sorghum. They reported greater *in vitro* dry matter disappearance, total VFA production, and degree of gelatinization of micronized sorghum compared to dry-rolled sorghum. They further observed a reduced AP ratio produced for steers fed micronized sorghum. In addition, Douglas et al. (1991) observed improved performance for broilers fed micronized sorghum, and Ahmed et al. (1976) reported a trend for higher feed efficiency for dairy calves fed micronized sorghum than dry-rolled sorghum.

1.5. Summary

As a staple food that transforms into a potential energy source for the livestock industry, especially the beef cattle industry, sorghum grain has a long history of reaching its maximum potential as a nutritious feed ingredient. Supported by its unique trait of being well-adapted to drought areas, sorghum grain has drawn significant attention from breeders who want to develop superior cultivars of sorghum grain through a breeding development program. The waxy endosperm type of sorghum grain is one of the traits that has been developed to fulfill the need for a highly digestible feedstuff. Despite its ability to grow well under arid environments, the

kernel structure has also been challenging when utilizing sorghum grain in feedlot settings because it is less digestible than corn and other conventional energy sources such as wheat and barley. Higher amylopectin content in waxy endosperm compared to hetero-waxy and high-amylose phenotypes does not guarantee a higher nutritive value of the grain because other factors, such as kernel structure and other chemical compounds, are involved in determining the digestibility of sorghum grain. Application of suitable and proper grain processing methods such as reconstitution and steam-flaking may improve the digestibility of sorghum grain. Because highly processed grain may harm animals through digestive disorders, a combination of superior sorghum cultivars and proper grain processing methods may maximize the nutritive value of sorghum grain and maximize animal performance.

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Chapter 2 - *In vitro* Screening of Sorghum Parental Lines for Digestibility as a Step Toward Development of Superior Sorghum Hybrids for Cattle Feeding

2.1. Abstract

Fifty-one cultivars of sorghum parental lines were used in a series of *in vitro* assays to assess fermentation by mixed ruminal microorganisms as a step toward the development of superior sorghum hybrids. Sorghum grains were milled to pass a 1-mm screen of a cyclone mill, and subsequently incubated for 30 hours with a mixture of artificial saliva and strained ruminal contents from fistulated cattle. The study was designed as an incomplete randomized block design due to the large number of sorghum cultivars tested. Maximum cumulative gas production (K) and time required to reach half the maximum gas production ($t_{½}$) were different ($P<0.01$) across sorghum cultivars. Similarly, terminal pH of cultures and *in vitro* dry matter disappearance (IVDMD) were also different among sorghum parental lines ($P<0.01$). Production of volatile fatty acids (VFA) also was assessed, and substantial differences among cultivars were noted for concentrations of propionate, iso-butyrate, isovalerate, valerate, and acetate:propionate ratio ($P<0.01$), but no differences were observed for concentrations of acetate, butyrate, and total VFA production ($P>0.05$). Furthermore, forty-eight sorghum cultivars of the parental lines from *in vitro* experiment were investigated to measure the gelatinization temperatures using differential scanning calorimetry (DSC). Results showed that onset temperature (T_o) ranged from 67.54 to 83.90ºC, peak temperature (T_p) ranged from 74.94 to 98.35ºC, and conclusion temperature (T_c) ranged from 74.53 to 105.32ºC. In addition, gelatinization enthalpies (ΔH_{gel}) were ranged from 1.06 to 6.49 J/g. Lower peak temperature and gelatinization enthalpies could

indicate higher digestibility of sorghum grain tested. Differences in results demonstrated from both *in vitro* assay and DSC analysis suggest there is potential for development of sorghum cultivars that are more suitable than current cultivars as feeds for ruminants.

Keywords: Sorghum, *in vitro*, gas production, gelatinization

Abbreviations: *in vitro* dry matter disappearance, IVDMD; volatile fatty acid, VFA; acetate: propionate, AP; differential scanning calorimetry, DSC; onset temperature, T_o; peak temperature, T_p; conclusion temperature, T_c.

2.2. Introduction

Sorghum grain has been used as livestock feed for many years due to its energy and protein content (Quinby & Martin, 1954; McCuistion et al., 2019). However, the revolutionary improvement of sorghum grain was due to its drought tolerance (Smith & Frederiksen, 2000) and suitability for growing under arid and semi-arid condition (Haussmann et al., 1998; Verma et al., 2018; McCuistion et al., 2019). Using sorghum grain in the diet is challenging because it has a unique kernel structure that is different from its compatriot energy source feed, corn (Rooney & Pflugfelder, 1986). The uniqueness of sorghum grain is mainly due to the starch-protein matrix in its kernel structure (Waniska, 2000; Watson, 1984), which is the crucial factor affecting starch digestibility (Streeter et al., 1991) and animal performance (Theurer et al., 1999).

In diet formulation, sorghum is a potential substitute for other conventional grains such as wheat, barley, and corn (Spicer et al., 1986; Hancock et al., 1992) as sorghum exhibits an average feeding value when appropriately processed of 92% that of corn (Zinn, 1991). Various processing methods have been applied to improve the starch digestibility of sorghum grain, including dry-rolling (Beauchemin et al., 2001), micronization (Croka & Wagner, 1975) reconstruction (Hibberd et al., 1985), and steam-flaking (Owens et al., 1997). However, applications of grain processing methods are limited by the kernel and chemical structure of the grain, making the sorghum grain less digestible than other grains (Spicer et al., 1986).

Genetic improvement offers a promising avenue to enhance the nutritive value of sorghum grain (Hoffman & Rooney, 2014). Through a continuous hybridization program, several superior sorghum grain varieties have been developed (Pfeiffer et al., 2019). The most significant of these is the waxy endosperm type, which boasts a high amylose content and a reduced starch-protein matrix, potentially making it more digestible than normal and hetero-

waxy endosperm types (Wong et al., 2009; Rooney & Pflugfelder, 1986). This potential for genetic improvement should inspire hope and excitement for the future of sorghum grain development (Stephen et al., 2012; Gizzi & Gambin, 2016).

The primary objective of this study is to assess the *in vitro* digestibility of sorghum parental lines, a crucial step in the development of superior sorghum hybrids. This research has significant contribution for further research to improve the nutritional value of sorghum grain, which is important in feedlot cattle nutrition.

2.3. Materials and Methods

Before the study's initiation, the Kansas State University IACUC committee reviewed and approved all procedures.

2.3.1. Estimation of starch and protein content

Estimation of starch, protein, and amino acid content of sorghum parental lines were conducted using near-infrared spectroscopy (NIR; Perten DA 7250 spectrometer Perten Instrument, Springfield, IL, USA) to assess whole grains and ground grains. These were then used to assess the correlation between the estimated protein, starch and amino acids content with other variables measured.

2.3.2. *In vitro* digestibility

Fifty-one cultivars of sorghum grain parental lines were milled using a Udy Cyclone sample mill–joined belt drive (Udy Corporation, CO) to achieve a particle size of 1 mm. Ground samples were then stored in a plastic bag until processing. Milled grain samples were dried in a 105°C forced-air oven for 24 hours for dry matter measurement. Due to the large number of samples, an incomplete randomized block design was used for eight runs of *in vitro* experiments. At the end of the experiment, each sorghum cultivar was tested in six runs. Each run included 45

individual Pyrex® bottles representing 42 milled sorghum cultivars and 3 blank cultures. While the 42 culture bottles were filled with substrate and mixture of ruminal fluid and McDougall's buffer solution, the blank cultures were only filled with mixture of buffered ruminal fluid without substrate and were used for the calculation of IVDMD as a correction factor.

2.3.3. Gas production

Substrate samples of as much as 1.5 grams (dry basis) were prepared and added to 250-mL culture bottles, which were then placed in a 39 °C incubator while waiting for the ruminal fluid and McDougall's buffer preparation. Urea was included in the buffer solution. Ruminal fluid was collected from steers fed 70% concentrate and 30% roughage, with corn as the primary concentrate. Two layers of cheesecloth were used to strain the ruminal fluid into pre-warmed insulated containers. The strained ruminal fluid was then transferred to a separate funnel and sparged with nitrogen gas to allow the stratification of the ruminal fluid. After discarding the bottom strata, the middle strata were then used as inoculants. As much as 30 mL of freshly strained ruminal fluid and 120 mL of McDougall's buffer solution, to achieve the 1:4 ratio of final buffer ruminal fluid solution, were then dispensed into the culture bottles containing substrate of ground sorghum grain. The cultures were purged with nitrogen gas and then sealed with an Ankom pressure sensing module (Ankom RF Gas Production System; Ankom Technology, Macedon, NY) and placed into an orbital bed incubator (Innova 4300/New Brunswick Scientific, Edison, NJ) for continuous agitation at 39°C. The Ankom modules recorded gas production every 15 minutes during incubation. After 30 hours, the culture incubation was terminated by cooling the culture bottles in ice for 10 minutes. A portion of the fluid contents (4mL) was combined with 1 mL of 25% w/v metaphosphoric acid solution in 10

mL plastic scintillation vials to facilitate deproteinization, sealed, vortexed, and frozen at -20°C for VFA and ammonia analysis.

The Ankom gas production system measured gas pressure in PSI. Gas pressure was converted to moles of gas produced using the ideal gas law and then converted to milliliters (mL) of gas produced using Avogadro's law (Ankom Technologies, 2021), according to the following:

Ideal gas law

$$N = p(V/RT)$$

where, n = amount of gas produced in moles (mol), p = pressure in kilopascal (kPa), V = head-space volume in culture bottle (L), R= gas constant (8.314472 $L \cdot kPa \cdot K^{-1} \cdot mol^{-1}$), and T = Temperature in Kelvin (K).

Using Avogadro's law, at atmospheric pressure measured in PSI (1 PSI = 6.894757293 kilopascal), 1 mole of gas occupies 22.4 L at 273.15°K and 101.325 kPa (standard condition). This conversion was used to calculate the total milliliters of gas produced from the moles of gas produced using the following equation.

Gas produced in milliliters = n x 22.4 x 1000

2.3.4. Volatile fatty acid (VFA)

Samples of acidified culture fluid were thawed, centrifuged at 30,000 x g for 20 minutes, and analyzed for VFA by gas chromatography (Agilent 7890A GC; Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector and capillary column (Nukol, 30 m X 0.53 mm; Sigma-Aldrich, St. Louis, MO) and using hydrogen as the carrier gas with flow rate of 45 cm/second. Injector and detector temperatures were 280°C; the initial oven temperature was 70°C and increased to 200°C at the rate of 30°C/minute.

2.3.5. pH measurement

As the culture bottles cooled, the pH of the content of the bottle was determined using a portable pH meter (Orion Star A221; Thermo Fisher Scientific, Waltham, Maine).

2.3.6. *In vitro* dry matter disappearance (IVDMD)

After taking the fluid content for VFA and ammonia analysis, the remaining content was transferred into an aluminum pan and dried at 105°C for 96 hours in a forced-air oven to determine residual dry matter. The IVDMD was calculated based on the formula below and was expressed as a coefficient.

$$\text{IVDMD} = \left[1 - \left[\frac{(\text{Dried residue weight,g} - \text{Pan weight,g}) - \text{Blank}}{\text{Initial grain sorghum weight,g (DM)}}\right]\right]$$

Blank = Dried blank residue, g – Pan weight, g

2.3.7. Ammonia

Ammonia concentration was determined as described by Broderick and Kang (1980) using a BioTek PowerWave XS Microplate Reader (BioTek, Winooski, VT).

2.3.8. *In situ* dry matter disappearance (ISDMD)

In situ dry matter disappearance was evaluated using ground sorghum grain samples with six replications for each cultivar. Since only 18 samples could be analyzed per mesh bag (40.5 cm x 51 cm; n=16 sorghum cultivars + corn + blank), ISDMD was designed as an incomplete randomized block design for 48 sorghum cultivars. Each sample (1.0 g DM) was weighed in an Ankom R510 concentrate bag (5 cm x 10 cm; ANKOM Technology Macedon, NY). Two canulated steers were utilized to incubate the samples. One mesh bag per steer was utilized to keep the Ankom concentrate bags in the rumen. Each mesh bag contained 16 nylon bags of different sorghum cultivars, a similar processed corn sample, and a blank sample. The blank sample was used to calculate the background DM from the rumen. Mesh bags were incubated

inside the rumen for 16 hours. At the end of the incubation period, concentrate bags were removed and placed into plastic containers filled with warm water. Residue adhering to the exterior of each concentrate bag was removed by continuous gentle agitation in the warm water. The cleaning process was repeated twice, removing the excess moisture using paper towels. Concentrate bags were then placed in a forced air oven at 100°C for 24 hours before weighing the dry concentrate bags.

In situ dry mater disappearance is expressed as a coefficient of disappeared dry matter of each sample. A blank bag devoid of substrate is used to calculate DM from the rumen. The formula used to calculate ISDMD is as follows:

$$\text{ISDMD} = \left[1 - \left[\frac{(\text{Dried nylon bag,g} - \text{Initial nylon bag weight,g}) - \text{Blank}}{\text{Initial grain sorghum weight,g (DM)}}\right]\right]$$

Blank = Dried blank residue, g – Nylon bag weight, g

2.3.9. Gelatinization temperature

Forty-eight sorghum cultivars of parental lines were analyzed in duplicate using the differential scanning calorimetry (DSC) method for gelatinization characteristics. The number of sorghum cultivars tested for DSC analysis was less than that of *in vitro* analysis due to the availability of some samples. Sorghum grains for DSC analysis were milled in the same way as for the *in vitro* experiment. Ground samples were then mixed with deionized water to achieve 50% moisture. Approximately 12 to 14 mg of mixture samples were weighed into DSC pans, and the pans were then sealed. Pans were allowed to equilibrate for 24 hours prior to analysis. Gelatinization properties measurement was conducted using DSC-60 (Shimadzu, Kyoto, Japan). Samples were heated in the DSC from 20°C to 140°C at the rate of 10°C/min. The endothermic profile was analyzed using TA-60WS thermal analyzer software (Version 2.30; Shimadzu,

Kyoto, Japan). Endothermic enthalpy was determined as the area under the peak divided by the dry weight of the sample.

2.3.10. Particle size analyses

Analysis of particle size for 47 ground samples of sorghum parental lines was performed using a single wavelength Beckman Coulter LS 13 320 Particle Size Analyzer (Miami, FL) with the Tornado Dry Powder System. The geometric mean diameter (d_{gw}) of the sorghum flour and the geometric standard deviation of the particle diameter (S_{gw}) were calculated according to Patwa et al. (2014).

2.3.11. Statistical analyses

Gas production data were modeled using both logistic and log-logistic functions with the NLIN procedure of SAS. In all cases, the log-logistic model provided a superior fit as determined by the mean squared error, which for the log-logistic model was on average 12% that of the logistic model. The log-logistic function yields the following parameters:

K, which is the maximum gas production measured in mL

t½, which is the time required to achieve 50% of K, measured in hours

r, describes shape of the curve, and is without unit

Log-logistic model

$$\text{Gas production} = \frac{K}{1+(t½ \div Time)^r}$$

Gas production: cumulative gas production (mL) at a given time interval (hour),

Time: Time interval in quarter-hour increments of the total time (0.25 hours),

The Glimmix procedure in SAS was used to assess the pH, VFA, ammonia production, IVDMD, and ISDMD. Data from the analysis of gelatinization temperatures and particle size analysis were analyzed descriptively in SAS using the average values observed. Calibration and

statistical models used to estimate starch, protein and amino acids content were based on previous study on sorghum cultivars (Peiris et al., 2019; Peiris et al., 2020; Peiris et al., 2021). In addition, the Pearson Correlation Coefficient test was used to assess the correlation between the estimated protein, starch, amino acid content, as well as particle size of sorghum grains and response variables.

2.4. Results and Discussion

Estimated protein, starch, and amino acids of 48 varieties of sorghum parental lines, gas production and fermentation parameters from i*n vitro* experiment of 51 varieties of sorghum parental lines, as well as ISDMD, gelatinization temperature, as well as particle size of 47 varieties of sorghum parental lines are presented in Figure 2.1 through Figure 2.26. Whole sorghum grains were used for protein, starch, and amino acids estimation; and ground sorghum grains were used for analysis of *in vitro* fermentation parameters, ISDMD, gelatinization characteristics, and particle size.

2.4.1. Starch, protein, and amino acid content

The protein content of sorghum plays a crucial role in the digestibility of starch and other nutrients. Although high protein levels may initially reduce digestibility (Rooney & Pflugfelder, 1986), proper processing can help alleviate these negative effects and enhance overall nutrient availability for ruminants (Theurer, 1986; Schake et al., 1972). The estimated starch content ranged from 68.44 to 78.30% (Figure 2.1), and estimated protein content of sorghum cultivars in this study ranged from 9.80 to 15.56% (Figure 2.2), meaning that each cultivar with different protein concentrations may affect the starch digestibility differently. Proteins and starches in sorghum are tightly bound in a protein-starch matrix (Belton et al., 2006). Higher protein content in sorghum can make this matrix more rigid, potentially reducing the digestibility of starch and

other nutrients (Belton et al., 2006; Hamaker & Bugusu, 2003). The encapsulation of starch granules by protein can limit access by ruminal microbes and enzymes, slowing down starch fermentation (Duodu et al., 2003; El Nour et al., 1998). Sorghum contains prolamin proteins, specifically kafirins (Wong et al., 2009), which are poorly digested by ruminants (Wang et al., 2009). The higher the concentration of these indigestible proteins, the more they hinder nutrient accessibility (El Nour et al., 1998; Hamaker et al., 1987), especially starch (Rooney & Pflugfelder, 1986), for enzymatic degradation.

Cereal species tend to contain inadequate amounts of the essential amino acids lysine, tryptophan, methionine, and threonine for maximal animal performance (Eggum, 1977; Ajakaiye, 1984). Of all cereals, sorghum contains the least amounts of lysine, tryptophan, and possibly arginine (Sikka & Johari, 1979; Ajakaiye, 1984). In this study, the estimated concentrations of lysine, methionine, and cysteine ranged from 0.26 to 0.35%, from 0.17 to 0.26%, and from 0.19 to 0.26% of DM (Figure 2.3, Figure 2.4, Figure 2.5) respectively. McCuistion et al. (2019) reported lysine, methionine, and methionine and cysteine content of 0.20%, 0.16%, and 0.34% of total amino acids, respectively. Another study by Deyoe & Shellenberger (1965) using 15 different hybrids of sorghum grain recorded amino acids lysine from 1.57-2.61% and methionine from 0.81 to 1.97% of protein, respectively. Variations in the amino acid content of sorghum cultivars could be due to the variation in grain maturity, genotype, and nitrogen fertilizer (Ajakaiye, 1984).

2.4.2. Gas production

Cumulative gas production (K) and time to attain half of the maximum gas production ($t_{½}$) across sorghum grains varied ($P<0.01$). The cumulative gas production of 51 sorghum cultivars (Figure 2.6) ranged between 359.50 mL to 481.81 mL, with pedigree PI534127

generating the least cumulative gas production and PI565123 producing the greatest. Greater cumulative gas production indicates greater feed digestibility, as Streeter et al. (1990) reported. In an *in vitro* study, after 12 hours of incubation, they observed greater gas production for waxy endosperm sorghum of 113.1 mL/g of CO_2 than produced by normal endosperm sorghum of 93.4 mL/g. Digestibility studies have demonstrated that waxy endosperm is more digestible than hetero-waxy or normal endosperm (Zhu, 2014; Lichtenwalner et al., 1978; Wester et al., 1992). Lower cumulative gas production observed from some cultivars in the current study could be due to tannins, which have a high affinity to amylose (Barros et al., 2012), reducing starch degradation (Rocchetti et al., 2020). The formation of a tannin-protein complex may also contribute to the lower cumulative gas production, as the tannin-protein association may strengthen starch-protein interaction (Gómez et al., 2016).

The excellent performance of pedigree PI565123 on cumulative gas production was not followed by its performance on time required to emit half of the maximum gas production. Pedigree PI565123 spent 10.72 hours to reach half of the maximum gas production, longer than PI585348, which was 2.21 hours faster ($P<0.01$) (Figure 2.7.). With a duration time of 8.51 hours, pedigree PI585348 recorded the fastest time in attaining half of the maximum gas production, while pedigree PI533927 spent 11.91 hours as the longest time ($P<0.01$).

Among those cultivars, sorghum pedigree PI656123 exhibited more excellent performance than other cultivars in gas production measurement because it produced greater cumulative gas production during incubation. Similar to the findings in cumulative gas production, Streeter et al. (1990) found a significantly greater cumulative gas production rate in waxy endosperm sorghum than in both waxy-bird resistant and normal endosperm sorghum, indicating grain sorghum with higher digestibility exhibits greater gas production.

There was no correlation observed between starch content and *K*. However, the *K* value in this study exhibited a positive correlation but with protein content but has negative correlation with lysine content of sorghum cultivars (Table 2.1). The correlation indicates that some sorghum cultivars contain higher protein concentrations than others, which may benefit microbial growth and fermentation activities. Dietary protein is essential as the main source of nitrogen required for optimum growth and development of ruminal microbes (Stern & Hoover, 1979; Griswold et al., 1996). In addition, particle size has positive correlation with $t_{½}$ which could be due to higher digestibility of some sorghum grains even though they have larger particle size. Digestibility of sorghum grain can be affected by protein-starch interaction, antinutritional factors, and physical form of the feed (Rooney and Pflugfelder, 1986).

2.4.3. Volatile fatty acid (VFA) production

Results from VFA production showed that acetate (Figure 2.9) and butyrate (Figure 2.12) concentration, and total VFA production (Figure 2.15) were unaffected by the sorghum grain cultivars ($P>0.10$). However, propionate production (Figure 2.10), iso-butyrate (Figure 2.11), iso-valerate (Figure 2.13), valerate (Figure 2.14), and A:P ratio (Figure 2.16) were different among cultivars ($P<0.01$). Greater VFA production represents the greater digestibility of feedstuff, because VFA produced in the rumen are derived from ruminal feed degradation and fermentation (Nagaraja, 2019; Opatpatanakit et al., 1994). Variations in VFA production observed from the current study might be due to variations in the structure and chemical composition of the grain, protein-starch interaction, and anti-nutritional factors (Rooney & Pflugfelder, 1986). As for the A:P ratio, the result from this study demonstrated the range of A:P ratio from 2.90 to 1.93, with pedigree PI585374 having the greatest A:P ratio and pedigree PI534097 having the least (Figure 2.16). Acetate propionate ratio is affected by feed intake, feed

efficiency (Van der Walt & Linington, 1989), and ruminal fermentation (Lin et al., 2020). A lower A:P ratio is preferred to improve animal performance in the fattening period since propionic acid is most efficient in capturing energy produced from the ruminal fermentation process (Ørskov, 1977).

Variation observed from propionic acid production in this study might be mainly affected by starch digestibility. High-concentrate feeding leads to higher ruminal propionic acid production. The greatest propionic acid concentration in the current study was 17.72 mM, produced by pedigree PI534097, while the lowest was 9.49 mM, produced by pedigree 5_Herds. Several factors contributing to the variation in propionic acid production could be similar to those in other fermentation parameters. However, starch-protein interaction could be the major inhibitor for starch digestibility since it forms a strong barrier for microbial and enzymatic degradation (Rooney & Pflugfelder, 1986).

There was no correlation observed between the protein and starch content of sorghum and general VFA production. However, a negative correlation was noted between particle size and propionate (Table 2.1). This could be due to the increase in surface area of feed particles that have smaller particle size which leads to the increase in enzymatic and microbial attachment, resulting in greater starch digestibility (Firkins et al., 1986; Nocek & Tamminga, 1991) and propionate production (Hatew et al., 2015).

2.4.4. pH

Results from the current study showed variation in pH across sorghum cultivars ($P<0.01$); pH values ranged from 6.62 to 6.80 (Figure 2.17). Some cultivars yielded lower pH than others, indicating that some sorghum cultivars are more digestible. However, the pH range is in the proper range for the optimum growth of ruminal microbes, especially for fiber-fermenting

bacteria (Shriver et al., 1986). The pH measurement is crucial in an *in vitro* experiment since pH can determine the extent of feed digestion by the ruminal microbes. Ruminal feed digestion produces organic acid, which can affect the rumen's acidity level. In contrast, a greater digestible feed ingredient produces a greater organic acid concentration, leading to a lower pH value (Dijkstra et al., 2012). Pearson's correlation test indicated positive correlation between pH and starch and lysine content, but negative correlation with protein content (Table 2.1).

2.4.5. *In vitro* dry matter disappearance (IVDMD)

Results from the current study showed that IVDMD across sorghum cultivars ranged from 48.87% to 65.09%, with pedigree PI534127 performing the lowest IVDMD and pedigree PI534021 exhibiting the highest (Figure 2.18.). Variation among cultivars ($P<0.01$), indicating that certain cultivars are more digestible than others. Pedigree PI534021 was developed from the durra race and is characterized as low protein, high digestibility, small seed, and white kernel (unpublished data from USDA), which may contribute to the high IVDMD. The digestibility of feedstuff can be determined through dry matter disappearance during incubation in an *in vitro* system (Pedersen et al., 2000; Defoor et al., 2000). Dry matters of feedstuff undergo ruminal degradation and fermentation process, producing VFA, microbial cell protein, and other nutrients such as vitamins to the host (Ørskov, 1977; Nagaraja, 2019). Since starch degradation might be the major contributor to IVDMD, as starch is the major component of sorghum grain, low protein content may result in higher digestibility of sorghum starch. High protein component might reduce starch digestibility as starch-protein interaction inhibits microbial and enzymatic degradation of starch (El Nour et al., 1998; Huntington, 1997). Pearson's correlation test indicated positive correlation between IVDMD and protein and lysine content, but negative correlation with particle size (Table 2.1). Protein is one of the predominant nutrients in sorghum

grain, representing 9.5% of DM (McCuistion et al., 2019). Some sorghum cultivars tested contain greater protein content (Figure 2.2) which could impact IVDMD. Furthermore, smaller particle size of feed particle increases nutrient digestibility as it provides larger surface area for microbial and enzymatic degradation (Firkins et al., 1986; Nocek & Tamminga, 1991), resulting in greater IVDMD.

2.4.6. Ammonia production

Production of ammonia resulting from the current study presented in Figure 2.19 showed no variation across sorghum cultivars (P=0.11). However, numerically, some sorghum varieties exhibit greater ammonia production than other cultivars. While pedigree PI585295 produced ammonia up to 23.76 mM, ammonia produced by pedigree PI569812 was the least among others, up to 13.49 mM. Ruminal protein degradation of sorghum grain depends on many factors, including grain structure, starch, polyphenols, starch, disulfide and non-disulfide bonds, and changes in protein structure (Duodu et al., 2003). The rate of ruminal protein digestibility can be measured through ammonia production (Broderick, 1978) since dietary protein is degraded into peptides, amino acids, and ammonia (Tamminga, 1979).

The presence of tannin in some sorghum cultivars might be the major factor affecting the variation in ammonia production in this study, as each of the cultivars may contain a different tannin concentration (Kaufman et al., 2013). Tannin significantly impacts protein degradation (Cousins et al., 1981). It can precipitate protein 12 times its weight, involving hydrogen bonding and non-polar hydrophobic association (Butler et al., 1984). Therefore, the binding capacity of tannin to the protein is determined by the concentration of tannin (Osborne & McNeill, 2001) in the grain. Protein high in proline content tends to associate with tannin > 100-fold higher than other proteins (Butler, 1981). Tannin concentration in each cultivar in the present study varies as

reported by USDA (2020, unpublished data), meaning that variation observed in ammonia production is expected. Pedigree PI569812, on the one hand, which exhibited the lowest ammonia production, may contain tannin in an appreciable amount sufficient to limit protein degradation. On the other hand, tannin content in pedigree PI585295 may not be sufficient to reduce protein digestibility.

Since sorghum grain is used in ruminant diets mainly for its starch component, starch-protein interaction could be another crucial factor affecting protein digestibility. This study's Pearson Correlation Coefficient test demonstrated no correlation between starch and protein content of sorghum grain and ammonia production. Thus, another factor, such as tannins, might affect the ammonia concentration. Some pedigrees with low tannin content are expected to exhibit greater ammonia production since tannin may not alter the protein degradation of those pedigrees. The inhibition capacity of tannin to protein degradation depends on tannin concentration (Broderick & Albrecht, 1997).

2.4.7. *In situ* dry matter disappearance (ISDMD)

Result from this study demonstrated that ISDMD varied across sorghum cultivars ($P<0.01$). The greatest ISDMD was exhibited by pedigree PI534021 of 91.56% and pedigree PI533927 having the least of 63.25% (Figure 2.20). Variation in ISDMD could be due to variations in the protein and starch content of sorghum cultivars tested which could affect the variation in starch-protein interaction (El Nour et al., 1998; Hamaker et al., 1987), leading to differences in ISDMD. In addition, Pearson's correlation test indicated negative correlation between particle size and ISDMD (Table 2.1) which can be explained that smaller particle size provides larger surface area for enzymatic and microbial digestion, resulting in greater digestibility (Firkins et al., 1986; Nocek & Tamminga, 1991).

2.4.8. Gelatinization characteristics

Results from this study on gelatinization peak temperature (T_p) showed that pedigree PI563454 required the highest temperature for complete gelatinization, up to 95.62 °C, while PI543123 required the least energy to be completely gelatinized, up to 74.92 °C (Figure 2.22). Surprisingly, the tested corn required energy for gelatinization up to 98.35 °C, higher than any tested sorghum cultivars. The gelatinization peak temperature is defined as the temperature required for the maximum rate of gelatinization to occur (Cooke & Gidley, 1992). The rate of starch gelatinization is an indicator of starch digestibility where, at the same temperature, isolated starch that is gelatinized in faster rate has greater digestibility than starch that is gelatinized in slower rate (Parada & Aguilera, 2009; Akingbala et al., 1981). Gelatinization is when starch loses its native structure due to heat application to cleave the intramolecular hydrogen linkage in the crystalline area (Rooney & Pflugfelder, 1986). Degradation of hydrogen bonds in the starch granule increases the accessibility for enzymatic digestion (Coral et al., 2009). Therefore, temperature plays a critical role in determining the digestibility of starch through the gelatinization process (Parada & Aguilera, 2009; Coral et al., 2009).

Onset temperature (T_o), which is the temperature required to initiate a gelatinization process (Akingbala et al., 1981), ranged from 67.54 °C to 78.49 °C (Figure 2.21). Pedigree PI656118 requiring the lowest, and pedigree PIPI613536 requiring the highest temperature to initiate the breakdown of hydrogen bond in the starch granule. As for T_c, which is the temperature required for a complete gelatinization (Li, 2022), pedigree PI585348 requires the lowest energy of up to 74.53°C, and PI656022 needs heat up to 105.32°C for completing the gelatinization process (Figure 2.23). Furthermore, corn starch requires higher energy to start the gelatinization process, up to 83.90°C, than any sorghum cultivar tested. However, corn requires

lower energy, up to 98.49 ºC, to complete the gelatinization process than some sorghum cultivars.

Enthalpy of gelatinization (ΔH_{gel}), which refers to the amount of energy required to disrupt the molecular order within the starch granule (Biliaderis et al., 1980; Wolters et al., 1992), was also observed. Pedigree PI585295 required the highest energy up to 6.49 J/g for a complete starch gelatinization, while PI613536 requires the lowest energy up to 1.06 J/g (Figure 2.24). The wide range of gelatinization temperatures and gelatinization enthalpy observed in this study might largely be due to the variation in the structural and chemical composition of the sorghum grain tested (Li, 2022). Other factors may contribute to gelatinization temperatures of starch, including moisture content and grain size, with higher moisture increasing it and larger grain size decreasing it (Fukuoka et al., 2002; Schirmer et al., 2011; Coral et al., 2009). Since moisture content for all the samples in the current study was set to achieve 50%, granule size could be one of the factors affecting the gelatinization peak temperature (Parada & Aguilera, 2009). The distribution of starch granules varying in size (Schirmer et al., 2015) may impact gelatinization temperature since larger size granule is gelatinized more rapidly than smaller ones (Coral et al., 2009). Characteristics of gelatinization exhibited positive correlation with particle size (Table 2.1) indicating that larger particle sizes require higher temperature to gelatinize starch (Marshall, 1992).

2.4.9. Particle Size

Particle size analysis was performed because particle size of grains that was ground using the same screen size (1 mm) may be different which is affected by milling method, fluor type, and fluor moisture (Liu, 2009). Particle size, calculated as the geometric mean diameter, of ground sorghum grain in this study ranged from 85.1 to 454.7 µm (Figure 2.25), the geometric

standard deviation of ground sorghum particle ranged from 7.0 to 60.4 µm (Figure 2.26). Pearson's Correlation test indicated correlation with some digestibility parameters (Table 2.1). The correlation between grain particle size and feed digestibility in ruminants is important in optimizing the efficiency of ruminants. Particle size affects the rate of digestion and absorption of nutrients in the rumen, influencing overall feed digestion (Theurer, 1986). Smaller particles increase surface area to the microbial attachment in the rumen, leading to more rapid fermentation (Firkins et al., 1986; Nocek & Tamminga, 1991) and improved nutrient availability (Huntington, 1997). However, smaller particles feed may pass the rumen faster, reducing the extent of fermentation in the rumen but might increase post-ruminal digestion (Nocek & Tamminga, 1997; Kazemi-Bonchenari et al., 2017).

Ground grains with smaller particle size, on one hand, can increase the fermentation rate leading to a rapid drop in ruminal pH (Fredin et al., 2015; Shipandeni et al., 2023). Low ruminal pH can cause acidosis, negatively affecting ruminant health and productivity (Devries et al., 2014; Nagaraja & Titgemeyer, 2007). On the other hand, larger grain particles are fermented slower, which may help stabilize the rumen environment and maintain optimal ruminal pH (Krause et al., 2002) by promoting chewing and rumination (Beauchemin et al., 2003). Increased chewing and rumination activities can stimulate saliva production (Beauchemin et al., 2003), which can act as a buffer in the rumen, helping maintain pH levels (Chibisa et al., 2016). Moreover, higher ruminal pH can benefit ruminants on high-concentrate diets to prevent acidosis (Owens, 1998). However, larger particle sizes may not be completely broken down in the rumen, which can lead to lower digestibility (Rémond et al., 2004). Grains with large particle sizes may pass through the digestive system with less fermentation and nutrient absorption (Goetsch et al., 1987; Ewing et al., 1986). Different processing techniques, such as rolling, cracking, and

grinding, can help achieve the desired particle size and improve digestibility without negatively affecting rumen health (Theurer, 1986)). Thus, ensuring the correct particle size through proper grain processing is crucial for maximizing feed efficiency in ruminants while maintaining health status of the animals (McCuistion et al., 2019).

2.5. Conclusion

Variation in the *in vitro* parameters of digestibility observed from fifty-one sorghum and gelatinization properties from forty-eight cultivars indicated a wide range of possibilities for sorghum parental lines to support development of new hybrids. The greater cumulative gas production, propionate, and IVDMD exhibited by some pedigrees compared to others, are significant contributors to the simultaneous effort to improve nutritional value of sorghum grain. Applying traditional and new technologies, including molecular genetic tools, transgenic technology, and cytoplasmic and genetic male sterility, could greatly support the invention of a superior sorghum grain cultivar. Furthermore, using suitable grain processing methods, such as reconstitution and steam-flaking, on the superior sorghum hybrid could be promising to provide high-quality feed for ruminant animals, especially beef cattle. Therefore, drought resistance traits combined with high digestibility characteristics may create a superior sorghum grain cultivar for the future livestock industry.

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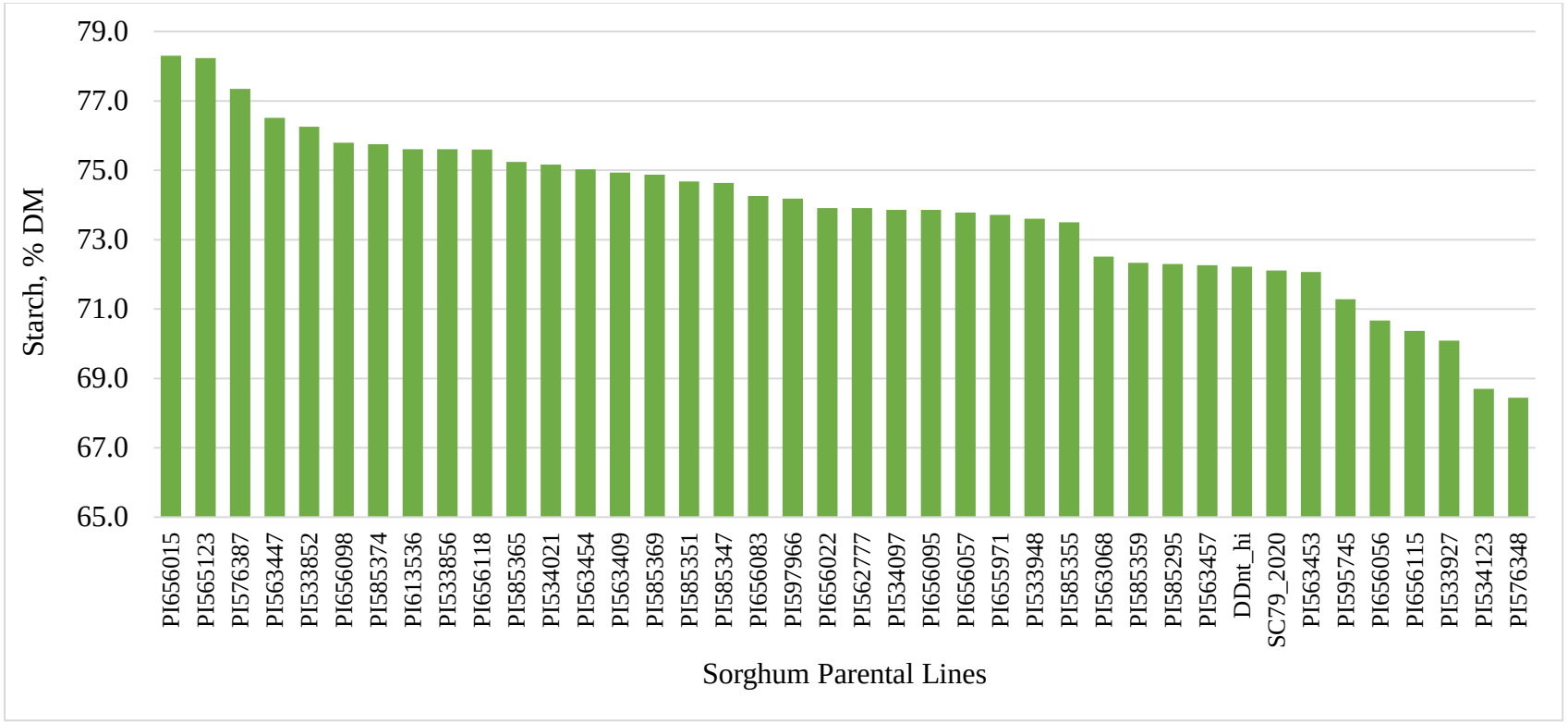

Figure 2.1. Estimation of starch content of each sorghum parental lines using near infrared spectroscopy (NIR).
A Perten DA 7250 spectrometer (Perten Instruments, Springfield, IL, USA) was used to scan each grain to estimate starch content. Calibration and statistical models used to estimate starch content were based on previous study on sorghum cultivars (Peiris et al., 2021).

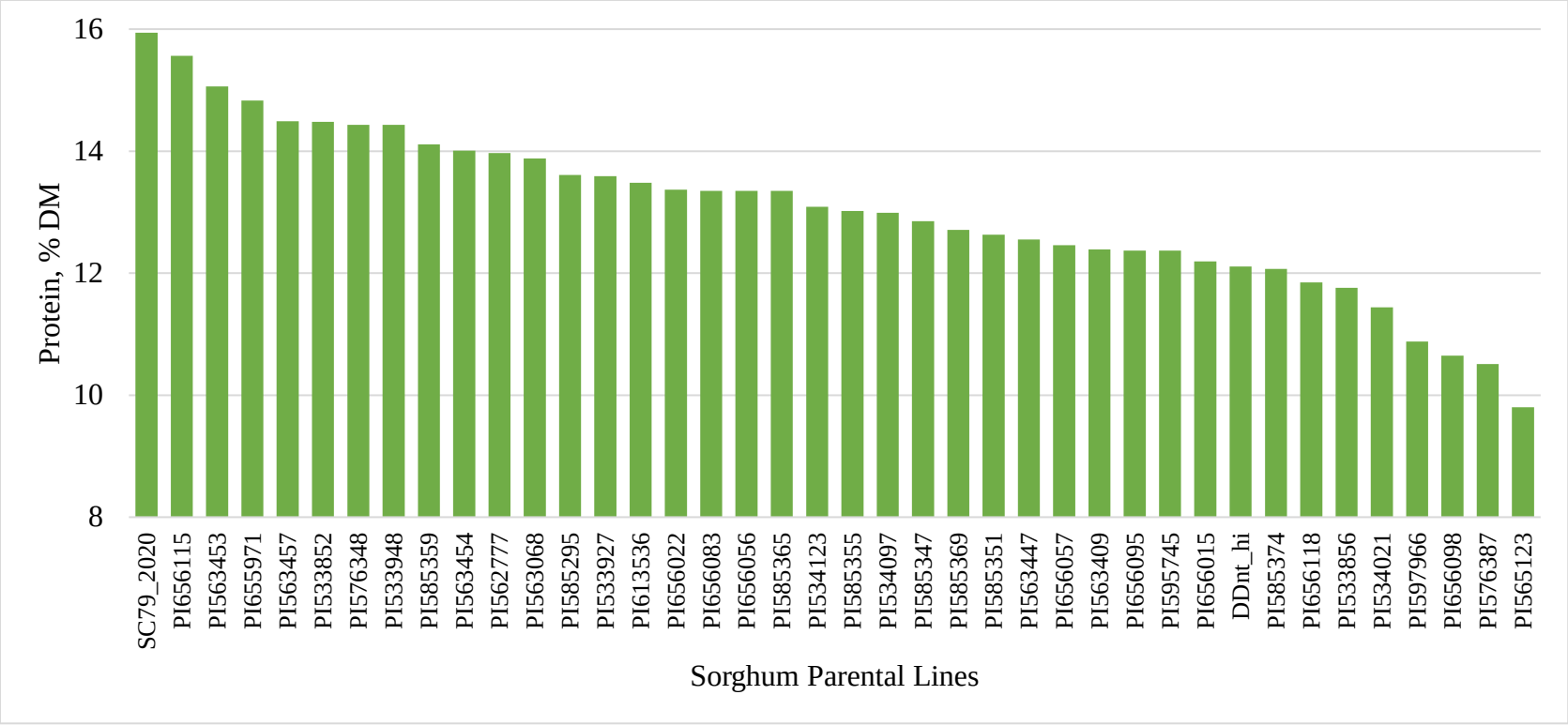

Figure 2.2. Estimation of protein content of each sorghum parental lines using near infrared spectroscopy (NIR).
A Perten DA 7250 spectrometer (Perten Instruments, Springfield, IL, USA) was used to scan each grain to estimate protein content. Calibration and statistical models used to estimate protein content were based on previous work study on sorghum cultivars (Peiris et al., 2019; Peiris et al., 2020).

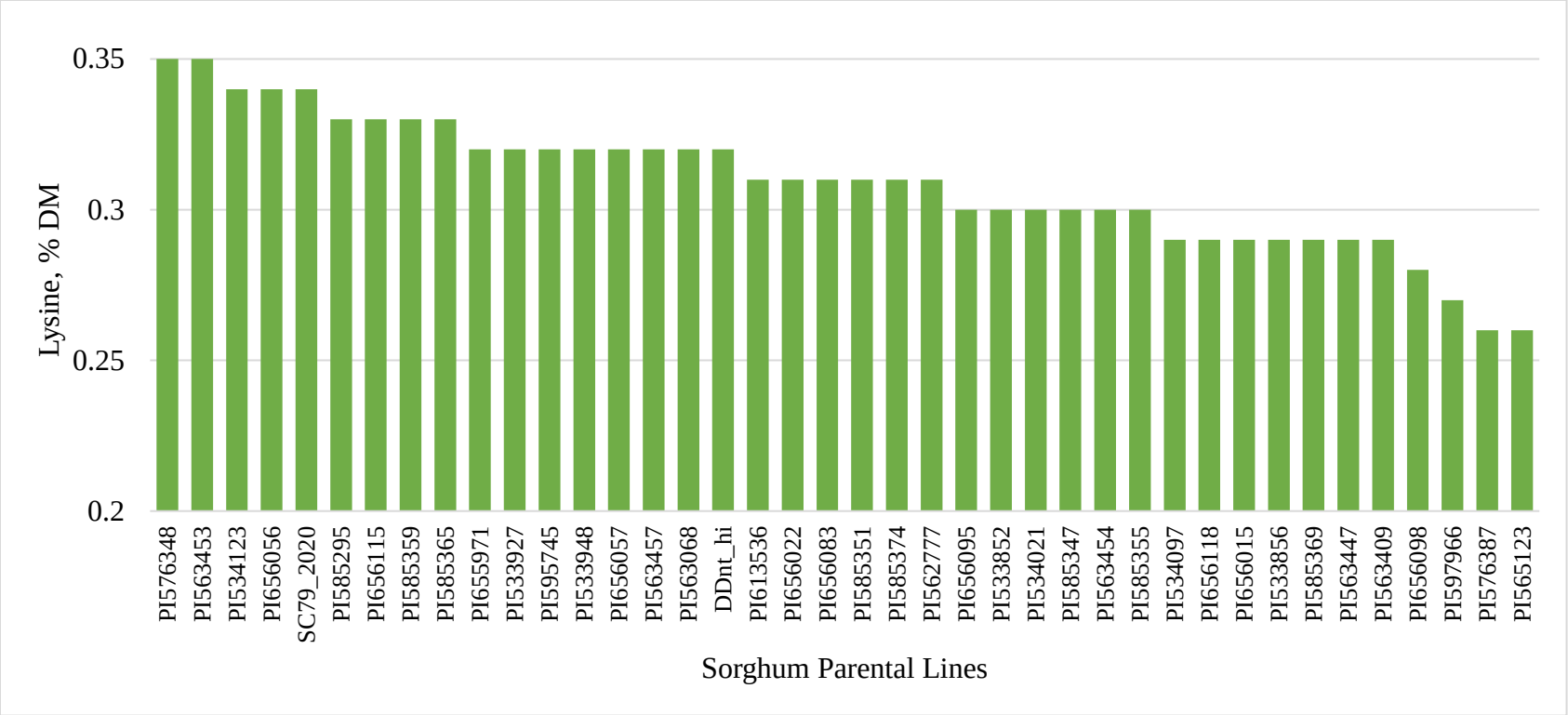

Figure 2.3. Estimation of lysine content of each sorghum parental lines near infrared spectroscopy (NIR).
A Perten DA 7250 spectrometer (Perten Instruments, Springfield, IL, USA) was used to scan each grain to estimate lysine content.

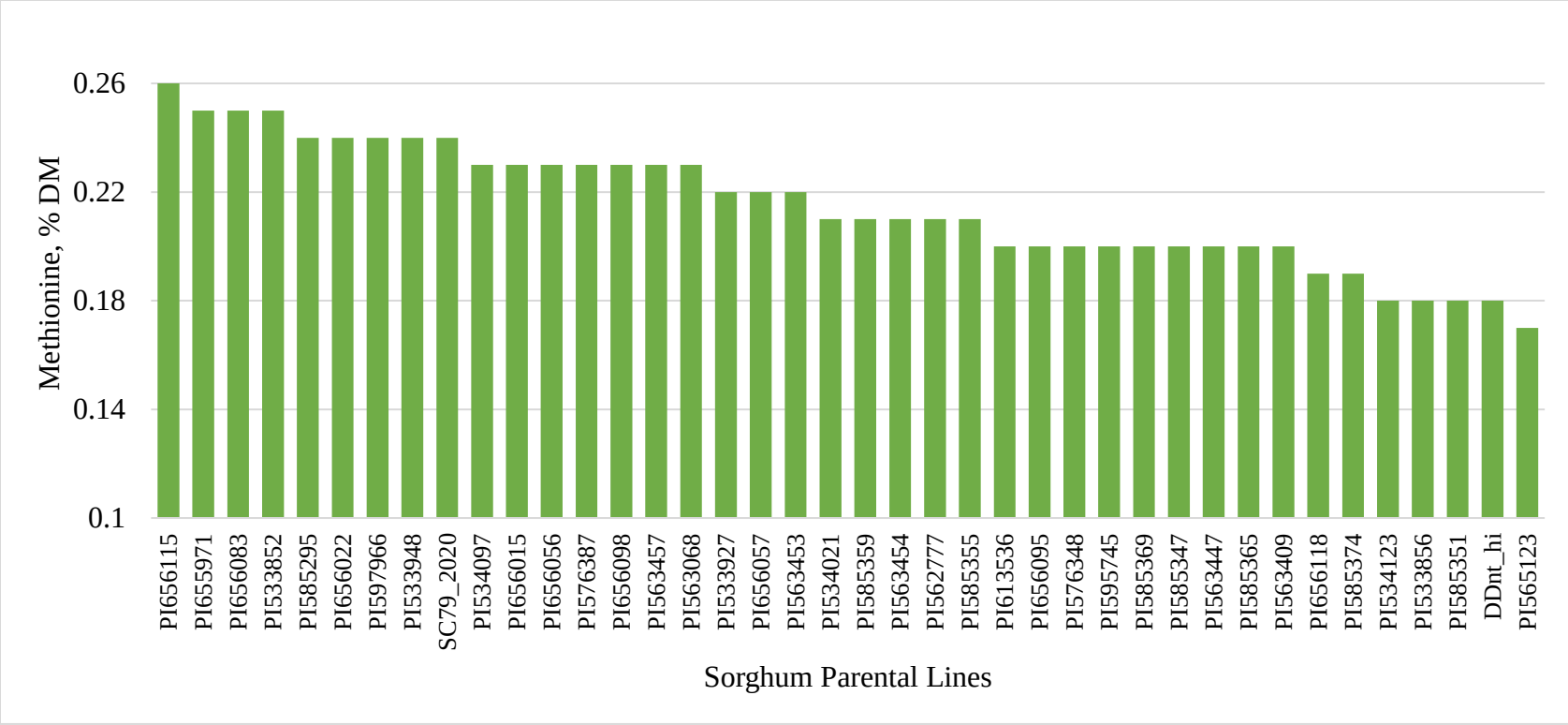

Figure 2.4. Estimation of methionine content of each sorghum parental lines using near infrared spectroscopy (NIR).
A Perten DA 7250 spectrometer (Perten Instruments, Springfield, IL, USA) was used to scan each grain to estimate methionine content.

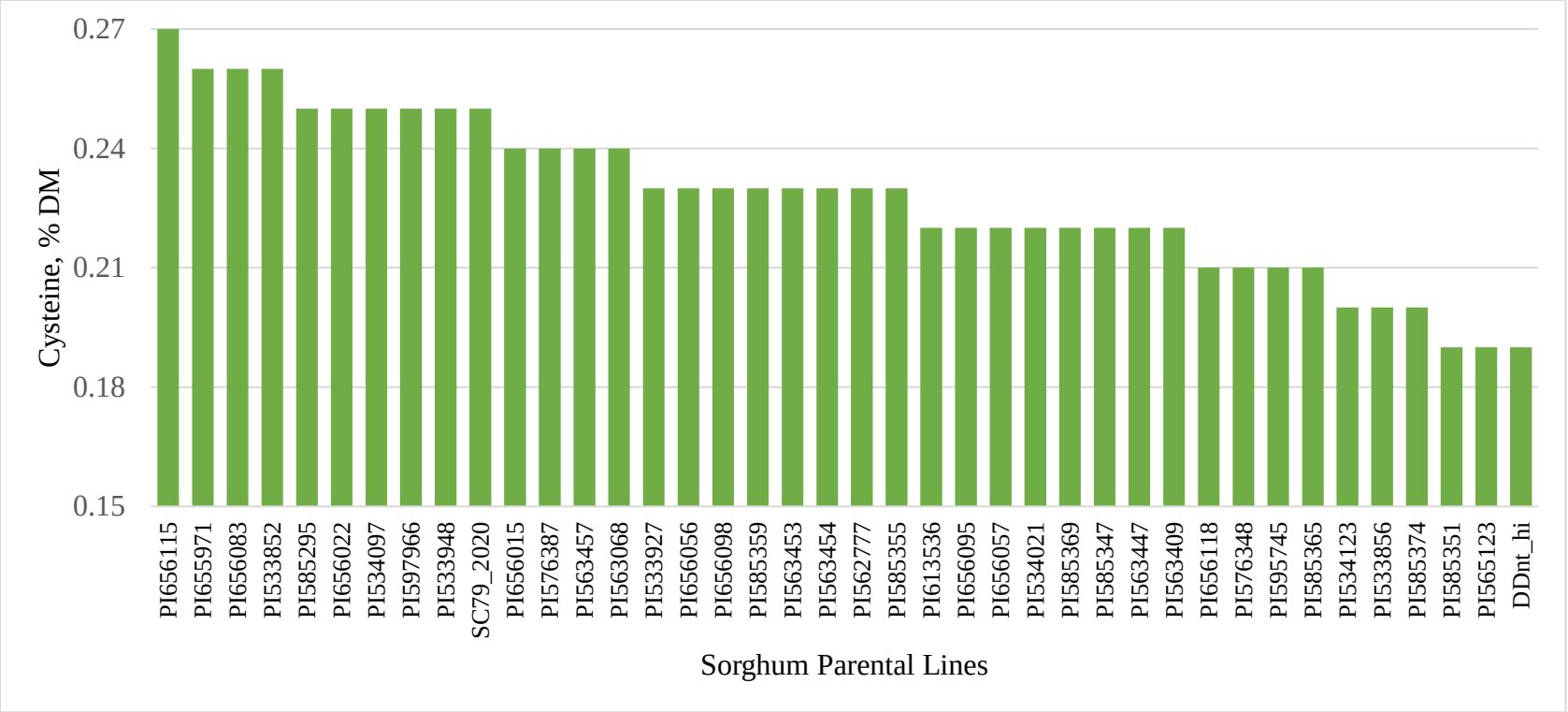

Figure 2.5. Estimation of cysteine content of each sorghum parental lines near infrared spectroscopy (NIR).
A Perten DA 7250 spectrometer (Perten Instruments, Springfield, IL, USA) was used to scan each grain to estimate cysteine content.

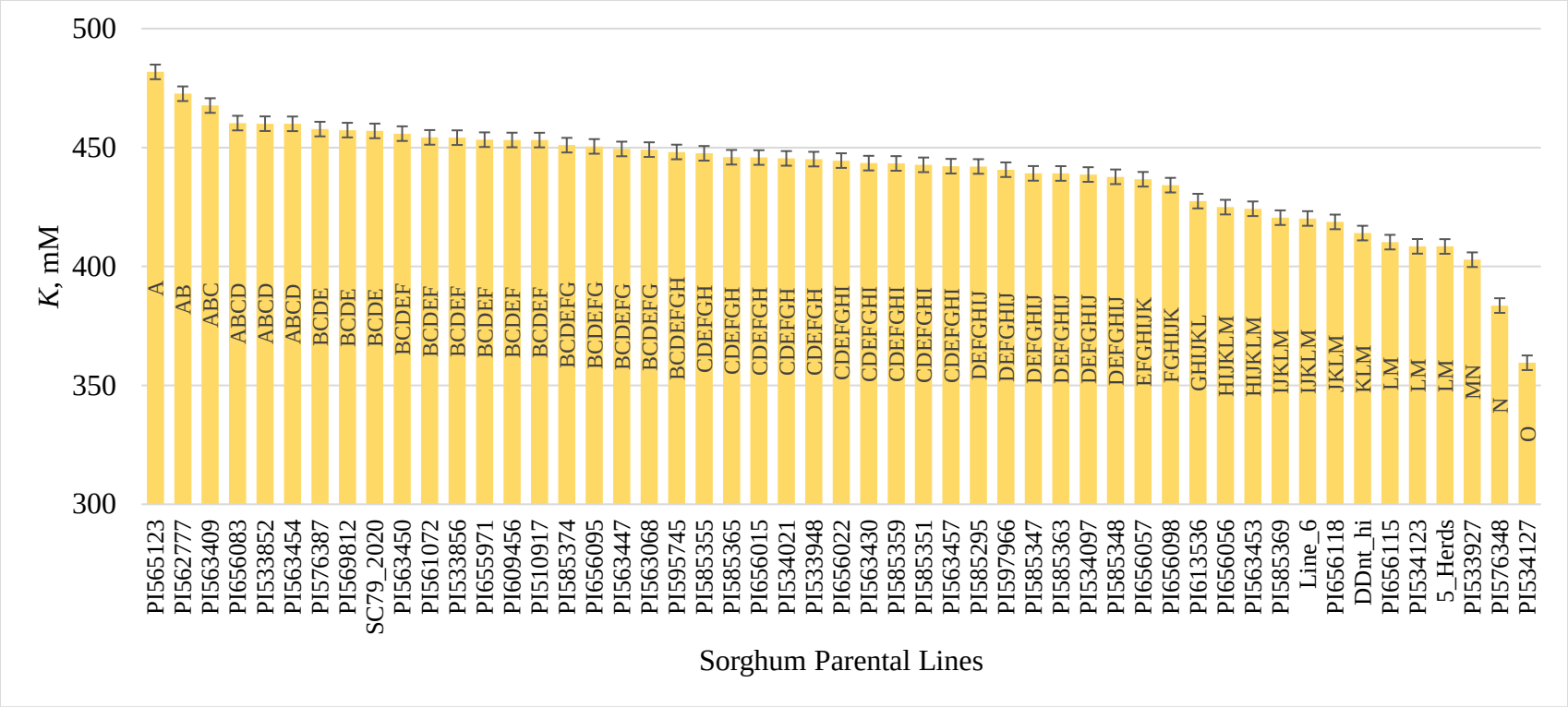

Figure 2.6. Maximum *in vitro* gas production (*K*) for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate.

Values are least-square means ± standard error of the means. Pedigree affected *K* ($P<0.01$). Bars without a common letter are different ($P<0.05$).

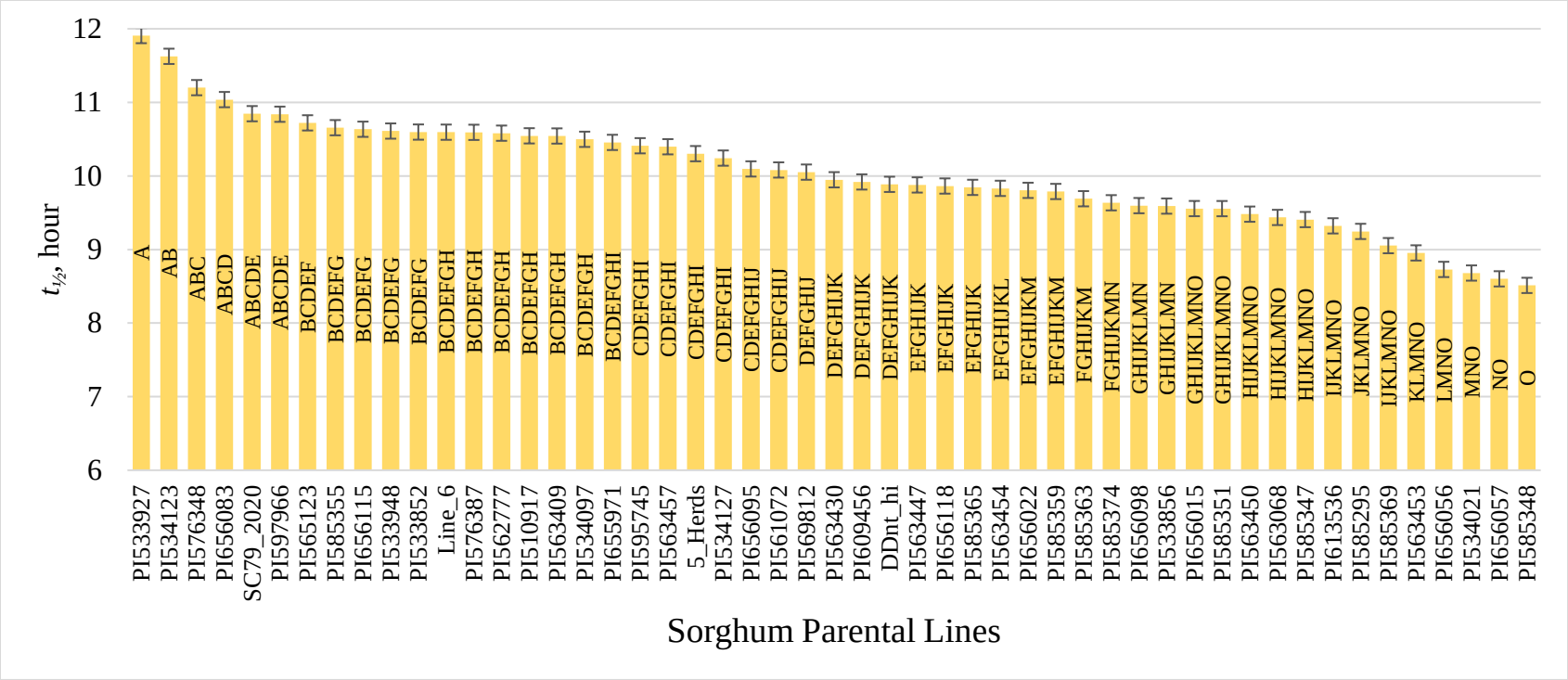

Figure 2.7. Time to reach half maximum gas production ($t_{½}$) for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate.
Values are least-square means ± standard error of the means. Pedigree affected $t_{½}$ ($P<0.01$). Bars without a common letter are different ($P<0.05$).

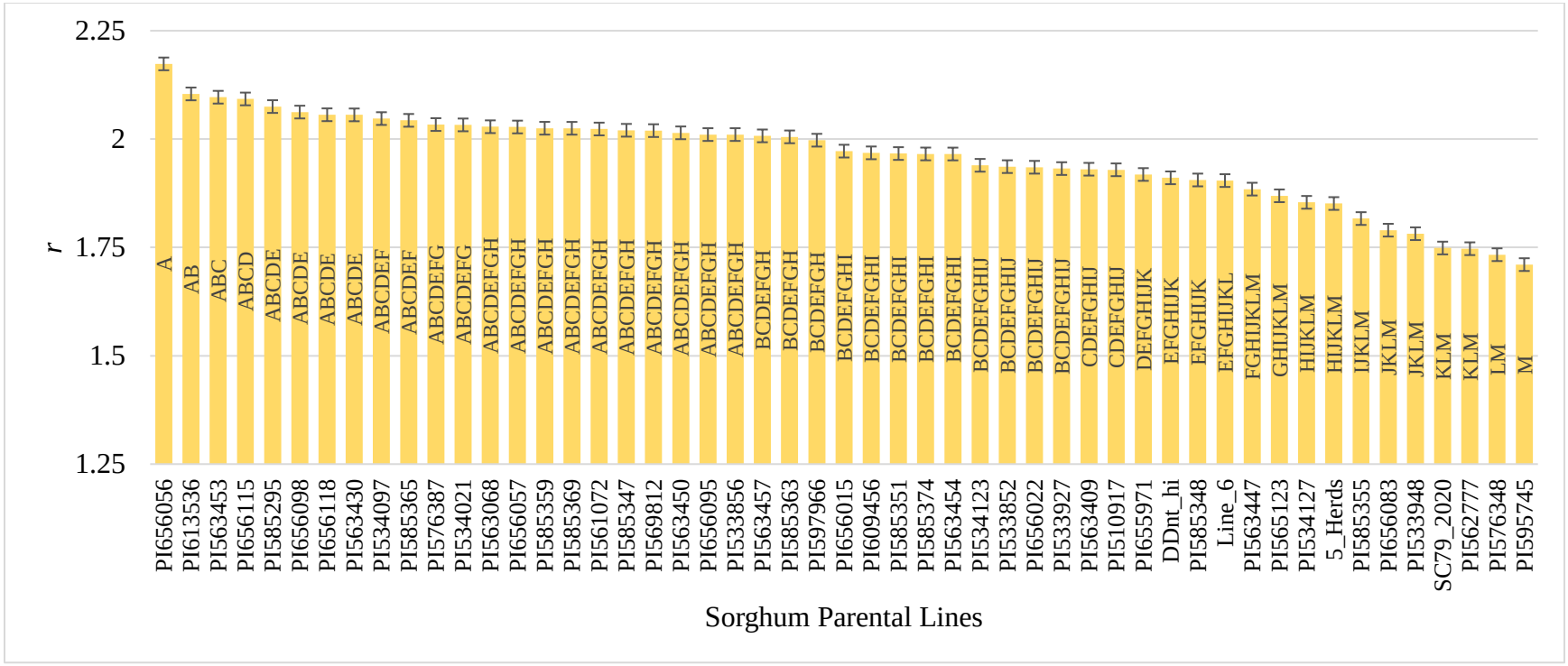

Figure 2.8. *r* for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate.
Values are least-square means ± standard error of the means. Pedigree affected *r* (P<0.01). Bars without a common letter are different (P<0.05).

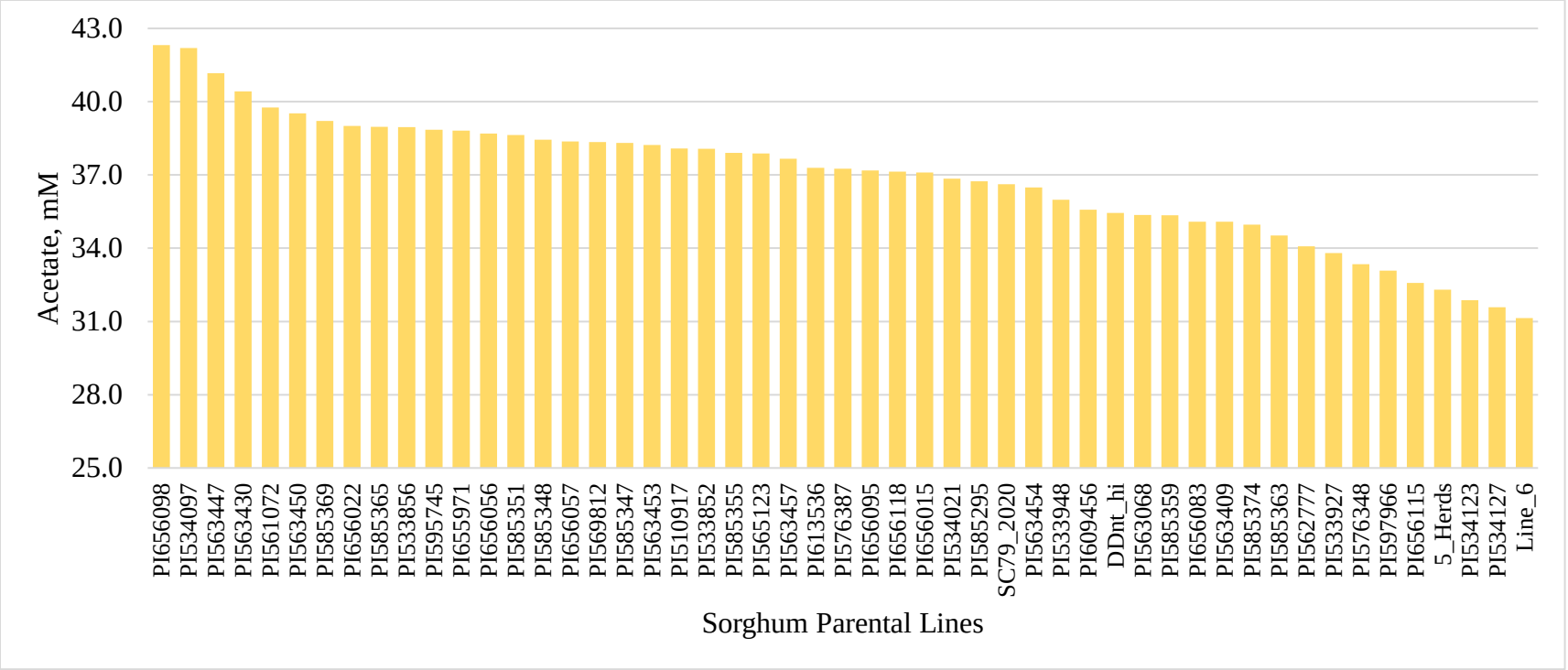

Figure 2.9. Acetate concentration for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate. Values are least-square means ± standard error of the means. Pedigree did not affect acetate concentration (P=0.71).

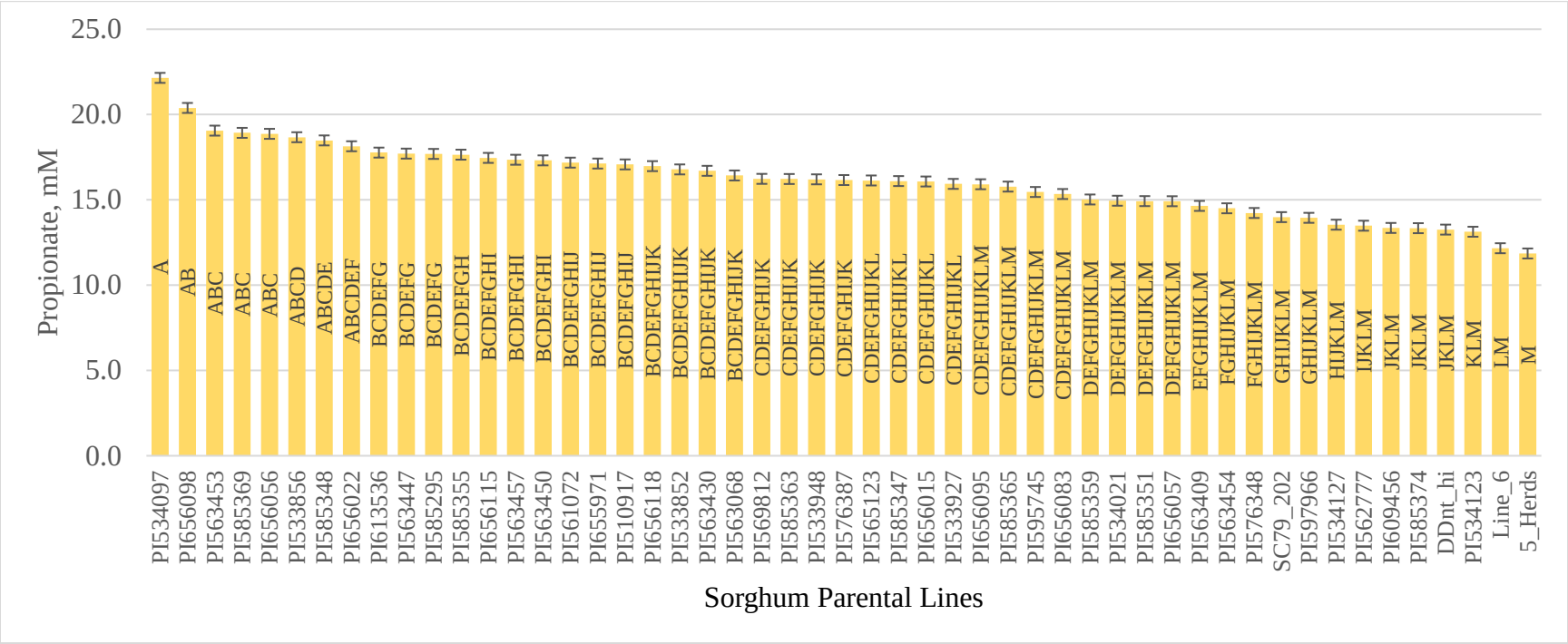

Figure 2.10. Propionate concentration for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate. Values are least-square means ± standard error of the means. Pedigree affected propionate concentration ($P<0.01$). Bars without a common letter are different ($P<0.05$).

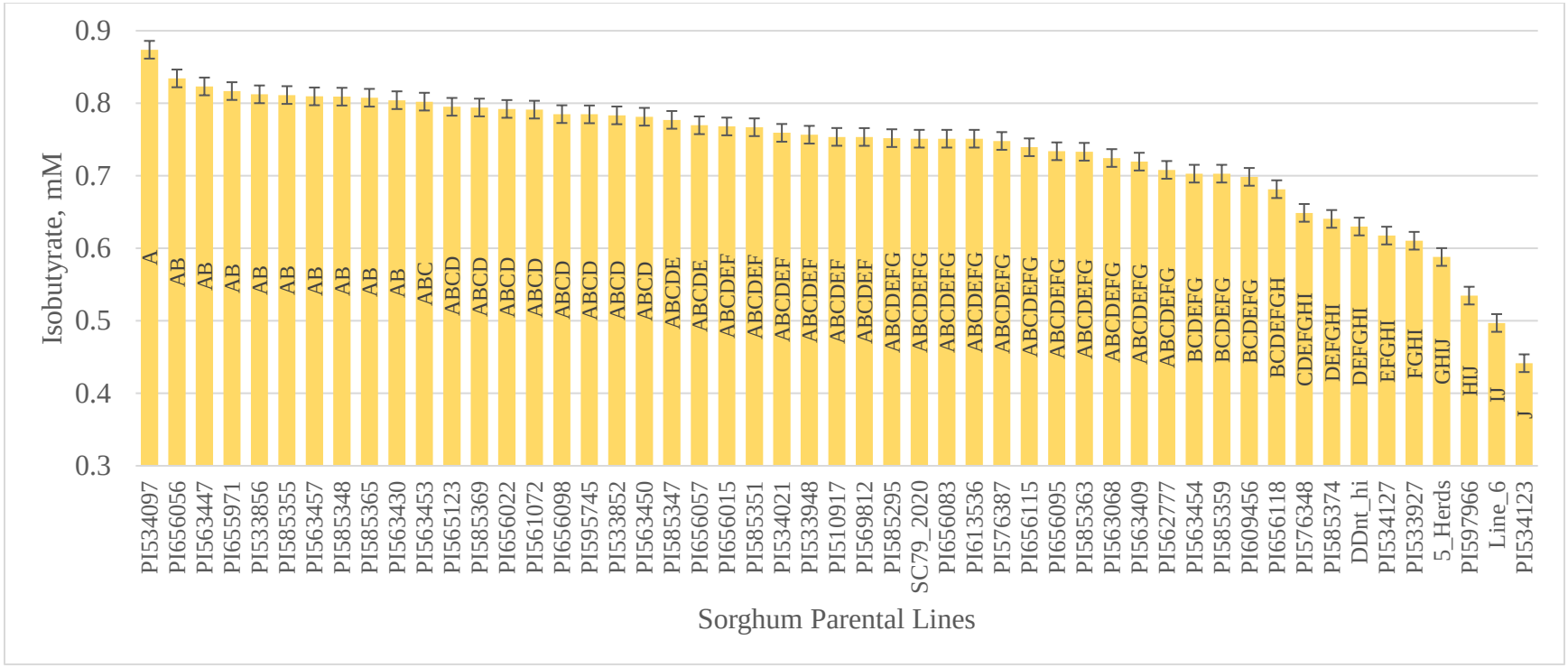

Figure 2.11. Iso-butyrate concentration for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate.
Values are least-square means ± standard error of the means. Pedigree affected iso-butyrate concentration ($P<0.01$). Bars without a common letter are different ($P<0.05$).

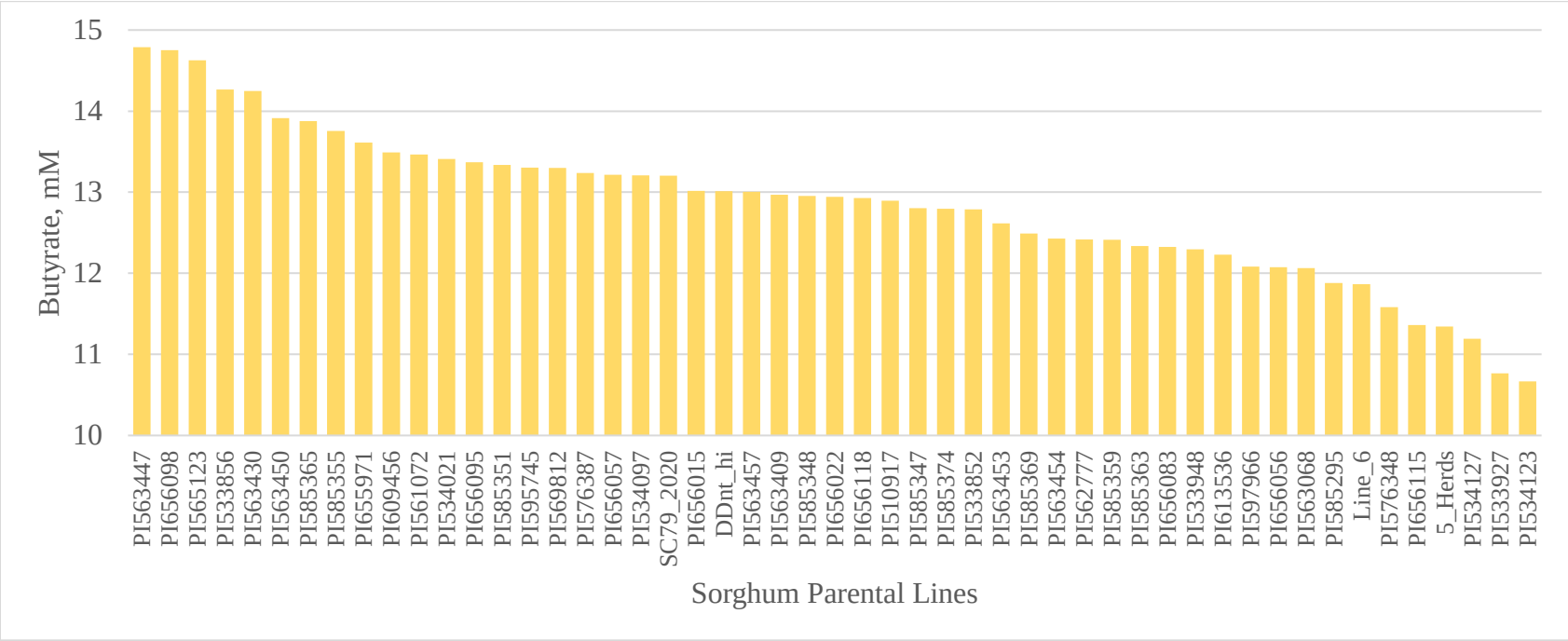

Figure 2.12. Butyrate concentration for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate. Values are least-square means ± standard error of the means. Pedigree did not affect butyrate concentration (P=0.24).

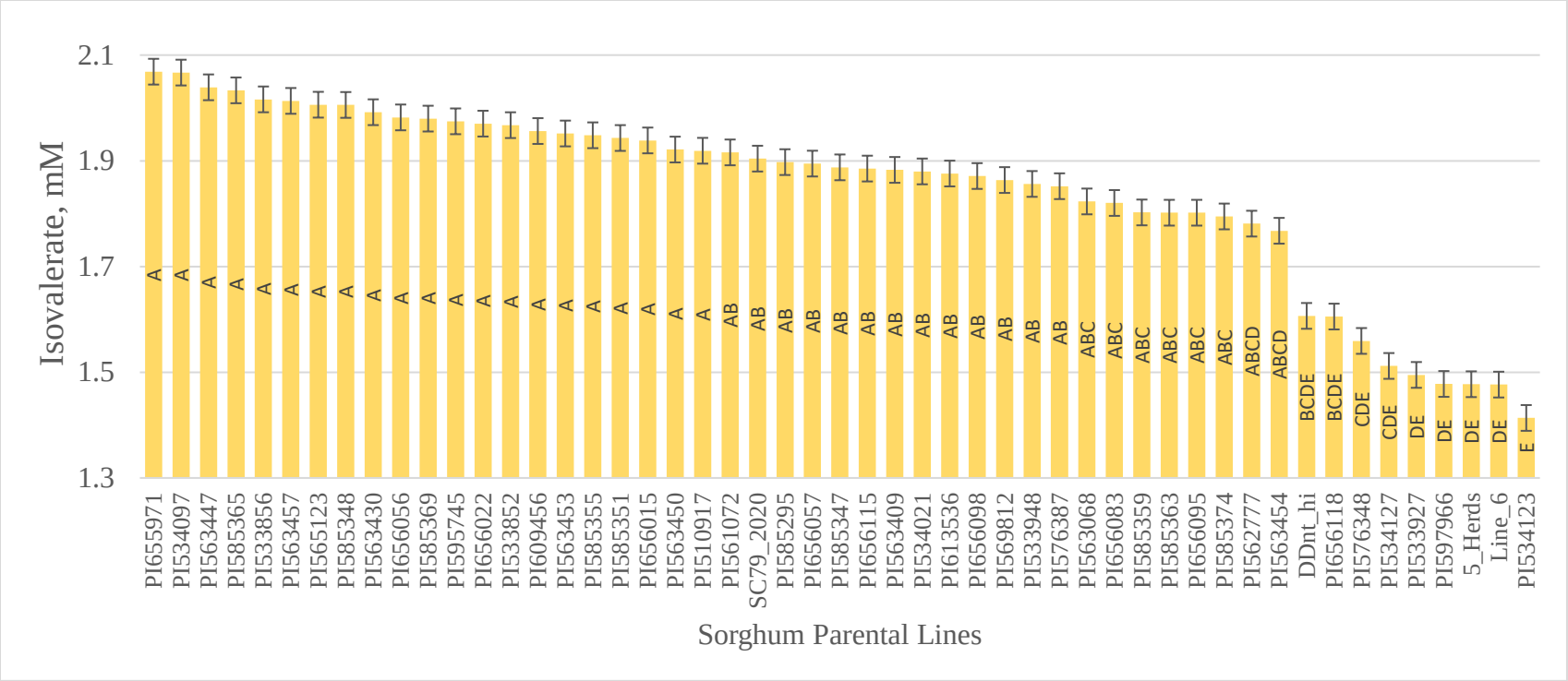

Figure 2.13. Isovalerate concentration for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate. Values are least-square means ± standard error of the means. Pedigree affected iso-valerate concentration ($P<0.01$). Bars without a common letter are different ($P<0.05$).

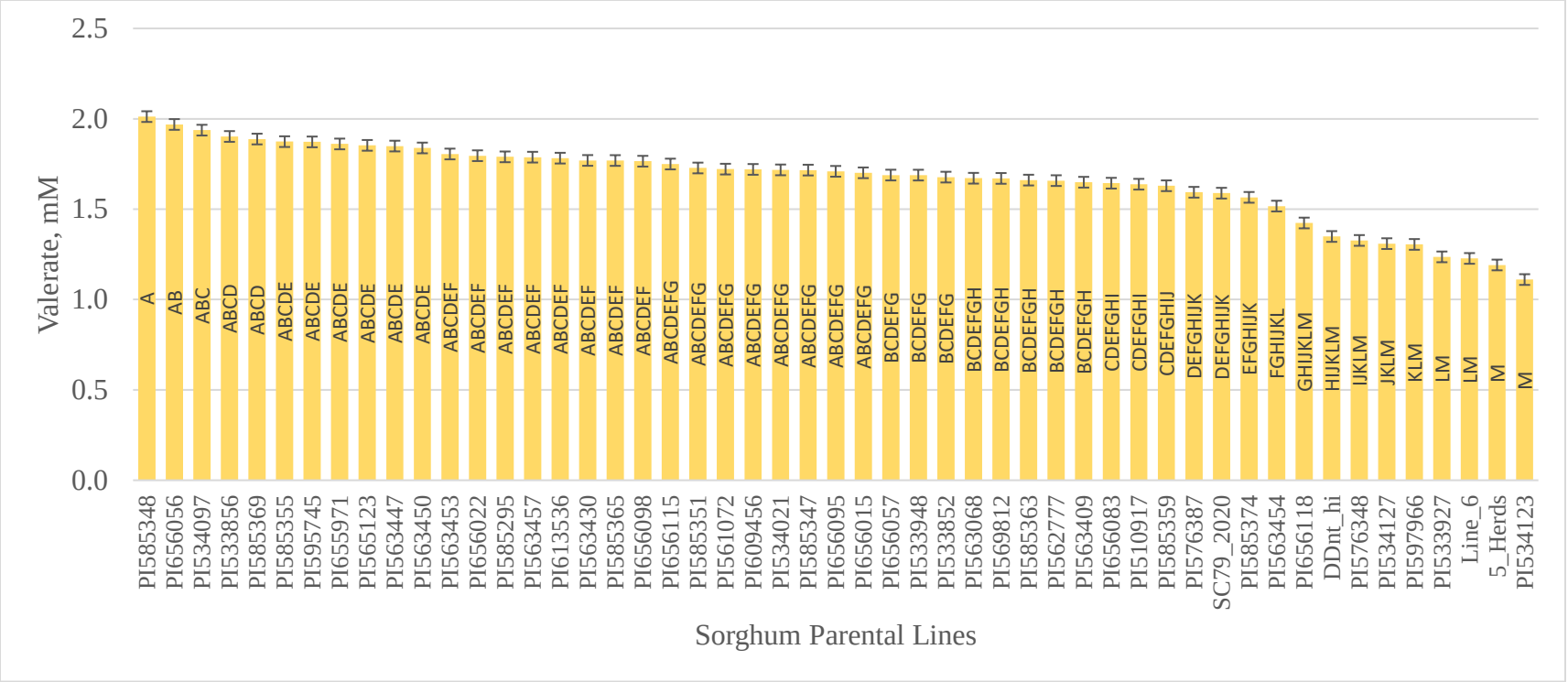

Figure 2.14. Valerate concentration for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate. Values are least-square means ± standard error of the means. Pedigree affected valerate concentration ($P<0.01$). Bars without a common letter are different ($P<0.05$).

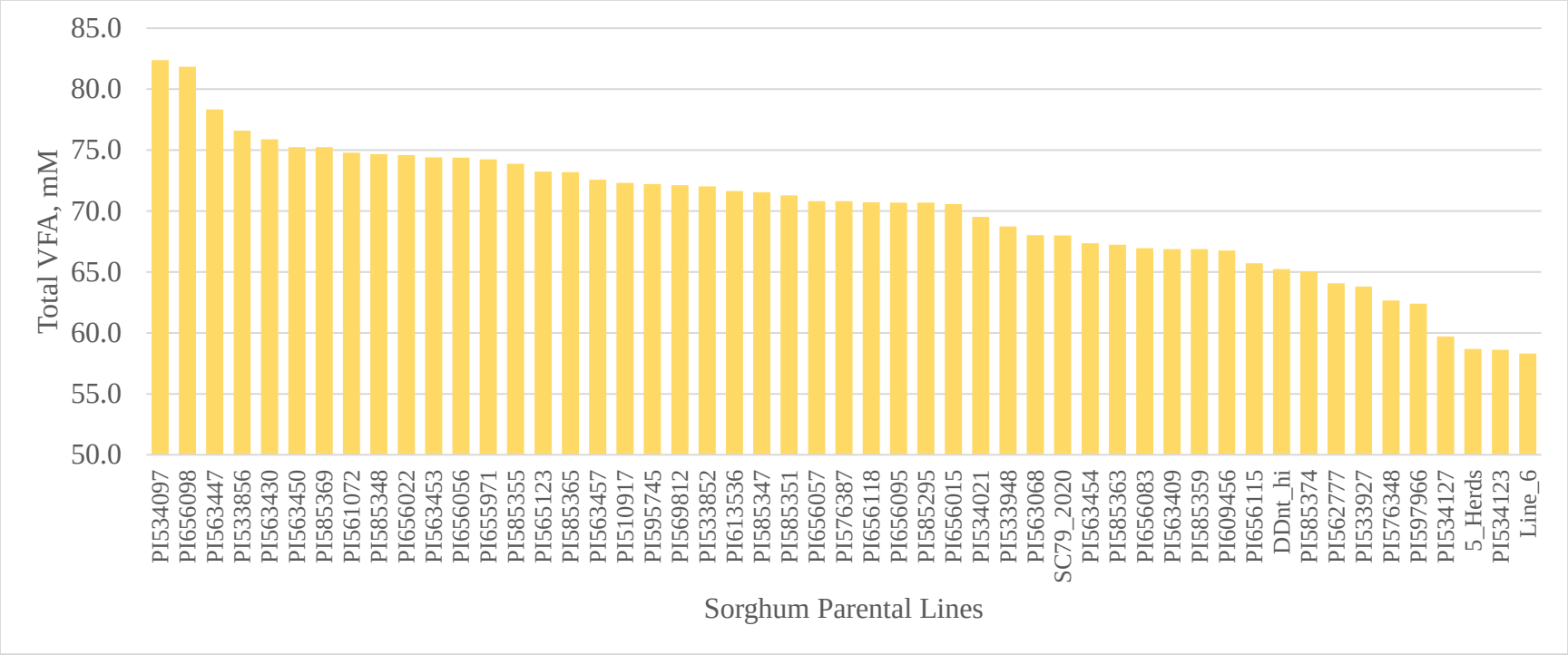

Figure 2.15. Total volatile fatty acid (VFA) concentration for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate.
Values are least-square means ± standard error of the means. Pedigree did not affect total VFA concentration (P=0.29).

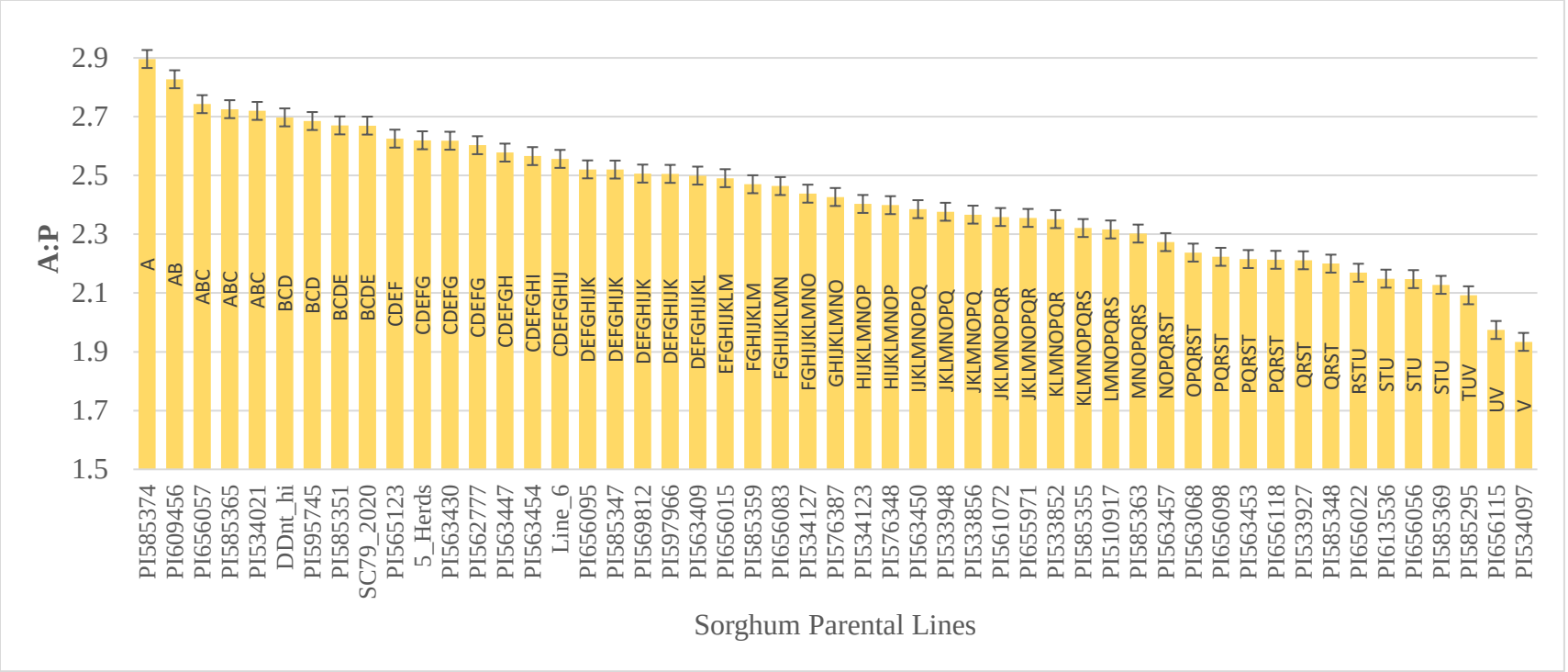

Figure 2.16. Acetate:propionate ratio for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate. Values are least-square means ± standard error of the means. Pedigree affected A:P ratio ($P<0.01$). Bars without a common letter are different ($P<0.05$).

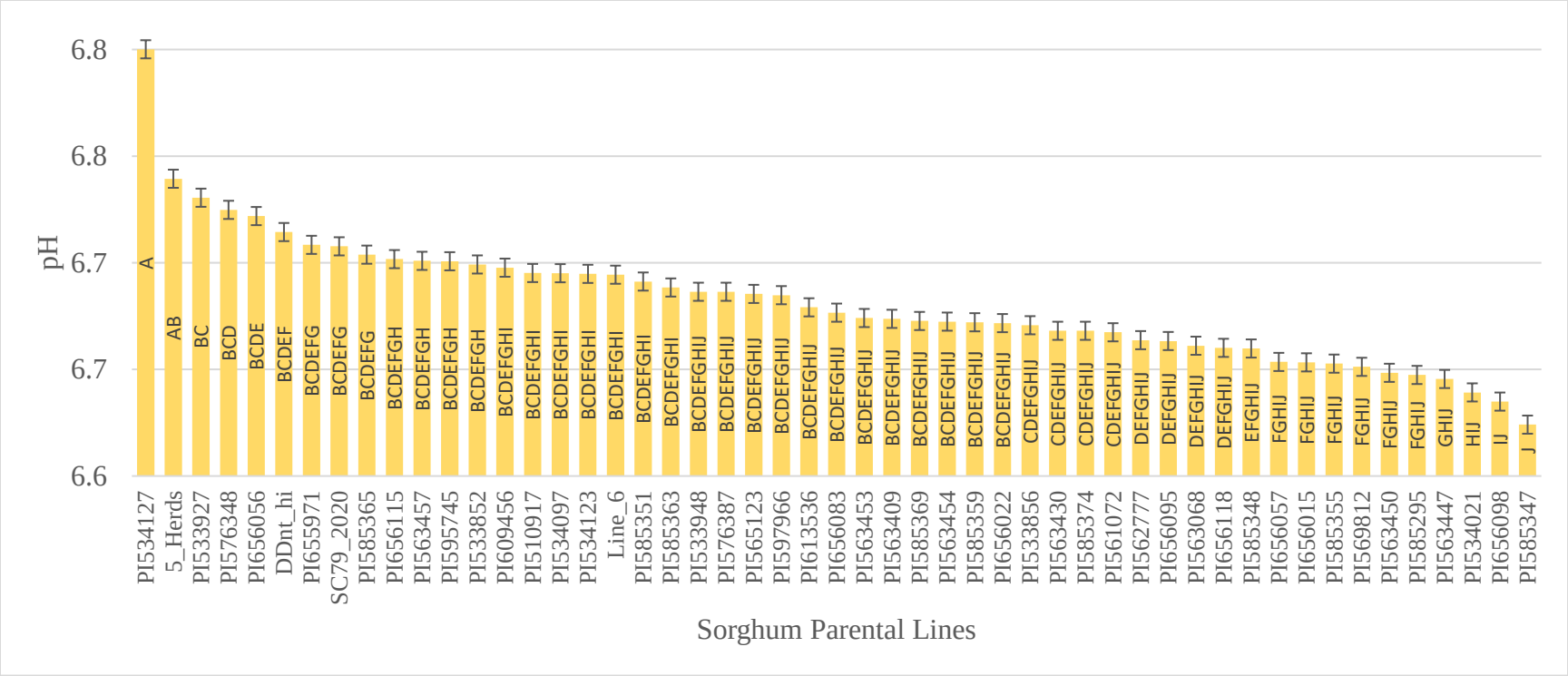

Figure 2.17. Terminal pH for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate. Values are least-square means ± standard error of the means. Pedigree affected terminal pH ($P<0.01$). Bars without a common letter are different ($P<0.05$).

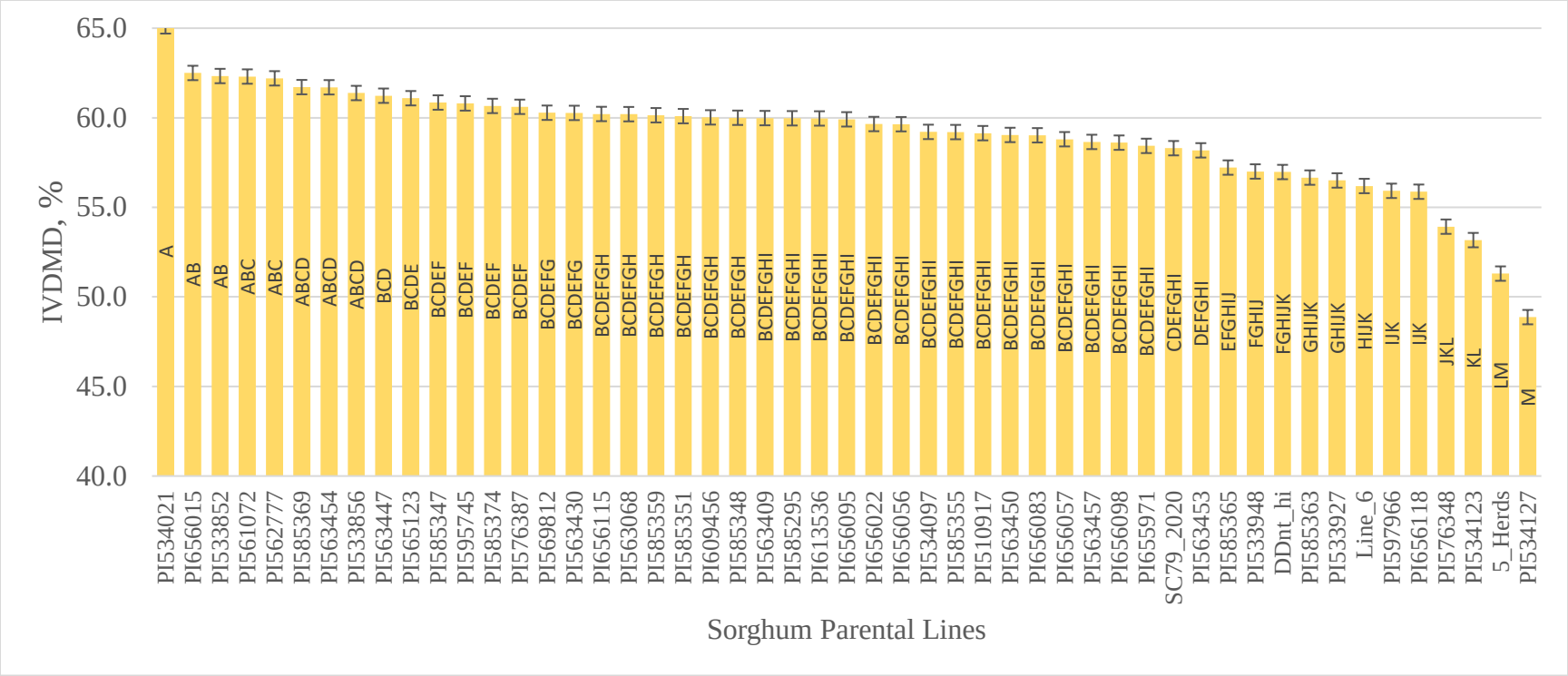

Figure 2.18. In vitro dry matter disappearance (IVDMD) for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate.
Values are least-square means ± standard error of the means. Pedigree affected IVDMD ($P<0.01$). Bars without a common letter are different ($P<0.05$).

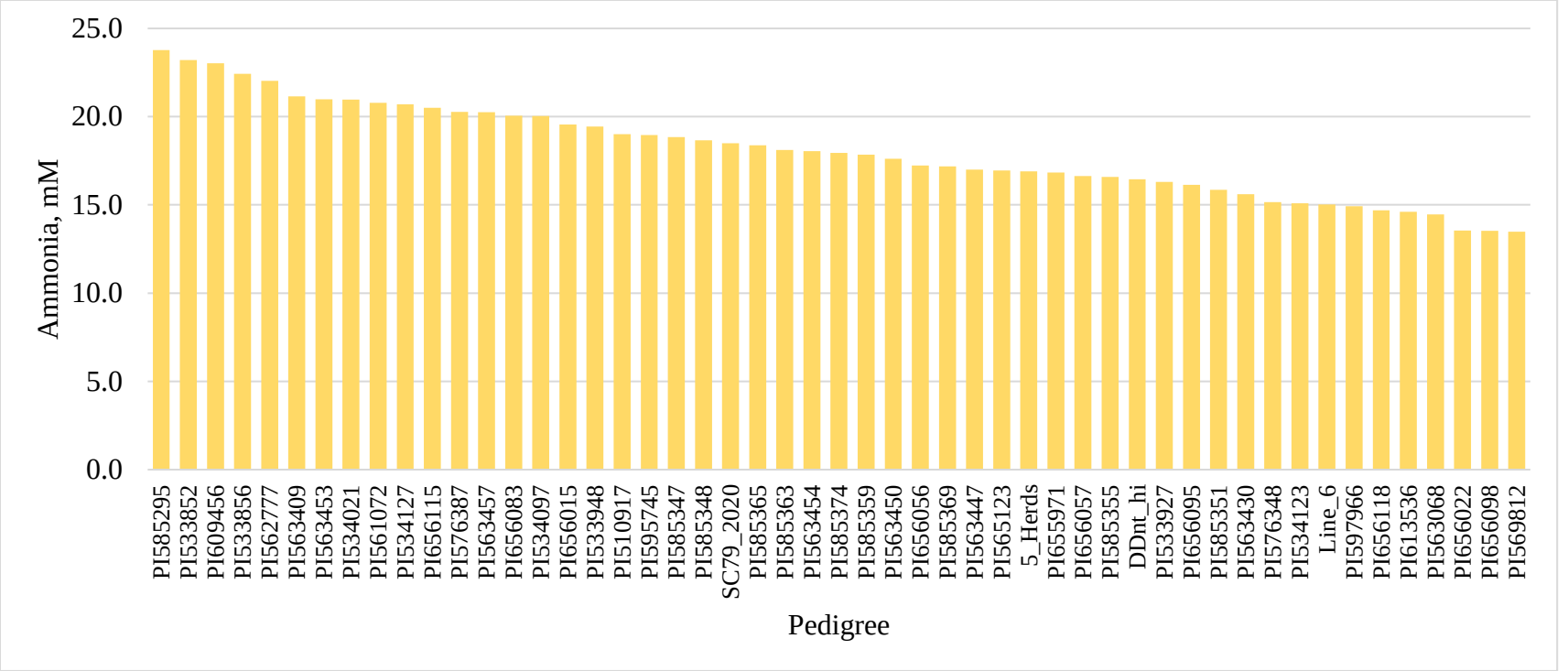

Figure 2.19. Ammonia concentration for *in vitro* cultures of mixed ruminal microorganisms using sorghum parental lines as substrate. Values are least-square means ± standard error of the means. Pedigree did not affect ammonia concentration (P=0.11).

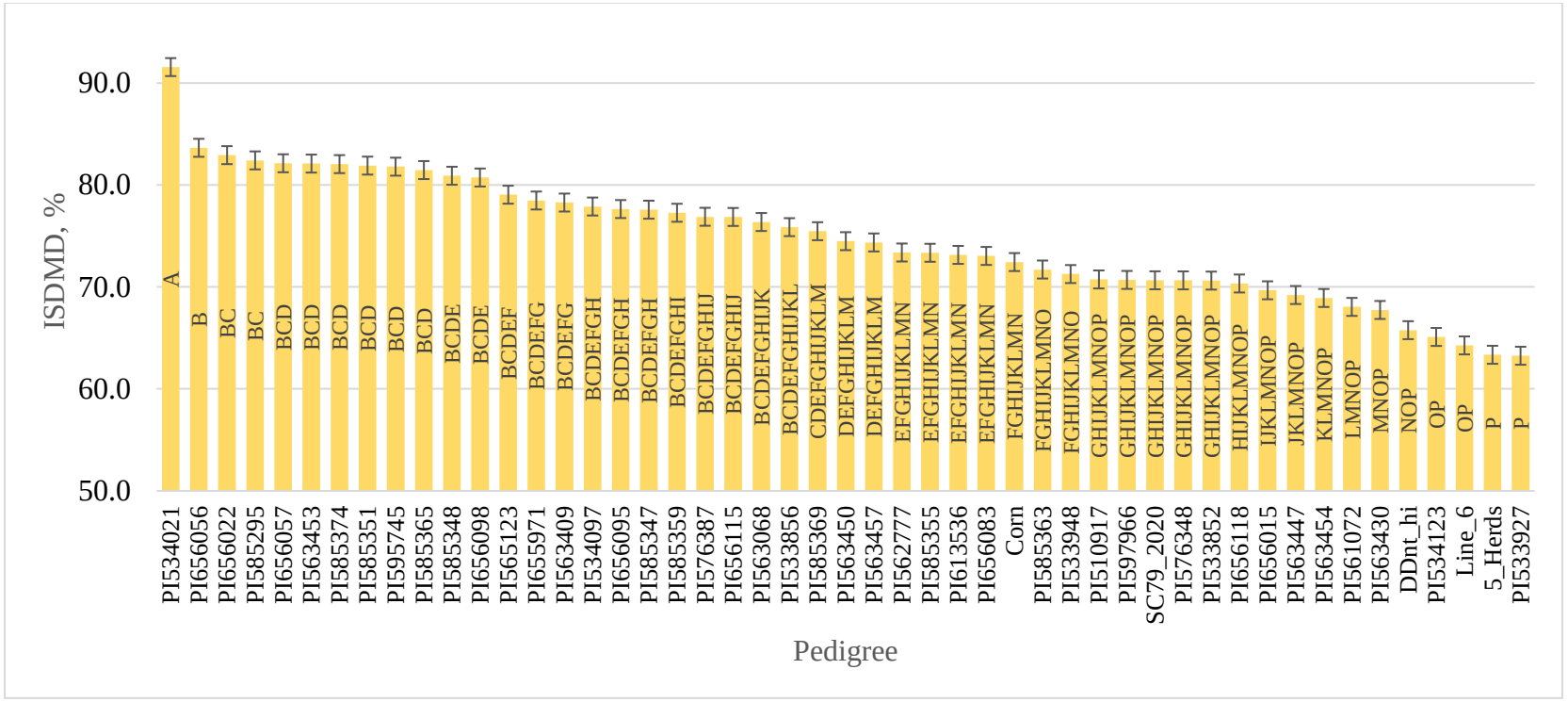

Figure 2.20. *In situ* dry matter disappearance (ISDMD) using sorghum parental lines as substrate.
Values are least-square means ± standard error of the means. Pedigree affected ISDMD (P<0.01). Bars without a common letter are different (P<0.05).

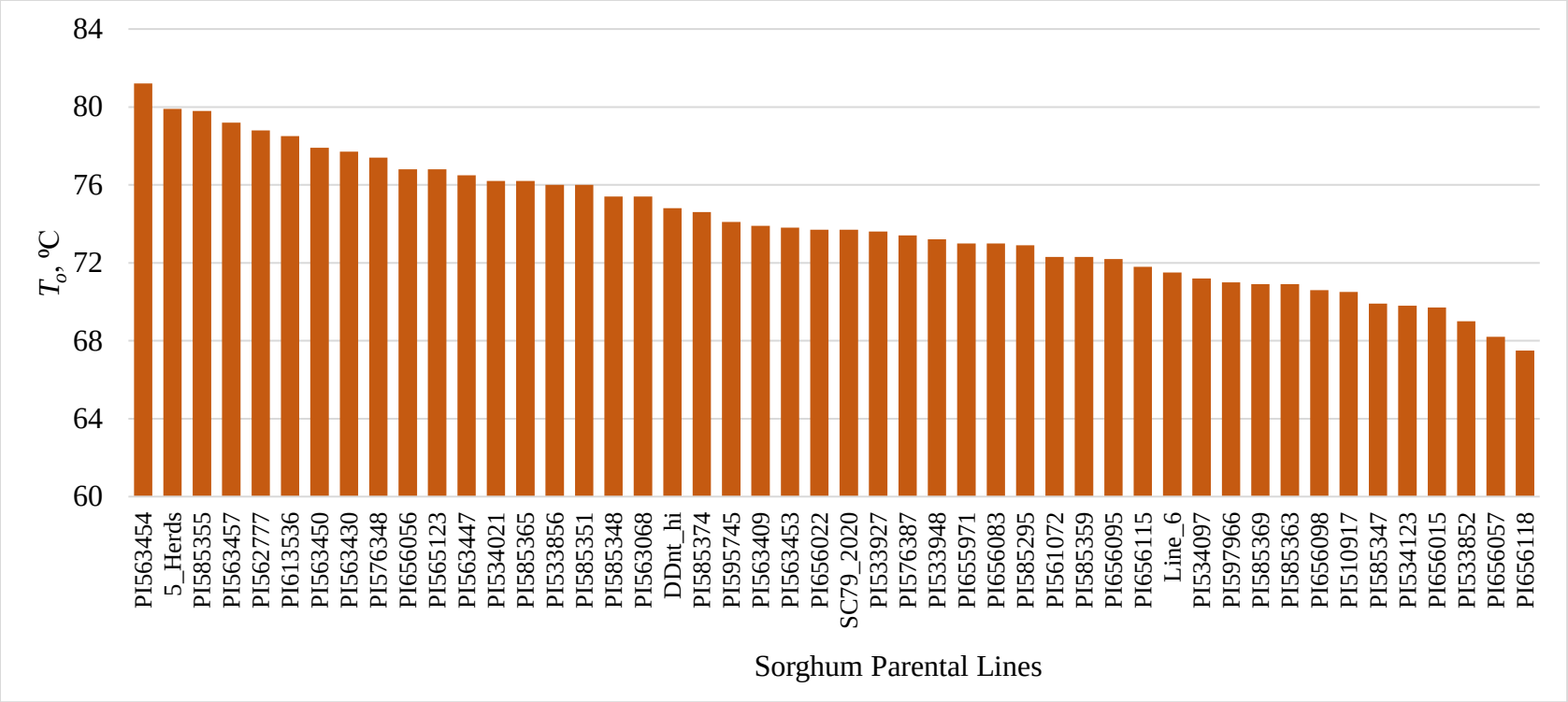

Figure 2.21. Onset temperature (T_o) of gelatinization for sorghum parental.
Since T_o measurement was only done in duplicate, data obtained was analyzed using descriptive statistic in SAS. Number of observation (n)=48; average value of T_o (mean)=74.01ºC; standard deviation=3.31, the highest temperature (maximum)=81.2ºC; the lowest temperature (minimum)= 67.5ºC

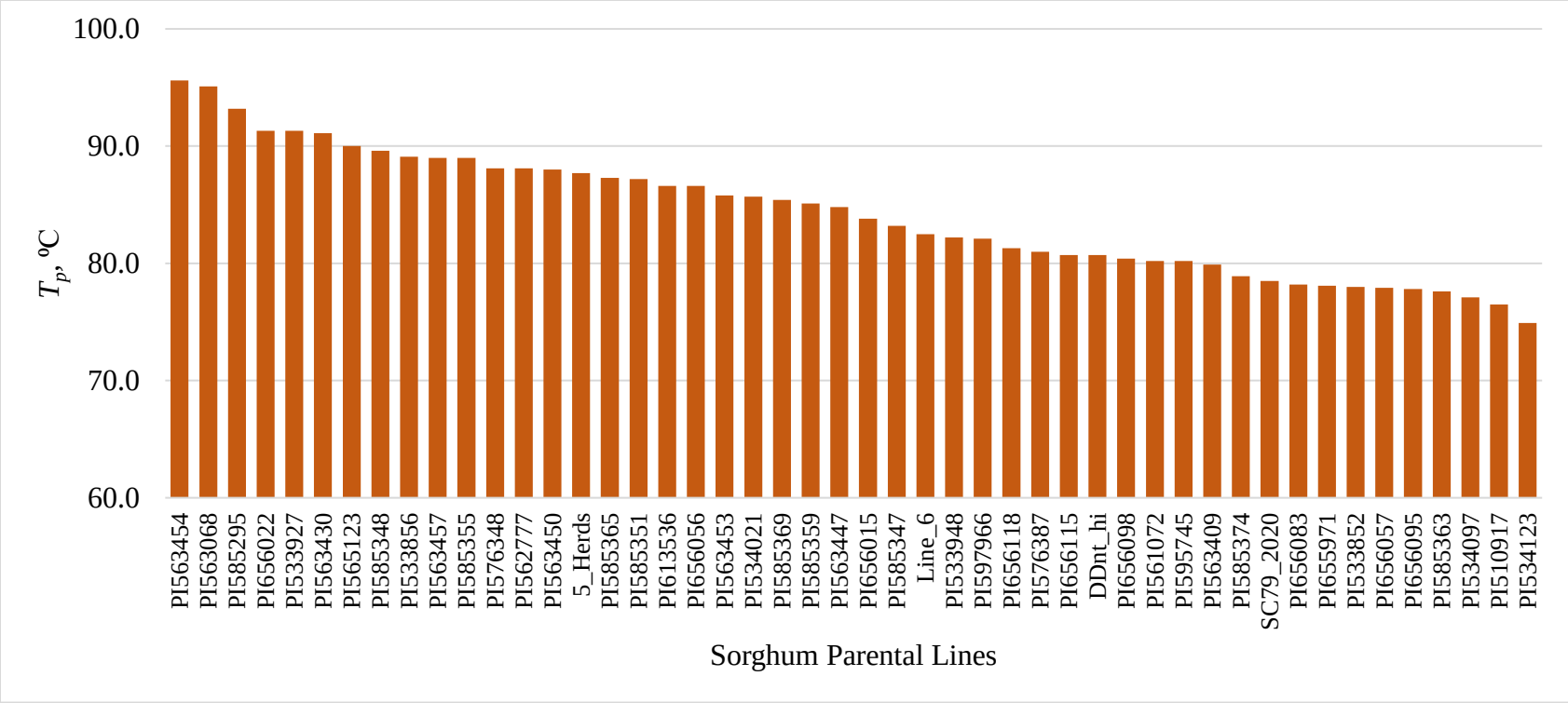

Figure 2.22. Peak temperature (T_p) of gelatinization for sorghum parental lines.
Since T_p measurement was only done in duplicate, data obtained was analyzed using descriptive statistic in SAS. Number of observation (n)=48; average value of T_p (mean)= 84.22°C; standard deviation=5.30, the highest temperature (maximum)=95.60°C; the lowest temperature (minimum)=74.90°C

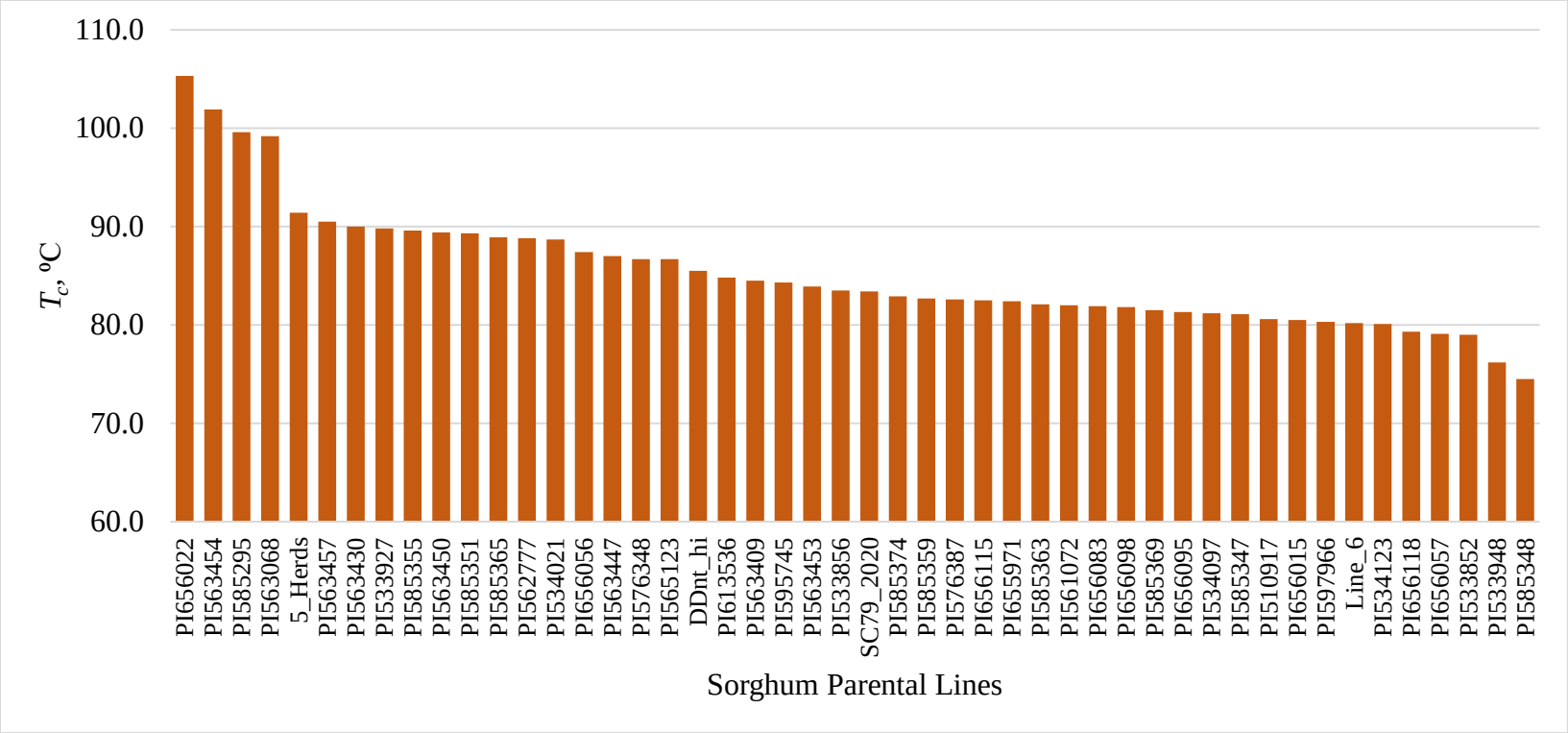

Figure 2.23. Conclusion temperature (T_c) of gelatinization for sorghum parental lines.
Since T_c measurement was only done in duplicate, data obtained was analyzed using descriptive statistic in SAS. Number of observation (n)=48; average value of T_c (mean)=83.33ºC; standard deviation=6.31, the highest temperature (maximum)=105.30ºC; the lowest temperature (minimum)=74.50ºC

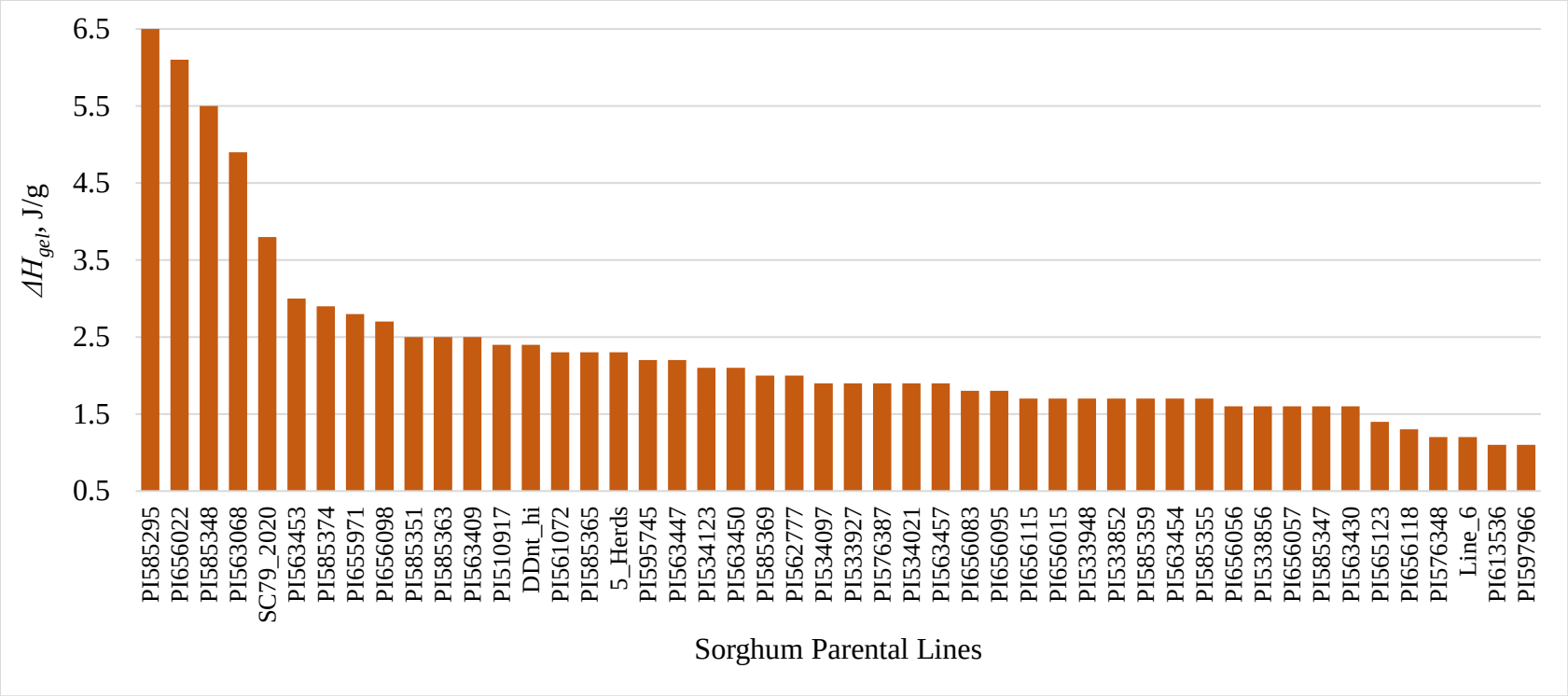

Figure 2.24. Gelatinization enthalpy (ΔH_{gel}) for sorghum parental lines.
Since ΔH_{gel} measurement was only done in duplicate, data obtained was analyzed using descriptive statistic in SAS.
Number of observation (n)=48; average value of ΔH_{gel} (mean)=2.30 J/g; standard deviation=1.19, the highest enthalpy gelatinization (maximum)=6.50 J/g; the lowest enthalpy gelatinization (minimum)= 1.10 J/g.

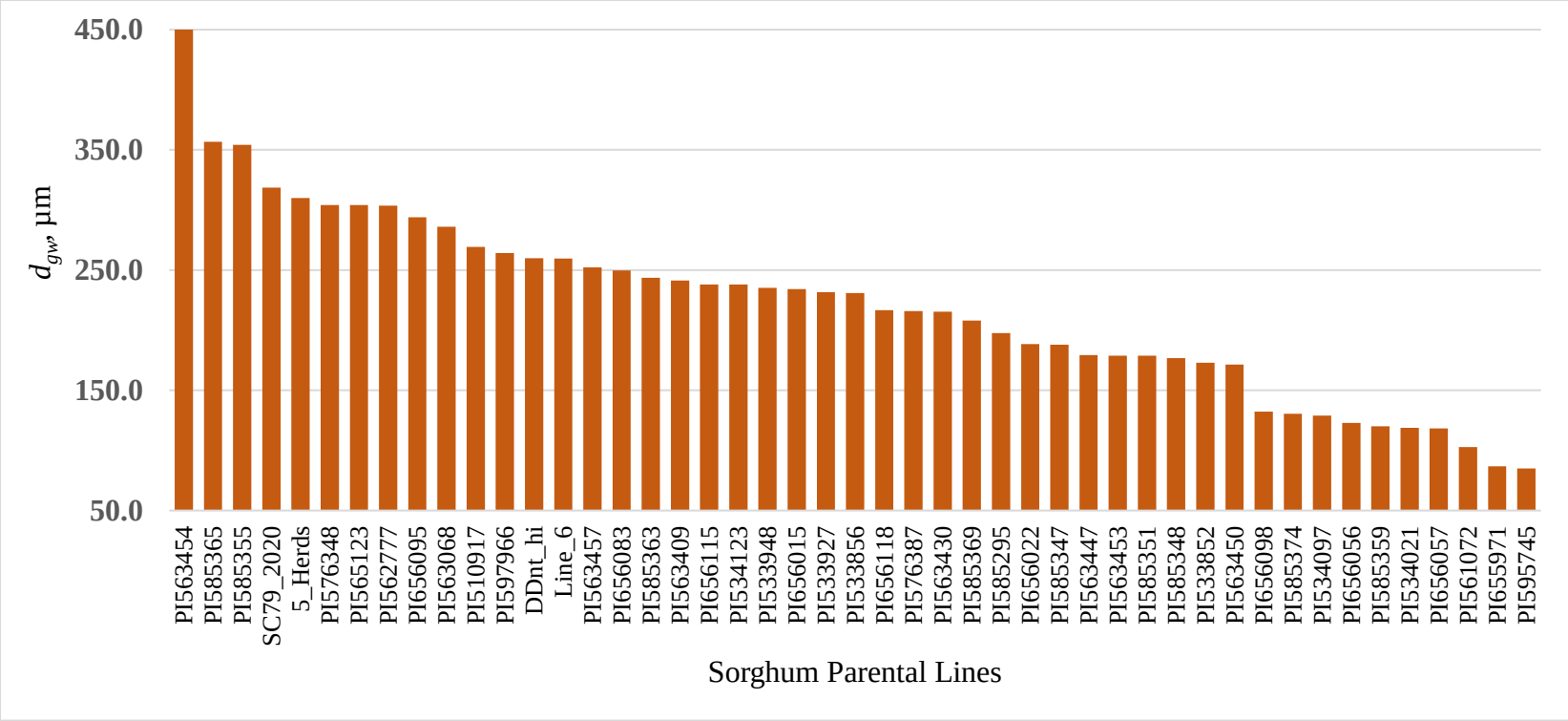

Figure 2.25. Geometric mean diameter (d_{gw})of particle size for sorghum parental lines**.**
Since d_{gw} measurement was only done in duplicate, data obtained was analyzed using descriptive statistic in SAS.
Number of observation (n)=47; average value of d_{gw} (mean)=220.60 µm; standard deviation=78.05, the greatest geometric mean diameter (maximum)=454.70µm; the least geometric mean diameter (minimum)=85.10 µm.

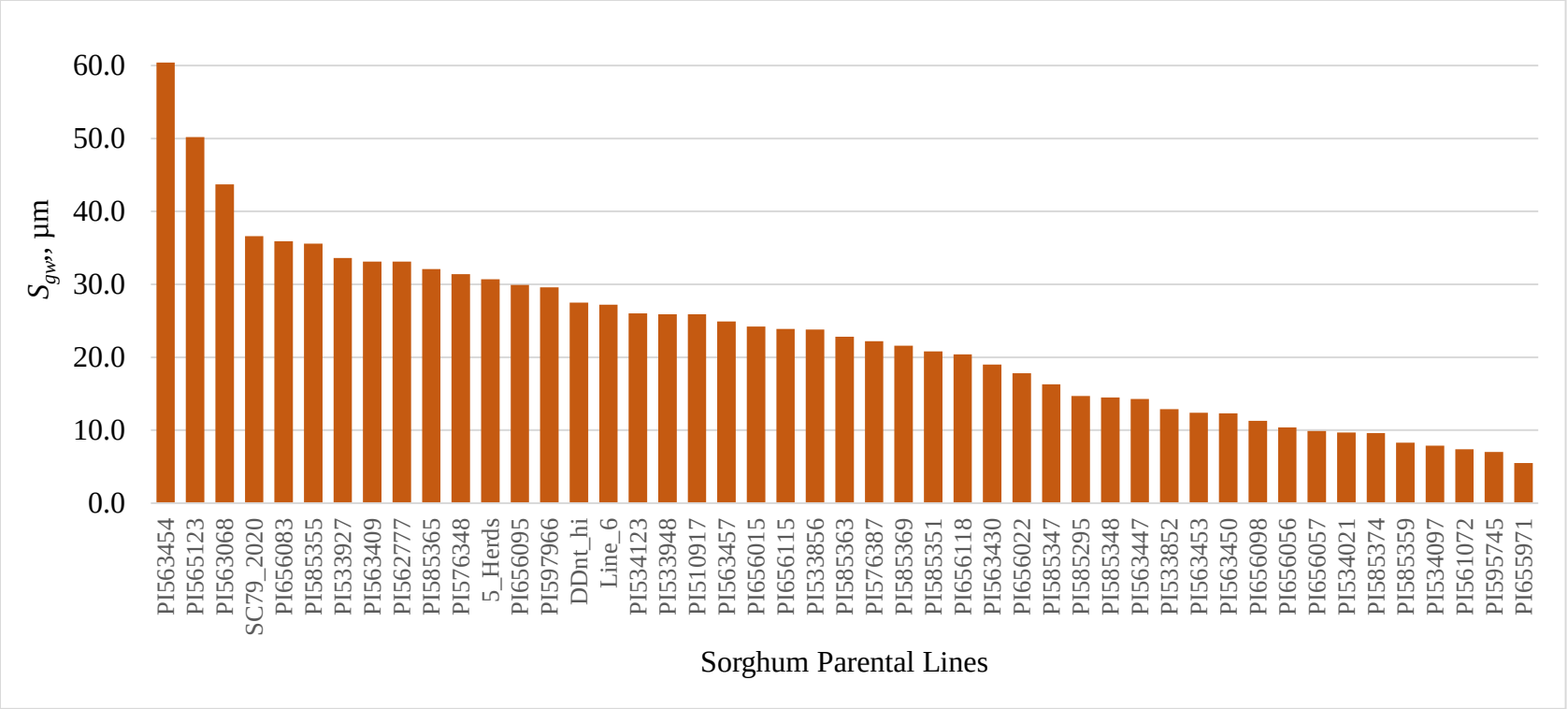

Figure 2.26. Geometric standard deviation (S_{gw}) of particle size from sorghum parental lines.

Since S_{gw} measurement was only done in duplicate, data obtained was analyzed using descriptive statistic in SAS.

Number of observation (n)=48; average value of geometric (mean)=22.86 µm; standard deviation= 11.88, the greatest geometric standard deviation (maximum)=60.40 µm; the least geometric standard deviation (minimum)=5.50 µm.

Table 2.1. Pearson correlation coefficient test between starch, protein, amino acids content, and particle size and variables measured

Parameters	Starch	Protein	Lysine	Methionine	Systeine	d_{gw}	S_{gw}
K	nc	r=0.64; P<0.01	r=-0.48; P<0.01	nc	nc	nc	nc
t½	nc	nc	nc	nc	nc	r=0.37; P<0.05	r=0.41; P<0.01
r	nc	nc	nc	nc	nc	r=-0.30; P<0.05	r=-0.34; P<0.05
pH	r=0.36; P<0.05	r=-0.47; P<0.01	r=0.37;P<0.05	nc	nc	nc	nc
Propionate	nc	nc	nc	nc	nc	r=-0.37; P<0.05	r=-0.39; P<0.01
A:P	nc	nc	nc	r=-0.44;P<0.01	r=0.50;P<0.01	nc	nc
IVDMD	nc	r=0.57; P<0.01	r=0.39;P<0.05	nc	nc	r=-0.27; P<0.10	nc
ISDMD	nc	nc	nc	nc	nc	r=-0.44; P<0.01	r=-0.39; P<0.01
T_o	nc	nc	nc	nc	nc	r=0.41; P<0.01	r=0.38; P<0.01
T_p	nc	nc	nc	nc	nc	nc	r=0.25; P<0.10
T_c	nc	nc	nc	nc	nc	r=0.32; P<0.05	r=0.34; P<0.05
ΔH_{gel}	nc	nc	nc	nc	nc	r=-0.11; P<0.05	nc

nc = no correlation

Chapter 3 - *greatOplus* (extruded blend of flaxseed and *Nannochloropsis oculata* biomass) Improves Finishing Cattle Performance and Carcass Characteristics

3.1. Abstract

Omega-3 fatty acid supplements such as flaxseed and microalgae in beef cattle diets have shown promising results for increasing the omega-3 content of beef, particularly ALA and EPA. This study aimed to investigate effects of supplementing an extruded blend of flaxseed and *Nannochloropsis oculata* microalgae (*greatOplus*, GOP) as a source of omega-3 fatty acids to determine impact on animal performance and carcass characteristics of finishing steers. Cattle fed GOP had greater (P<0.05) dry matter intake (DMI) and average daily gain (ADG) compared to cattle fed the control diet (CON), but feed:gain (F:G) was not affected by treatment (P>0.10). Cattle fed GOP had greater hot carcass weight (HCW) compared to those fed CON (422.7 vs 409.5 kg; P<0.01) with greater 12th rib backfat (1.55 vs 1.47 cm; P<0.02) and greater USDA yield grades (2.91 vs 2.75; P<0.01). GOP treatment did not affect Prime, Premium Choice, Low Choice, Select, and Sub-select grades (P>0.10). Marbling score (488 vs 491), longissimus muscle area (81.6 vs 81,0 cm^2), and liver abscess incidence (13.9 vs 16.3%) for CON and GOP, respectively, were unaffected by treatment (P>0.10). Carcass values were calculated using base prices, premiums, and discounts published by USDA during the week of harvest and were greater for cattle-fed GOP than cattle-fed CON diet ($2,122 vs. $2,059; P<0.01). Including *greatOplus* at 10% of the diet dry matter improved cattle performance, largely as the result of its impact on dry matter intake and average daily gain.

3.2. Introduction

Beef is crucial in human nutrition, providing high-quality protein and essential micronutrients (Wu, 2021; Pereira & Vicente, 2013). It is a rich source of highly bioavailable iron, zinc, vitamin B12, and other B vitamins (Agarwal & Fulgoni, 2022; Wyness, 2016). Beef also contains compounds like taurine, creatine, and carnosine, which are absent or negligible in plant-based food (Wu, 2021). Beef consumption contributes significantly to daily nutrient intake, with lean fresh beef being particularly efficient in providing nutrients per calorie (Agarwal & Fulgoni, 2022). When consumed in moderation as part of a balanced diet, beef can contribute to optimal growth, development, and health throughout the life span (Murimi, 2022; Miles & Caswell, 2008).

Recently, concern about beef consumption has increased as people consider beef fat as the major stimulant of cardiovascular-related diseases (CVD Briggs et al., 2017) and certain types of cancer (Kolonel, 2001; Fergusson, 2010). Research on dietary fats and CVD has evolved over time. While earlier studies linked increased overall fat intake to CVD risk, recent evidence suggests that the type of fat is critical (Glassner, 2018; Fergusson, 2010), where saturated and trans fats have been associated with increased CVD risk (Erkkilä et al., 2008; Zhu et al., 2019). While trans fatty acids generated from partial biohydrogenation of unsaturated fats may possess negative effect (Bhardwaj et al., 2011), trans fatty acids produced from ruminal fermentation, such as conjugated linoleic acid (CLA), benefits human health (Whigham et al., 2000). Replacing saturated fats with polyunsaturated fats as part of an overall healthy dietary pattern has been shown to reduce CVD risk, as recommended by the American Heart Association (Sacks et al., 2017).

Demand for beef with enriched levels of omega-3 fatty acids has increased significantly as the interest in dietary components as possible protection for various fat-related diseases increases (Scollan et al., 2014). Omega 3 fatty acids, particularly EPA and DHA, have been associated with numerous health benefits (Corino et al., 2022). These essential fatty acids, primarily found in fish and fish oil supplement, plays crucial roles in cardiovascular health, reducing morbidity and mortality (Yashodhara et al., 2009; Calder &Yaqoob, 2009; Mori, 2017). Enhancing omega-3 content in beef has been of interest to the producers since beef consumers have been targeted daily with destructive commentaries concerning the negative contribution of beef to human health disorders (Ponnampalam et al., 2021). Increasing omega-3 deposition in meat has been focusing on the search for feedstuff able to modify the fatty acids profile in beef (Corino et al., 2022) since the metabolism of dietary fat in ruminants is a complex mechanism (Jenkins, 1994). Meat produced from animals fed pasture-based diets contains greater omega-3 fatty acids than animals fed grain-based diets (Duckett et al., 2013; van Elswyk & McNeill, 2014; Razminowicz et al., 2008). Thus, for beef cattle under a concentrate-based feeding program, it is important to identify proper feed ingredients or supplements to increase beef's omega-3 long-chain fatty acids concentration.

Omega-3 fatty acid supplementation in beef cattle diets has shown promising results in improving meat quality and nutritional value. Dietary supplementation with omega-3 sources such as flaxseed and microalgae can significantly increase the omega-3 content in beef, particularly EPA and DHA (Kucuk et al., 2023; Demeda et al., 2020). Flaxseed contains a relatively high proportion of alpha-linolenic acid, which is an essential omega-3 fatty acid, and has been successful in promoting the health and performance of cattle in previous research (Pouzo et al., 2015; Kim et al., 2009). Alpha-linolenic acid is a precursor for the formation of the

longer polyunsaturated fat, EPA, which in turn serves as a precursor for the formation of several important reproductive hormones and immune compounds that regulate inflammatory responses in animals (Moallem, 2018; Liermann et al., 2021; Fabjanowska et al., 2023). However, the rate of conversion of linolenic acid to EPA is minimal. Some species of marine algae, including Nannochloropsis species, are known to produce a substantial amount of EPA. However, the challenge remains in protecting these fatty acids from ruminal biohydrogenation to maximize their incorporation into beef tissues (Scollan et al., 2014; Dewhurst et al., 2013).

Several techniques have been developed to improve essential fatty acid retention in the ruminal environment and increase their absorption in the small intestine (Gonthier et al., 2004). Extrusion is a method to protect essential fatty acids from ruminal biohydrogenation by applying pressure and temperature (Dewhurst et al., 2013). Protection includes a physical barrier to avoid microbial attachment, modification of fatty acid profile, and formation of complexes between fatty acids and protein or other compounds which are more resistant to ruminal biohydrogenation (Mustafa et al., 2003; Kennelly, 1996; Schingoethe et al., 1996). Thus, supplementing protected omega-3 long-chain fatty acids can be expected to increase omega-3 deposition in meat. This study aimed to investigate the effect of supplementing extruded blend of flaxseed and *Nannochloropsis oculata* microalgae (*greatOplus*) as a source of omega-3 fatty acids on animal performance and fatty acid profile of beef of finishing steers.

3.3. Materials and Methods

3.3.1. Animals and diets

The animals used in this study were yearling steers (n=700; 374 ± 8.2 kg initial body weight) and were blocked by initial body weight and assigned randomly, within the block, to 28 feedlot pens containing 25 animals/pen. Cattle were identified with uniquely numbered ear tags

and RFID tags, vaccinated against viral and clostridial pathogens, and treated for internal and external parasites (Bovishield Gold 5, Ultrabac 7 Somubac, and Dectomax injectable; Zoetis Animal Health), and implanted with a combination implant of trenbolone acetate and oestradiol (Component TE-200; Elanco USA). The control diet (CON) consisted of 58.3% steam-flaked corn, 20% wet corn gluten feed, 2.56% soybean meal, and 4.17% vitamin/mineral/feed additive premix. For the *greatOplus* (GOP) diet, a portion of the corn and all the soybean meal were replaced with 10% GOP (dry basis) to create isonitrogenous diets. Cattle were fed once daily for *ad libitum* intake. After 175 days on feed, animals were weighed and transported to a commercial abattoir for harvest. Animal performance measurements included average daily gain (ADG), dry matter intake (DMI), and feed:gain (F:G).

Ingredients and nutrition composition of diet in this study are presented in Table 3.1 and fatty acid composition of *greatOplus* supplement is presented in Table 3.2.

3.3.2. Carcass evaluation and liver abscess incidence and severity

Hot carcass weight (HCW) and incidence of abscessed livers were assessed on the day of harvest, and marbling score, 12th rib fat thickness, longissimus muscle area, and USDA yield and quality grades (USDA, 2017; USDA, 2011; USDA, 2006) were determined following 48 hours of refrigeration. Livers were characterized concerning incidence and severity using the scoring system described by Brown et al. (1975), where a score of 0 is assigned to livers with no abscess, A^- to a mild abscessed liver (one or two small abscesses or with inactive scars), A^0 to a moderate abscessed liver (with one or two large abscesses or multiple small abscesses), or A^+ to a severely abscessed liver, defined as containing various large abscesses, with inflammation surrounding the abscess, and often seen adhered to adjacent tissue. Carcass values in this study

were calculated using base prices, premiums, and discounts published by USDA (https://www.ams.usda.gov/mnreports/lsddcbs.pdf) during the week of harvest.

3.3.3. Fatty acid analysis of meat

Fatty acid compositions were analyzed based on the method by O'Fallon et al. (2007). **FAME synthesis**. Twenty-eight samples of chuck and round cuts from both CON and GOP treatments were ground at room temperature using a meat grinder for 3-5 minutes. Each sample was prepared in two duplicates. As much as 1.0 g wet sample was placed into a 16 x 125 mm screw-cap Pyrex culture tube to which 1.0 mL of the C13:0 internal standard (0.5 mg of C13:0/mL of MeOH), 0.7 mL of 10N KOH in water, and 5.3 mL of MeOH were added. All tubes were incubated in a 55°C water bath for 1.5 h and shaken for 5 seconds every 20 minutes to permeate, dissolve, and hydrolyze the samples properly. After incubation, tubes were transferred to a cold tap water bath container for 10 minutes. After cooling below room temperature, 0.58 mL of 24N H_2SO_4 was added. The tubes were mixed by inversion, and with the presence of precipitated K_2SO_4, the tubes were incubated again in a 55°C water bath for another 1.5 h and then vortexed for 5 seconds every 20 minutes. After the second incubation, the fatty acid methyl esters (FAME) synthesis was completed, and tubes were cooled in a cold tap water bath container. Into each tube, 3 mL of hexane was added, and tubes were vortex-mixed for 2 minutes. Tubes were centrifuged at 1500 x g for 5 minutes. The hexane layer containing the FAME on the top layer of the solution in the tubes was then transferred to the gas chromatography (GC) vials. The vials were then capped and placed at -20 °C until GC analysis. **GC analysis**. Fatty acid methyl esters were quantified using an Agilent 7890 gas chromatograph (Agilent Technologies; Santa Clara, California) equipped with Supelco SP-2560 capillary columns (100 m long x 0.25 mm internal diameter x 0.2 μm film thickness; Sigma-Adrich, St.

Louis, MO) for dual simultaneous injections. Inlet temperatures were 240 ºC, injection volumes were 1µL with a 200:1 split ratio; initial oven temperature was 100 ºC with a 2-minute hold, followed by a ramp rate of 4ºC/minute to a final temperature of 240 ºC followed by a 2-minute hold. Hydrogen was used as the carrier gas with a constant column flow rate of 2.2 mL/minute. Detector temperatures were 280 ºC. Tridecanoic acid (Product No. 91988, Sigma-Adrich) was added to samples as an internal standard, and Supelco 37 Component FAME Mix (Product No. CRM47885, Sigma-Adrich) was used as the external standard.

3.3.4. Statistical analyses

Data were analyzed as mixed models using the GLIMMIX procedure of SAS (ver. 9.4), with fixed effects of diet, location of grind (i.e. chuck or round), and their interaction. Pen was the random effect. Dependent variables include animal performances, carcass characteristics, USDA carcass quality grade, death loss and condemned carcass, liver abscess incidence, and fatty acid composition of meat, and carcass value.

3.4. Results

3.4.1. Animal performance

Cattle fed GOP had greater ($P<0.05$) DMI and ADG compared to cattle fed the CON diet. However, feed:gain (F:G) was not affected by the treatment ($P>0.10$) (Table 3.3).

3.4.2. Carcass characteristics

Cattle feed GOP had greater HCW ($P<0.01$) and 12th rib fat thickness ($P<0.02$) compared to those fed CON treatment. However, longissimus muscle area and marbling score were unaffected by the treatment ($P>0.10$) (Table 3.4).

3.4.3. USDA carcass quality grade

Cattle fed GOP had greater USDA yield grades (P<0.01) compared to those fed CON treatment. However, GOP treatment did not affect Prime, Premium Choice, Low Choice, Select, and Sub-select grades (P>0.10) (Table 3.5)

3.4.4. Death loss and condemned carcass

Feeding GOP resulted in less death loss and condemned carcasses of 1.00% compared to CON treatment with 1.75%. However, given the low incidence it is not possible to determine if this is a meaningful decrease.

3.4.5. Liver abscess incidence

Liver abscess incidence was not affected by the GOP treatment (P>0.10) (Table 3.6).

3.4.6. Carcass values

Carcass values of animals from the GOP treatment was greater (P<0.01; SEM=27.99) compared to those in the CON treatment ($2,122 and $2,059, per carcass, respectively).

3.4.7. Fatty acid composition of beef

There were effects (P<0.01) of GOP treatment on C18:2 and C18:3(ω-3) concentrations in meat samples (Table 3.7). However, GOP treatment also decreased (P<0.01) the C15:0, C16:0, C17:0, C17:1, C18:2(ω-6), and C23:0 concentrations. Interactions (P<0.01) between treatment and carcass location were observed on C18:2 and C18:3(ω-3) concentration. Most fatty acids observed were significantly (P<0.01) affected by the carcass location (Table 3.7).

3.5. Discussion

Supplementing omega-3 long-chain fatty acids (LCFA) in beef cattle diets is promising to produce beef with enriched omega-3 FA content (Kucuk et al., 2003; Demeda et al., 2020). Omega-3 has currently drawn significant attention from people concerning a healthier lifestyle

due to the fact that omega-3 possesses a biological function in reducing risk of CVD (Briggs et al., 2017) and certain types of cancer (Kolonel, 2001; Fergusson, 2010). Omega-3 supplement does not only provide health benefits for humans through beef rich in omega-3 but also benefit the immune system and inflammatory protection for animals (Moallem, 2018; Liermann et al., 2021; Fabjanowska et al., 2023). Effect of omega-3 supplement on animal performance has been reported by several studies. Puozo et al. (2015) conducted a study on the effect of corn grain and flaxseed as a source of omega-3 supplement on finishing beef cattle where flaxseed was included up to 0.250% of body weight (BW). After 70 days of feeding, they found a significant effect of treatment observed on DMI but no effect on ADG. Another study was conducted by Wistuba et al. (2006) using fish oil as source of omega-3 up to 3% of the diet for 70 days of treatment period. They reported decrease in DMI but no effect on ADG or F:G ratio. Furthermore, Kim et al. (2009) demonstrated a decrease in DMI for animals fed whole flaxseed up to 15% but significantly improved feed conversion ratio.

Result from the current study demonstrated that GOP supplement improved ADG and DMI which could be to the improved ability of the animals fed GOP supplement to maintain feed intake during the study. Fluctuated temperature which tended to be hot during the study may affect dry matter intake of the animals since cattle are susceptible to heat stress, which can impair their productivity and welfare (O'Brien et al., 2010; Brown-Brandl et al., 2006). However, the presence of omega-3 fatty acids in GOP supplement may help to mitigate heat stress by enhancing antioxidant status and reducing oxidative stress (Liermann et al., 2021; Lima et al., 2014). Effect of omega-3 supplement on animal performance has also been reported by Pouzo et al. (2015) who demonstrated improved DMI of steers as affected by flaxseed supplement up to 0.250% of body weight (BW). In addition, Kim et al. (2009) demonstrated a decrease in DMI for

animals fed whole flaxseed up to 15% of the diet DM but significantly improved feed conversion ratio of Hanwoo steers. Another study by Witsuba et al. (2006) reported that using fish oil as source of omega-3 decreased DMI but no effect on ADG and G:F ratio.

The positive effect of GOP supplements on animal performance in the present study could also be due to the supplement's processing method, which uses the extrusion technique. Extruded GOP improve propensity of omega-3 to escape ruminal biohydrogenation and increase intestinal flow and absorption (Kennely, 1996). Extrusion is a method to protect essential fatty acids and other dietary nutrients from ruminal biohydrogenation and degradation with the application of certain pressure and temperature (Dewhurst et al., 2013; Mustafa et al., 2003). Protection includes physical barrier to avoid microbial attachment, modification of fatty acid profile, and formation of complexes between fatty acids and protein or other compounds which are more resistant from ruminal biohydrogenation (Mustafa et al., 2003; Kennelly, 1996; Schingoethe et al., 1996). When comparing an extruded blend of flaxseed and *Nannochloropsis oculata* microalgae (*greatOplus*) supplement with control treatment, Thorn & Drouillard (2024) observed a significant increase of ruminal outflows of total fatty acids and α-linolenic acid (ALA), the precursor for EPA and DHA (Cholewski et al., 2018). This finding indicated that extrusion effectively protects the essential fatty acids from ruminal biohydrogenation (Kennely, 1996). Thus, the increase of nutrients absorbed in the intestine may lead to an increase in animal performance.

Since consumer preference is the final target of the beef cattle industry, it is crucial to ensure that the meat characteristics and quality marketed fulfill the consumer's needs. Including GOP in the present study significantly increases hot carcass weight (HCW) but has no effect on the rib eye area and marbling score. Greater HCW in GOP treatment might correspond to the

ADG, which is also significantly greater in GOP treatment. Positive correlation between ADG, HCW, and quality grade (QG) has been reported by Knox (1957) and Reinhardt et al. (2012) as they conducted studies to investigate relationships between feedlot health, animal performance, and carcass characteristics. In addition, when demonstrating a correlation between live weight and carcass weight, Orme et al. (1959) further highlighted a strong correlation between live weight and rib eye area.

The 12th rib fat thickness from animals fed GOP treatment was greater than CON treatment which suggested that omega-3 supplements might affect the carcass quality. Kim et al. (2009) investigated the effect of the whole flaxseed supplement up to 15% of the diet DM on carcass characteristics of Hanwoo steers. They reported that 12th rib fat thickness and marbling score in beef treated with whole flaxseed were greater than in control animals. The marbling score from the present study and previous studies indicated that flaxseed supplement may improve carcass quality (Maddock et al., 2006). In a study comparing the effect of different flax processing on beef carcass characteristics from animals fed a concentrate-based finishing diet, Maddock et al. (2006) reported a marbling score from 456 to 478 for flaxseed supplements and 423 for control. From different regime of feeding system, Kronberg et al. (2011) observed a non-significant effect of flaxseed supplement up to 0.20% of BW on carcass characteristics of beef from steers finished on grasslands. They observed a marbling score of 367 and 366 for beef from animal-fed flaxseed and control, respectively. Lower marbling scores observed in beef from animals finished with a grasslands-based diet than in a concentrate-based diet suggests that the feeding system could affect the effect of fat supplements on carcass characteristics (Duckett et al., 2013; Kronberg et al., 2007; van Elswyk & McNeill, 2014).

Cattle fed GOP had greater USDA yield grades compared to those fed CON treatment. However, GOP treatment did not affect Prime, Premium Choice, Low Choice, Select, and Sub-select grades. Days on feed in the present study might affect the carcass quality since the animals were fed for 175 days which increased the propensity for fat deposition and altered fatty acids profile (Rincker et al., 2008; Noci et al., 2005; Wood et al., 2008). The effect of fat and ground flaxseed supplements on USDA quality grade has also been investigated by Labrune et al. (2008), who reported no differences in the percentage of carcass grading USDA choice and KPH. Wistuba et al. (2006) demonstrated no effect in quality grade, KPH, and yield grade of beef from cattle-supplemented fish oil up to 3% of the diet DM. Differences in the USDA quality grade from different studies might be due to the differences in the physiological status of the animal, as the carcass quality might be affected by the physiological response of the animal to the diet (Owens et al., 1993; Park et al., 2018)

Supplementing ruminant diets with fat can influence the physiological processes and alter the fatty acid composition of meat and milk products (Hess et al., 2008). The GOP treatment in the present study increased C18:2 and C18:3(ω-3), but decreased C15:0, C16:0, C17:0, C17:1, C18:2(ω-6), and C23:0. Greater concentration C18:3(ω-3) in GOP than CON treatment was expected since GOP is an extruded blend of flaxseed and microalgae *Nannochlopsis oculata* which high in α-linolenic acid. The C18:3(ω-3) deposition in meat might also be due to the extrusion process, which protected the omega-3 fatty supplement from ruminal biohydrogenation (Dewhurst et al., 2013). The ability of GOP supplement to escape ruminal degradation increases the availability of the omega-3 supplement for intestinal digestion and absorption, and eventually omega-3 deposition in meat (Kennely, 1996). Interestingly, concentration of C18:2(ω-6) in the present study decreased in GOP treatment which means that ratio of omega-6 to omega-3 also

decreased. A lower omega-6/omega-3 ratio in meat is recommended since it provides biological function and benefits human health (Simopoulos, 2002; Moallem, 2018).

The role of omega-3 fatty acids as anti-inflammatory substances might be further involved in reducing the number of dead animals and condemned carcasses. Number of dead animals and condemned carcass were numerically less for cattle fed GOP supplement compared to the CON treatment, but given the low incidence it is not possible to determine if this is a meaningful decrease. The finishing period in this study was conducted during the summer seasons, and omega-3 may be involved in the body temperature regulation of the animals. Cattle are susceptible to heat stress, which can impair their productivity and welfare (O'Brien et al., 2010; Brown-Brandl et al., 2006). Omega-3 fatty acids can mitigate heat stress by enhancing antioxidant status and reducing oxidative stress (Liermann et al., 2021; Lima et al., 2014). The omega-3 fatty acid helps cattle cope better in hot conditions by improving their thermoregulatory mechanisms, maintaining feed intake, and reducing the effects of heat stress on body temperature (Fabjanowska et al., 2023; Teama & El-Tarabany, 2016). During the study, the temperature differences between the eye ball (similar with core body temperature) and ear tip were used as an indicator of blood flow to peripheral tissues, which is an important mechanism for controlling body temperature during environmental extremes. The temperature differential observed was approximately 0.3°C less for cattle fed the GOP diet, suggesting improved peripheral blood perfusion and greater capacity to regulate body temperature. Proper body temperature regulation enables animals to maintain their feeding behavior, which eventually positively affects their performances (Lima et al., 2014).

Combination of energy source, omega-3 fatty acids content, and anti-inflammatory properties exhibited by the GOP treatment in this study finally contribute to the carcass price

which was higher in GOP than CON treatment. There was a $63 per carcass increase in value for cattle fed GOP compared to the CON treatment. In this study, cost benefits were offset by a similar magnitude increase in cost of the GOP diet, suggesting that increased market value of the meat must be realized to make this profitable for producers.

3.6. Conclusion

Attempts to produce healthier beef products have directed many studies to find naturally available omega-3 fatty acids to be included in beef cattle finishing diets. However, use of omega-3 fatty acids in ruminant diets is limited due to the biohydrogenation process, leading to limited omega-3 deposition in meat. The extrusion process may protect the omega-3 from ruminal biohydrogenation, and an increase in meat's omega-3 fatty acid content can be expected. The *greatOplus,* an extruded blend of flaxseed and *Nannochloropsis oculata* supplement is combination of α-linolenic acid and EPA sources which can provide substantial amounts of EPA in the diet. This study demonstrated that including *greatOplus* at 10% of the diet dry matter improved cattle performance, largely due to its impact on dry matter intake and average daily gain.

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Table 3.1. Ingredients and nutritional composition of control diet (CON) and diet supplemented with *greatOplus* (GOP)

Item	CON	GOP
Diets, %DM		
Steam-flaked corn	58.27	50.84
Corn silage	15.00	15.00
Sweet bran	20.00	20.00
Dehulled soybean meal	2.57	-
greatOplus[1]	-	10.00
Supplement[2]	4.17	4.17
Nutrients, %		
Dry matter	65.41	66.1
Crude protein	14.00	14.00
NDF	16.05	16.28
ADF	8.15	8.36
Ether extract	3.18	5.19
Calcium	0.68	0.70
Phosphorus	0.38	0.42
Total C18:3(ω-3)	0.43	1.38

[1]Extruded flaxseed-algae blend supplement

[2]Supplement was formulated to provide 2,205 IU/kg of vitamin A; 10 mg/kg of copper; 30 mg/kg of zinc; 20 mg/kg of manganese; 0.50 mg/kg iodine; 0.1 mg/kg of selenium; and 0.15 mg/kg of cobalt.

Table 3.2. Composition of *greatOplus* supplement (g/kg DM)

Item	Composition
Crude protein	211.20
Net energy for maintenance, Mcal/kg	2.27
Net energy for gain, Mcal/kg	1.54
Neutral detergent fiber	319.80
Omega-3 fatty acid	94.84
Ash	60.92
Ether extract	187.90
Fatty acids*	
C14:0	0.15
C15:0	0.12
C16:0	14.19
C16:1 (ω-7)	0.19
C17:0	0.18
C18:0	7.70
C18:1 (ω-9t)	0.10
C18:1 (ω-7t)	0.03
C18:1 (ω-9)	42.42
C18:1 (ω-7)	1.51
C18:2 (ω-6)	39.90
C20:0	0.39
C18:3 (ω-3)	94.71
C20:1 (ω-9)	0.49
CLA 9c,11t (ω-7)	0.06
C20:2 (ω-6)	0.11
C22:0	0.44
C20:3 (ω-3)	0.13
C24:0	0.38
Total fatty acid	203.50

*Fatty acid nomenclature was used to identify each fatty acid. The first number in the name is the number of carbons, followed by the number of double bonds in the fatty acid.

Table 3.3. Effect of *greatOplus* treatment on animal performance.

Item	CON	GOP	SEM	P-value
ADG, kg/d	1.78	1.85	0.03	<0.01
DMI, kg/d	10.41	10.92	0.36	0.03
Feed:gain	0.17	0.16	0.07	0.51

Table 3.4. Effect of *greatOplus* treatment on carcass characteristics.

Item	CON	GOP	SEM	P-value
HCW, kg	409.5	422.7	12.27	<0.01
Longissimus muscle area, cm^2	81.6	81.0	0.22	0.27
Marbling score[1]	488	491	7.01	0.61
12^{th} rib fat thickness, cm	1.47	1.55	0.02	<0.02

[1]Marbling score assesses the amount and distribution of fat within the ribeye muscle between the 12^{th} and 13^{th} ribs of the carcass

Table 3.5. Effect of *greatOplus* treatment on USDA carcass quality grade.

Item	CON	GOP	SEM	P-value
Prime, %	2.6	3.7	0.97	0.410
Premium Choice, %	36.0	34.5	3.69	0.676
Low Choice, %	44.4	49.1	3.41	0.219
Select, %	14.6	11.5	1.91	0.223
Sub-select, %[1]	1.5	0.9	0.62	0.457
Yield grade	2.75[a]	2.91[b]	0.66	<0.01

[1]Sub-select is a lower grade than Select grade which may have less marbling, less tenderness, or a slightly different texture or flavor compared to Select grade.

Table 3.6. Effect of *greatOplus* treatment on incidence of abscessed liver.

Abscess severity	CON	GOP	SEM	P-value
A^-	1.8	3.4	0.99	0.17
A^0	5.8	6.6	1.30	0.68
A^+	6.3	6.3	1.25	0.26
Total	13.9	16.3		

Table 3.7. Effect of *greatOplus* treatment on fatty acid composition of chuck and round locations of beef.

Fatty acid (µg/g)	Chuck		Round		SEM	P-value		
	CON	GOP	CON	GOP		Trt	Cut	Trt x Cut
C13:0	1,553	1,559	1,574	1,561	52.9	0.94	0.82	0.86
C14:0	9,627	9,090	5,068	4,587	367.8	0.09	<0.01	0.93
C14:1	2,128	2,219	1,358	1,360	126.5	0.55	<0.01	0.57
C15:0	1,750	1,357	975	826	71.2	<0.01	<0.01	0.54
C16:0	87,626	76,382	43,670	38,869	3,511.5	<0.01	<0.01	0.27
C16:1	12,381	11,853	7,511	6,851	486.9	0.17	<0.01	0.88
C17:0	5,230	4,051	2,544	1,990	217.1	<0.01	<0.01	0.06
C17:1	3,363	2,790	2,132	1,663	129.7	<0.01	<0.01	0.63
C18:0	50,784	47,277	21,674	20,754	2,091.2	0.24	<0.01	0.49
C18:1	4,242	3,055	1,515	1,941	539.5	0.47	<0.01	0.13
C18:1*cis*-9	114,614	115,955	65,244	60,876	4,269.9	0.67	<0.01	0.42
C18:2	613[a]	1,729[b]	523[a]	625[a]	177.9	<0.01	<0.01	<0.01
C18:2 (ω-6)	6,728	2,742	6,600	3,550	583.4	<0.01	0.54	0.39
C18:3 (ω-3)	774[a]	2,456[b]	502[c]	1,277[d]	78.2	<0.01	<0.01	<0.01
C20:0	496	454	-	-	28.94	0.3	-	-
C20:1	663	740	430	403	44.8	0.49	<0.01	0.15
C20:3	411	480	386	358	65.8	0.64	0.12	0.3
C21:0	1,257	1,373	763	771	66.6	0.26	<0.01	0.33
C23:0	1,181	1,035	1,249	1,101	39.8	<0.01	0.07	0.97

[a,b,c,d] means in the same row without a common superscript are different (P<0.01)