In vitro screening of commercial sorghum hybrids and omega-3 supplementation in Holstein steers

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Abstract

Sorghum hybrids were evaluated for their susceptibility to both *in vitro* and *in situ* microbial digestion. Sorghum hybrids were sourced from institutional and commercial sources and 39 hybrids were evaluated. Grains were analyzed using near-infrared spectroscopy (NIR) to estimate each grain's protein and starch content using an Perten DA 7250 spectrometer (Perten Instruments, Springfield, IL, USA). Grains were split into subsamples and processed by grinding, reconstitution followed by ensiling (RE), and steam-flaking. Each processed grain was used as substrate and was combined with a buffered ruminal fluid solution, before being capped with an Ankom IR gas measuring device (Ankom Technology, Macedon, NY). *In vitro* cultures using ground sorghum as substrate were incubated for 28.25 hours, whereas *in vitro* cultures using RE and SF grain were allowed to incubate for 30 hours. Cumulative gas production, volatile fatty acids concentrations from *in vitro* cultures, and *in vitro* dry matter disappearance were measured and used to assess how well microbes digested each grain. *In situ* dry matter disappearance (ISDMD) was measured for ground and RE processed grain to provide an additional assessment of ruminal microbial digestion of the grain. *In vitro* gas production data were analyzed using non-linear models to estimate four gas production parameters: maximum cumulative gas production (*K*), time to reach half of the cumulative gas production ($t_{1/2}$), rate of gas production (*r*) , and maximum rate of gas production (*m*). Methane yield was estimated from each *in vitro* culture, using the VFA concentrations from the *in vitro* cultures solution using the formula Methane Yield (g/kg DM) = 4.08×(Acetate (mol/100 mol)/Propionate (mol/100 mol) + 7.05 (Williams et al., 2019). Each grain's estimated protein and starch content were correlated to *K*, $t_{1/2}$, *r*, *m*, VFA concentrations from *in vitro* cultures, methane yield, IVDMD and ISDMD. Starch and protein content correlated with *K*, *m*, and methane yield in ground sorghum. Starch

and protein content also correlated with *m* and ISDMD in RE sorghum, while starch and protein content correlated with *K* in steam-flaked sorghum. These results indicated that there was a noteworthy variation in susceptibilities of sorghum grain to *in vitro* microbial digestion; however, more extensive processing of the grain (RE or steam-flaking) decreased the differences among the grain with respect to *in vitro* microbial digestion.

A backgrounding study using an extruded blend of flaxseed and microalgae product (FAB; greatO*plus*), was fed to 11 ruminally and duodenally fistulated Holstein steers to assess ruminal microbial modification of the fatty acids present in FAB, as well as their post-ruminal disappearance. Steers were housed in a facility equipped with an Insentec feed and water monitoring system (Hokofarm;, Emmeloord, the Netherlands). A cross over design was utilized and treatments consisted of a control diet without the FAB and a treatment diet with FAB included at 10% of the diet dry matter. Dry matter intake by day was different ($P<0.001$); steers consuming the FAB had greater feed intakes on day 2, 6 and 7 compared to the steers fed the control diet. Water consumption was not different ($P>0.10$). Ruminal acetate concentrations were not different between treatments ($P>0.10$). Ruminal propionate concentrations were affected by hour ($P=0.026$); steers fed the FAB supplement, had greater ruminal propionate concentrations at hour 8 ($P<0.05$) and tended to have greater propionate concentrations at hour 0 ($0.05<P<0.10$). Ruminal butyrate concentrations were also affected by hour ($P=0.036$); steers fed the FAB supplement tended to have greater ruminal butyrate concentrations at hours 8 and 10 ($0.05<P<0.10$). Steers fed the control diet had greater ruminal butyrate concentrations at hours 18 and 24 than steers fed FAB ($P<0.05$). Ruminal pH and ammonia concentration were not different between treatments ($P>0.10$). Duodenal flow of several fatty acids, (g/d) were greater for steers fed the FAB and in particular, the ruminal flow of α-linolenic acid (ALA) was four

times greater for steers fed the FAB supplement compared to steers fed the control diet (6.3 g/d vs 1.6 g/d; P=0.001) The coefficient of intestinal disappearance of each fatty acid was not different between treatments for the vast majority of the fatty acids (P>0.05), except for ALA. There was a reduction in the coefficient of intestinal disappearance of ALA for steers fed the FAB supplement compared to steers fed the control diet (0.64 vs 0.41; P=0.039).

Feeding this FAB supplement may increase the ruminal flow of omega-3 fatty acids to the small intestine, allowing the potential for increased absorption of these essential fatty acids; however, extensive biohydrogenation will occur which leads to a small proportion of dietary omega-3 fatty acids reaching the small intestine, compared to the amount of omega-3 fatty acids in the basal diet.

Keywords: GreatO*plus*, *In vitro*, *In situ*, Sorghum, Omega-3, and α-linolenic acid

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1 Literature Review: Sorghum starch chemistry and its impact on processing for beef and dairy cattle

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1.1 Abstract

Water usage in the High Plains region will need to decrease due to decline of water resources. Sorghum is grown throughout this region but plays a relatively minor role for beef and dairy cattle rations compared to corn. Sorghum grain chemistry can vary, with some cultivars having high amylopectin, high protein, or high concentrations of polyphenols which give it a variety of colors (Taylor and Emmambux, 2008). These characteristics can have positive or negative effects on functionality in cattle diets. Waxy or high amylopectin cultivars of sorghum may have greater digestion in the rumen and small intestines when compared to normal sorghum cultivars (Streeter et al., 1990b). High protein sorghum cultivars have been associated with greater concentrations of kafirins, which have been observed to have negative effects on starch digestion due to disulfide bond formation when exposed to heat (Rooney and Pflugfelder, 1986; Rom et al., 1992). Polyphenols in sorghum, including flavonoids, can give cattle greater antioxidant capacity when fed sorghum silage without affecting milk yield (Khosravi et al., 2018). Dry rolling, ensiling and micronizing are some processing methods utilized in processing sorghum for cattle diets but steam flaking is the most common method utilized when incorporating sorghum in finishing cattle diets (Richards and Hicks, 2007). *In vitro* screening techniques have been utilized to identify cultivars with traits that have superior starch digestion when incubated with ruminal fluid (Wester et al., 1992). Our goal was to utilize an *in vitro* screening procedure to identify sorghum hybrids with traits that have potential for use in finishing cattle diets.

Key words: *in vitro*, grain chemistry, sorghum, steam flaking

1.2 Introduction

Protein consumption is increasing around the world. It is estimated that by 2030, global demand for meat and milk products will be 376 million and 874 million tonnes respectively (Ronda et al., 2019). Improvements in crop production will be essential for meeting this demand. Current agricultural output in the United States relies heavily on irrigation to sustain high crop yields in certain regions. Regions characterized by low rainfall and depleting aquifer sources will need to adopt more sustainable practices if they are to sustain competitive crop yields. Sorghum is a cereal grain known for its ability to grow in arid and semi-arid areas. Sorghum grain is grown mostly in the High Plains region where irrigation is more limited due to a lack of water or high costs associated with its recovery.

1.3 Starch Chemistry and Digestion

Carbohydrates constitute the major nutrient class found in cereal grains. Carbohydrates are classified based on their chemistry and structure. Neutral detergent fiber carbohydrates include hemicellulose, and cellulose while non-fiber carbohydrates include starch, which is the predominant carbohydrate in cereal grains (National Academies of Sciences, Engineering, and National Academies of Sciences, Engineering, and Medicine, 2016). Synthesizing starch involves linking multiple glucose molecules together through alpha 1,4 and sometimes alpha 1,6 bonds. Starch is structured in two different forms: amylose and amylopectin. Amylose is comprised of glucose molecules joined exclusively with alpha 1,4 bonds. Amylopectin is formed as a combination of glucose molecules connected by alpha 1,4 and alpha 1,6 bonds. Most cereal grains have greater proportions of amylopectin than amylose. Cereal grains with greater concentrations of amylopectin are usually denoted as being a waxy-type (National Academies of Sciences, Engineering, and Medicine, 2016). Due to amylopectin being branched, mammalian and microbial enzymes can better access glucose polymers and hydrolyze them into

monosaccharide, disaccharide, and oligosaccharide units compared with amylose. There tends to be greater extent of starch digested in waxy varieties than in non-waxy varieties due to relative ease of hydrolyzing amylopectin. Streeter et al. (1990b) compared four dry-rolled sorghum varieties: normal non-tannin, normal high tannin, normal waxy sorghum, and waxy high tannin, with respect to site and extent of digestion using Angus heifers. Starch digestion was greater for waxy varieties compared to normal types of sorghum, (92.8 vs 86.88 %; $P<0.10$) when starch disappearance in the hindgut was calculated as a percentage of the total tract starch digestion. This indicated certain traits, such as tannins and endosperm structure, can change the site of digestion in ruminants.

Starch is digested in ruminants through microbial fermentation or through action of mammalian enzymes. Ruminants ferment starch in the reticulorumen and cecum, whereas small intestinal digestion is facilitated by enzymes synthesized within enterocytes and the pancreas. Most dietary energy cattle receive is from volatile fatty acids produced during microbial fermentation of carbohydrates, and proteins in the reticulorumen and hindgut. Wheat and barley are the most rapidly fermented cereal grains in the rumen, followed by corn and then sorghum (Richards and Hicks, 2007). Rapid fermentation of some cereal grains can lead to metabolic disturbance in ruminants such as acidosis. Since corn and sorghum grains are less fermentable in the rumen, some of the starch in the grain can escape ruminal digestion. This can allow the starch to be digested in the small intestines or fermented in the hindgut (Ørskov, 1986). Starch digestion is affected by its interaction with proteins in the grain endosperm. This is referred to as the protein-starch matrix, and this structure is organized in a way that can make enzymatic attack of the amylose or amylopectin less efficient (Richards and Hicks, 2007).

When starch is hydrolyzed, it can be fermented in the reticulorumen or further digested into monosaccharides and absorbed by enterocytes in the small intestine. Energetically, enzymatic digestion in the small intestines is more energetically favorable to the host animal than microbial digestion in the rumen (97% vs 80%, respectively; Harmon and McLeod, 2001; Huntington et al., 2006). When starch is fermented, glucose is converted into different organic acids with the most prevalent being acetic, propionic, and butyric acids. Feeding cattle with greater proportions of cereal grains yield greater ruminal concentrations of acetic and propionic acids compared to cattle consuming greater proportions of forages. Propionic acid formation in the rumen is more energetically favorable than acetic acid formation due to propionic acid's ability to act as a hydrogen sink, which reduces energy lost due to methane production in the rumen. Butyric acid is utilized by ruminal epithelial cells as an energy source (National Academies of Sciences, Engineering, and Medicine, 2016). Lactic acid is an organic acid produced through fermentation, with greater amounts produced in cattle fed high-concentrate rations. Sometimes when an excess of carbohydrates is consumed rapidly, uncontrolled fermentation occurs, causing lactate levels to rise rapidly leading to a rapid decline in pH. If lactate concentrations in ruminal fluid rise above 50 mM, then acidosis can occur leading to death of the animal or cellular damage in the rumen (Nagaraja and Titgemeyer, 2007).

Mammalian enzymatic digestion utilizes pancreatic alpha-amylase and brush border enzymes on the enterocyte to degrade starch. These enzymes hydrolyze starch molecules into trisaccharide's, disaccharides, and glucose. The glucose is then absorbed by transporters on the enterocyte. From there, glucose can be released from the enterocyte to the blood, and it can be used by different body cells for energy, stored in the liver as glycogen or converted to triacylglycerols to be stored. It has been observed that ruminants are limited in their ability to

digest starch in the small intestine. Research has indicated that starch digestion is limited by the digestion of oligosaccharides, trisaccharides, and disaccharides in the small intestines and not the whole polymer itself (Mayes and Orskov, 1974; Ørskov, 1986; Owens et al., 1986).

1.4 Sorghum History and Grain Characteristics

Sorghum is known by several names worldwide, including milo, jowar, and kaoliang (Taylor and Emmambux, 2008). Sorghum originates from Northeast Africa where it has been grown for thousands of years. This area is known for its arid conditions and sorghum has adapted to this environment (Taylor and Emmambux, 2008). Today, the United States is a major producer of sorghum with production concentrated in the Great Plains region from South Dakota to Texas. This area is collectively known as the Sorghum Belt. Sorghum kernels are spherical in shape and lack an exterior husk to protect the kernel. Sorghum berry size varies, which affects its susceptibility to processing. Sorghum kernels have three main compartments including the pericarp, germ, and endosperm. Pericarp covers the entire seed and protects internal components from the outside environment. The germ is the embryo of the seed. Lastly, the endosperm layer contains starch as a storage carbohydrate to support growth of the developing embryo during germination (Taylor and Emmambux, 2008). Proteins associated with the endosperm cause it to have either a vitreous or floury texture (Richards and Hicks, 2007). Phenolic compounds, therein, cause various colors to be expressed, from red to white, to almost black, but color alone cannot be used to determine nutrient content or digestive characteristics of sorghum grain (Taylor and Emmambux, 2008).

Sorghum kernel size can also impact the digestive properties of the grain which could impact the adoption of sorghum in cattle diets. Variations in the kernel size of sorghum could present a challenge when processing the grain since the feed processing equipment will need constant adjusting to account for the variation in kernel size. Sorghum kernel size may also affect the

milling properties of the grain as well. Lee et al. (2002) used six sorghum seedlots and separated each sorghum seedlot using a Tyler Rotap shaker equipped with three sieve fractions (>3.35, >2.80, and > 2.36 mm). Kernels were graded into large, medium, and small kernel size classes to determine the relationship between kernel size and their respective physiochemical, milling, pasting, and cooking traits. Large and medium sized sorghum kernels had greater harness and less flour loss when kernels were dehulled. Small sorghum kernels had greater percentages of fine particles than medium or large kernels. Likewise, milling yields were greater for large kernels compared to medium and small kernels (Lee et al., 2002). This suggest that feedlots milling smaller sorghum kernels could have a greater losses of grain material and potentially greater risks for metabolic disturbances in steers fed this sorghum since greater amounts of fine material would be present after grain processing.

1.5 Sorghum Protein, Polyphenols, and Tannins

Sorghum grain has disadvantages with respect to its utilization in animal feeding systems. This has led to different levels of adoption by animal feeding industries. Poultry producers are the greatest utilizers of sorghum grain, followed by swine and beef cattle producers (McCuistion et al., 2019). Sorghum is more readily digested by monogastrics compared to ruminants (McCuistion et al., 2019).

Sorghum typically contains greater amounts of protein than maize. Kafirin is the main protein found in sorghum, followed by glutelin (McCuistion et al., 2019). Kafirin can adversely affect digestibility of other nutrients due to its ability to form crosslinks with other proteins and starch (Rooney and Pflugfelder, 1986). When exposed to heat, Kafirin can form disulfide bonds that can decrease digestibility of sorghum starch (Rom et al., 1992). Taylor et al. (1984) evaluated different low-tannin commercial sorghum cultivars grown in South Africa. As protein content

increased, Kafirin concentrations were found to increase as well ($r = 0.47$; $P<0.01$), whereas glutelin decreased ($r = -0.40$; $P<0.01$; Taylor et al., 1984; McCuistion et al., 2019).

Polyphenols are responsible for the diversity of colors found in sorghum grain. They are a broad range of organic compounds with a common benzene ring structure at their core and can interact with free radicals. Flavonoids and tannins are two classes of polyphenolic compounds found in sorghum which provide plants with metabolic and defensive characteristics (McCuistion et al., 2019). Flavonoids have the capability to act as antioxidants and regulators of cellular processes and been hypothesized to mitigate production of methane and to improve immune function in cattle (Kalantar, 2018). Certain flavonoids can also target specific pathogenic species (Cushnie and Lamb, 2005).

Tannins are another type of polyphenol that can bind to protein and starch. Many bird-resistant sorghum cultivars incorporate tannins to prevent depredation by birds and other animals. Formation of tannin-protein complexes can adversely affect digestion and nutrient utilization in animals (Adamczyk et al., 2017). Depending on tannin and protein structure, these complexes can be reversible or irreversible when exposed to low pH environments within the stomach or abomasum. Streeter et al. (1990b) compared bird-resistant (high-tannin) sorghum cultivars (BR) with sorghum cultivars low in tannins (non-BR) and concluded that total tract starch digestibility was not different ($P>0.05$) among the four cultivars evaluated. Nitrogen availability in the rumen decreased for BR types of vs non-BR types ($P<0.01$). Waxy sorghum variety's starch had improved starch digestion before the hindgut compared to normal endosperm varieties ($P<0.1$; Streeter et al., 1990b). Streeter et al. (1990a) also compared BR-normal and BR-waxy sorghum using an *in vitro* technique and found greater *in vitro* dry matter disappearance in BR waxy cultivars compared to BR non-waxy cultivars ($P<0.01$). Growing BR

sorghum cultivars with waxy characteristics could be advantageous since there is a greater extent of *in vitro* digestion of these cultivars compared to normal BR sorghum cultivars.

Some sorghum cultivars contain condensed tannins that attach to proteins, starches, or microbes. Condensed tannin complexes can resist enzymatic hydrolysis in stomach acid and thus, are irreversible. The binding of tannins can affect microbial digestion and have an inhibitory effect on bacterial and methanogen growth, which has potential to be exploited. A meta-analysis by (Jayanegara et al., 2012) concluded that, as tannin concentrations increase, methane emissions in ruminants decreased. Conversely, when using sorghum in ruminant diets to reduce methane emissions, the results seem inconclusive. Mavasa et al. (2022) compared partial replacement of maize meal with increasing amount of high tannin sorghum meal (i.e., 10%, 20% and 30% of the maize meal inclusion of the diet) in goats. The 10% sorghum meal treatment group emitted less methane compared to the control group (no supplementation of sorghum meal; $P<0.05$) while having no effects on body weight or live weight gain ($P>0.05$). de Oliveira et al. (2007) researched the inclusion of sorghum silage with differing levels of tannins on methane emissions in beef cattle. A 2 × 2 factorial arrangement in a 4 × 4 Latin square design was utilized where the four treatments included a high tannin and low tannin sorghum silage coupled with either a concentrate or urea-based supplement. Cattle consuming the treatments with high tannin sorghum silages, had similar methane emissions compared to cattle consuming the low tannin sorghum silage treatments ($P>0.10$). Soltan et al. (2021) compared four different inclusion rates of sorghum with low concentrations of tannins, (i.e., S_{25}, S_{50}, S_{75}, and S_{100}) with a maize-based control diet in lambs. Tannin concentrations in lamb diets were 1.11, 2.23, 3.35, and 4.46 g/kg (DM) for the S_{25}, S_{50}, S_{75}, and S_{100} diets, respectively. When total methane production was compared between treatments (L/day), there were no differences among diets ($P = 0.240$).

Methane produced per kg of body weight gain (L/kg) was least for lambs fed the S_{25} diet (203 L/kg) compared to the lambs fed the control diet with corn (316 L/kg; $P<0.05$). Dry matter and organic matter digestibility were less for lambs consuming sorghum compared with maize, ($P<0.05$), but average daily gain of lambs fed $S_{25,}$ and S_{50} were greater than for lambs fed the control diet (96.3, 81.5, and 74.9 g/day, respectively; $P<0.05$). Colombini et al. (2015) compared sorghum silages and corn silages fed to dairy cows and found that cows consuming sorghum silage produced more methane per kg of NDF consumed ($P<0.05$).

1.6 Cereal Grain Processing Methods

Sorghum appears to have a more complicated protein starch matrix compared to other cereal grains (Theurer, 1986; Herrera-Saldana et al., 1990). This matrix can impact digestion of sorghum which presents a challenge when compared to other grains like wheat or barley. Disruption of the protein-starch matrix through processing can increase susceptibility of grain to digestion. Roller mills comminute cereal grains by passing kernels between a pair of steel rolls operating at different speeds of rotation. This compressing and shearing of the grain yields small fragments which can be digested more extensively by livestock. Hammer mills consist of hammers rotating at high speeds inside a drum and grain is broken into small fragments as it contacts the hammers. A screen is fitted on the bottom of the device to achieve a desired particle size (Pulva Corporation, 2019). These methods are utilized widely to process grain for poultry and swine.

Ensiling methods utilize fermentation of cereal grains to improve their digestibility. High-moisture grain processing is the more common method utilized for ensiling grain, and it is accomplished by harvesting cereal grain when its moisture content is greater than 24% (Defoor et al., 2006). Grain can be stored whole or processed to expose the starch in the grain before it is stored. The most common structures to store high moisture grain are oxygen-limiting silos or

bunkers. These structures allow the grain to ferment over time while maintaining low oxygen levels. Fermentation times vary but usually require at least three weeks before ensiled grain can be fed. Reconstitution is a similar method that utilizes ensiling to process the grain and involves addition of water to dry grains to achieve final moisture contents of 30% prior to ensiling. Grain is usually physically processed to help facilitate moisture assimilation and fermentation. After the grain is processed and sealed within an oxygen limiting structure, the grain is allowed to ferment for three weeks before being fed. High-moisture grain processing is more common for corn since it has an extended period when the grain's moisture levels are greater than 24%. Sorghum frequently is ensiled following reconstitution methods since its harvest window is much shorter relative to corn (Bailey, 2017).

Hydrothermal processing techniques are utilized extensively in the United States and other developed nations to process grains. Steam rolling is a technique in which grain is subjected to steam for one to eight minutes and before the grain is passed between two corrugated steel rolls (Richards and Hicks, 2007). Typically, the density of the resulting flake is variable, since grain is subjected to heat for a short amount of time (Richards and Hicks, 2007). Usually, this technique is used on barley and wheat since much of the starch is digested in the rumen (99%) when utilizing this method, obviating the need for further processing (Theurer, 1986). Another grain-processing method is micronization. Micronization utilizes infrared radiation to heat the grain for a set time varying from 30 seconds to a few minutes. After heating, grains are either flaked or rolled to achieve a final product (Sajjadi et al., 2022). This processing method can increase digestibility and gelatinization in some grains, such as sorghum, but does not affect others, such as barley. Croka and Wagner (1975) utilized three animal trials to compare micronized and dry-rolled sorghum and found that cattle eating micronized sorghum ate less dry matter ($P<0.10$) but

had similar average daily gains. Dry heat processing is also utilized to process certain grains for feed. Dry heating certain grains like sorghum can cause rapid expansion of kernels (i.e., popping). Riggs et al. (1970) compared dry-rolled sorghum to grain that was popped and then rolled in finishing cattle. Dry matter intake was less for cattle fed popped sorghum ($P<0.01$), and apparent digestabilties of dry matter, organic matter, nitrogen-free extract, and nonprotein organic matter were greater for popped sorghum compared to dry-rolled sorghum ($P<0.01$). Dry heating of sorghum led to decreased bulk density because popped sorghum grain can expand to eight times its original size which could present handling challenges in feedlots.

One commonly employed method of hydrothermal grain processing is steam-flaking which utilizes steam to alter the starch structure in cereal grains. Many factors influence improvements in digestibility of starch after steam flaking, including chest temperature, cooking time, bulk density, and amount of added moisture. Steam-flaking has been observed to increase the net energy of gain value of normal corn and sorghum by as much as 18% (Zinn et al., 2002; Zinn et al., 2008). Starch gelatinization is critical to increase nutritive value. Gelatinization causes the starch granule to swell, which disrupts the protein-starch matrix. This causes an increase in the surface area of the starch molecule and allows enzymes from either microbial or endogenous sources to access polymers of starch. The shear force applied to grain in the process also is important. Pressure applied while grain is hot is accomplished by adjusting the gap between flaker mill rolls. More pressure exerted by rolls causes a greater compressive force to be applied to the grain and increases susceptibility to digestion. Steam flaking also shifts starch digestion from the hindgut to the rumen by the disruption of the protein-starch matrix of corn which allows greater microbial digestion of the starch (Zinn et al., 2002).

1.7 Sorghum Use in Cattle Diets

Sorghum can be fed either as grain or as a source of roughage and its flexibility has allowed the plant to be utilized in various beef and dairy cattle diets. Sorghum is believed to be the second most common grain fed to feedlot cattle in the United States (National Academies of Sciences, Engineering, and Medicine, 2016). It is usually fed during times of the year when it is sold at a discount relative to corn. Texas and Kansas are the two primary growers of sorghum grain.

Steam-flaked sorghum grain has been observed to yield similar cattle performance, lactation performance, and digestibility characteristics as steam-flaked corn (Brandt et al., 1992; Theurer et al., 1999). Brandt et al. (1992) compared cattle fed steam-flaked sorghum to those fed steam-flaked corn and found no differences in average daily gain, feed consumption, or gain efficiency ($P>0.1$). Degree of processing influences performance of steam-flaked sorghum. Flake density is a method commonly employed to measure degree of processing: as degree of processing increases, flake density (g/L) becomes lighter. Swingle et al. (1999) compared flake weights of 412, 360, 309, and 257 g/L in an experiment utilizing growing and finishing cattle. Starch availability and apparent starch digestibility increased linearly with decreased flake density ($P<0.05$). As flake density decreased, electricity requirements to process grain increased ($P<0.05$); moreover, the electricity requirements to flake sorghum from 412 g/L to 309 g/L increased by 50%. In contrast, electricity requirements to process sorghum grain from 412 g/L to 360 g/L required only an 8% increase. When average daily gain, feed efficiency, and electricity consumption by the flaker mill were collectively considered, a flake density of 360 g/L was deemed ideal for both growing and finishing diets (Swingle et al., 1999).

Steam-flaked sorghum also can be used in dairy rations. Chen et al. (1994) compared steam-flaked corn or sorghum with dry-rolled sorghum and steam-rolled corn in dairy cattle diets.

Dairy cows on steam-flaked sorghum diets had equivalent dry matter intake, milk yield, and milk fat ($P>0.05$) to cows eating steam-flaked corn diets. Cows fed steam-flaked sorghum diets had greater intake of dry matter, milk yield, milk fat, and milk protein than cows fed steam-rolled corn or dry-rolled sorghum diets ($P<0.05$; Chen et al., 1994).

Between 2000 and 2010, ethanol production increased rapidly in the United States (Newes et al., 2022). Starch in corn and sorghum are used to produce ethanol (Newes et al., 2022). Following ethanol extraction, the leftover bran, pericarp, and other non-starch components are the primary components in distiller's grains. Distiller's grains typically have greater concentrations of protein and fat than whole grains and can be excellent sources of protein in cattle diets. Most ethanol plants sell distiller's grains as either wet distiller's grains (WDG), where the distiller's grains are recovered from the ethanol production process without any additives, or dry distiller's grains, where condensed solubles are mixed with the wet distiller's grains before the mixture is dried (DDGS; Buenavista et al., 2021). Distiller's grains have potential drawbacks when used in cattle diets. They can be high in sulfur since the ethanol production process uses sulfuric acid. Metabolic disorders and diminished mineral absorption have been documented when high-sulfur diets were fed (Drewnoski et al., 2014). Also, since these products are made at different ethanol plants, there are variations in protein, starch, and fat concentrations which make formulating diets with these products challenging.

Sorghum and corn-based distiller's grains have no effect on USDA quality and yield grades of beef carcasses when they are used exclusive of one another in cattle diets. Depenbusch et al. (2009) compared sorghum and corn distiller's grains, either wet or dried, in a finishing ration consisting of steam-flaked corn. Average daily gain, gain:feed, and USDA quality and yield grades were not different among treatments. Stelzleni et al. (2016) compared distiller grains, corn

gluten feed, and soybean meal inclusion in finishing rations and observed that cattle consuming distiller's grain had greater average daily gain ($P=0.05$) when compared to cattle fed soybean meal and greater gain:feed ($P<0.05$) compared to cattle fed soybean meal or corn gluten feed as protein sources.

Sorghum can be an excellent source of roughage for beef and dairy cattle. Many forage sorghum hybrids are utilized for silage or haylage production since sorghum-sudangrass hybrids exist. Using sorghum silage has some drawbacks, including reduced starch concentrations and sometimes lower dry matter content when compared to corn silage. Khosravi et al. (2018) replaced corn silage with sorghum silage in dairy cattle diets (25% of diet dry matter) and observed no differences in digestabilties of dry matter, organic matter, crude protein, neutral detergent fiber, or acid detergent fiber ($P>0.1$). Likewise, dry matter intake and milk yield ($P>0.1$) were not different among cows consuming either treatment. There was an increase in total antioxidant capacity in blood and milk from cattle-fed sorghum silage ($P<0.05$), which could be due to polyphenols in silage.

1.8 *In Vitro* Gas Production

Assessing various feed ingredients through *in vivo* techniques can be expensive, as it involves animal handling, feeding, and labor for conducting experiments. Techniques utilized in laboratory settings to estimate digestibility and performance attributes have been developed, which can decrease costs of exploratory work with new feed ingredients or drugs. Tilley and Terry (1963) were the first to describe an *in vitro* procedure to screen different herbages for their susceptibility to ruminal degradation. Utilizing ruminal fluid combined with McDougall's buffer as inoculant, digestion inside test tubes was allowed to occur for 48 hours. Residual biomass was treated with pepsin to remove protein. *In vitro* dry matter digestibility was then calculated to determine susceptibility of the herbages to ruminal degradation. Later revisions to this method by

Menke et al. (1979) utilized total gas production to estimate metabolizable energy content of feedstuffs (R=0.98). This refinement improved laboratory techniques to cost-effectively evaluate many different feed ingredients.

Many different physical, chemical, and thermal treatments and processes for sorghum have been tested using *in vitro* techniques. Acosta and Schake (1992) compared different alkaline and acid treatments to whole plant sorghum grain silage using both *in vitro* and *in vivo* techniques, to see how the chemical treatments affected nutrient quality of the grain and its effects on cattle performance. Chemical treatments included application of anhydrous ammonia, sodium hydroxide, or sulfuric acid to whole-plant sorghum grain silage. A 3 × 4 factorial design was utilized, and i*n vitro* dry matter disappearance and *in vitro* volatile fatty acid culture concentrations were measured on each chemically treated whole plant sorghum. A follow-up study was used to evaluate cattle performance with diets containing chemically treated grains. *In vitro* dry matter disappearance was found to be greater for sodium hydroxide and sulfuric acid treated whole plant sorghum compared to anhydrous ammonia treated and control whole plant sorghum ($P<0.05$). When measuring *in vitro* organic acid production, anhydrous ammonia treated whole plant sorghum yielded more total volatile fatty acids, and acetate, ($P<0.05$) than the other chemically treated whole plant sorghum. When chemically treated whole plant sorghum were analyzed *in vivo*, steer consuming the anhydrous ammonia treated whole plant sorghum had the greatest average daily gain, adjusted final weight, and gain:feed when compared to cattle consuming the other chemically treated sorghum diets ($P<0.05$). Thermal processing techniques have been assessed using *in vitro* techniques to evaluate combinations of heat, moisture, and roller pressure with respect to effects on grain digestion. Osman et al. (1970) utilized an *in vitro* enzymatic starch digestion method to evaluate how moist heat processes

affected starch digestibility and concluded that added heat and moisture alone decreased starch degradation ($P<0.05$). In contrast, when the grain was subsequently flattened or sheared, starch degradation increased ($P<0.05$).

In vitro techniques have been used to study rates of sorghum grain digestion. Sorghum hybrids vary widely in the time required to hydrolyze starch incorporated in the endosperm. Since most concentrates stay in the rumen for a limited amount of time, sorghum hybrids containing starch that is more rapidly fermented should perform better and be selected for breeding (Sniffen et al., 1992; Pedersen et al., 2000). Wester et al. (1992) compared 68 different sorghum hybrids for their rates of starch digestion using an *in vitro* method. Two sorghum hybrids with the fastest and slowest rates of *in vitro* starch disappearance were utilized in a follow-up animal study. Feed efficiency was improved in lambs consuming the sorghum hybrids with the fastest *in vitro* starch disappearance compared to lambs consuming hybrids with the slowest *in vitro* starch disappearance ($P<0.1$). Based on these two experiments, the rate of starch digestion in sorghum can influence how well the grain will be digested and utilized by ruminants.

1.9 Conclusion

Water usage by farms and feedlots is a challenge and must be addressed if we are to continue leading the world in agriculture output. Sorghum could be one of the crops that helps farms and feedlots meet this challenge. Greater cultivation of sorghum in the High Plains region could reduce the amount of irrigation used by farms and the resulting grain and fodder could be used as feedstuffs by feedlots. Sorghum's potential as a source of concentrate or roughage in cattle diets allows cattle producers flexibility in utilizing a sorghum crop in their production systems. Steam-flaked sorghum has a feed value similar to steam-flaked corn which allows feedlots to adopt the grain with minimal modifications to existing steam-flaking infrastructure (Brandt et al., 1992).

Challenges with adoption of sorghum grain in finishing cattle diets include effects of kernel size, and selective breeding of sorghum varieties with superior traits for ruminal digestion and ease of processing. Studies conducted using *in vitro* screening methods have revealed that sorghum varieties exhibit a wide range of susceptibility to microbial digestion (Wester et al., 1992; Pedersen et al., 2000). These findings have potential to impact the future selection of superior sorghum hybrids intended for ruminant feeding since these studies indicate that the rate of digestion of the grain can impact the feeding value (Wester et al., 1992).

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2 Using *in vitro* gas production and *in situ* techniques to identify sorghum hybrids with superior susceptibilities to microbial digestion

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2.1 Abstract

Our objective was to evaluate 39 sorghum hybrids for their potential use in cattle diets using *in vitro* and *in situ* techniques to measure extent and rate of digestion of each grain. Sorghum grain was processed using three methods: grinding, reconstitution followed by ensiling (RE), and steam-flaking. *In vitro* gas production, volatile fatty acids concentrations, *in vitro* dry matter disappearance (IVDMD), and *in situ* dry matter disappearance (ISDMD) were measured for each processed grain. *In vitro* gas production was analyzed using non-linear models to estimate four different gas production parameters: maximum cumulative gas production (*K*), time to reach half of the cumulative gas production ($t_{1/2}$), rate of gas production (*r*), and maximum rate of gas production (*m*). Predicted methane yield was estimated utilizing VFA concentrations and the equation Methane Yield (g/kg DM) = 4.08×[Acetate (mol/100 mol)/Propionate (mol/100 mol)] + 7.05 (Williams et al., 2019). In addition, each sorghum hybrid was scanned utilizing near-infrared spectroscopy (NIR) to predict each grain's starch and protein content. These predictions were correlated to our response variables *K*, $t_{1/2}$, *r*, *m*, VFA concentrations, IVDMD, and ISDMD. Starch and protein content correlated with *K*, *m*, and methane yield in dry-rolled sorghum. Starch and protein content of our sorghum grain also correlated with *m* and ISDMD in RE sorghum and *K* in steam-flaked sorghum. These results were interpreted to indicate these sorghum hybrids had a significant variation in susceptibility to *in vitro* microbial digestion, and that more extensive forms of processing (RE and steam-flaking) decrease the differences among grains with respect to *in vitro* microbial digestion.

Keywords: extent of *in vitro* gas production, *in situ* dry matter disappearance, *in vitro* gas production, sorghum

Abbreviations: DM, dry matter; ISDMD, *in situ* dry matter disappearance; IVDMD, *in vitro* dry matter disappearance; MY, methane yield; NIR, near-infrared spectroscopy ; RE, reconstitution followed by ensiling

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2.2 Introduction

The High Plains region of the United States is known for its beef cattle industry, as it hosts the largest concentration of cattle in the United States (United States Department of Agriculture, January 2023). Cattle feeding operations located in the High Plains use corn, primarily irrigated, as their primary grain. The Ogalala aquifer is located in Texas, Oklahoma, Kansas, Nebraska, South Dakota, Wyoming, Colorado, and New Mexico, and it historically has provided water to irrigate crops grown in this region (Taghvaeian et al., 2017). By 2100, it has been predicted that 22,000 km^2 of farmland in the High Plains region will be unable to sustain irrigated crop production due to a lack of sufficient water (Deines et al., 2020). Sorghum is an alternative crop with characteristics similar to corn that is known for its ability to grow in arid environments (Taylor and Emmambux, 2008). Historically, sorghum has been utilized in the feedlot industries in Texas and Kansas, where sorghum crop production is concentrated. Variations in sorghum cultivars and perception of inferior starch digestion have led to feedlots preferring corn over sorghum in cattle diets (McCuistion et al., 2019).

Sorghum cultivars have a wide range of grain characteristics. Some sorghum cultivars exhibit high concentrations of flavonoids which act as antioxidants, while others have different starch structures (i.e., waxy varieties that have greater amylopectin content). Many sorghum cultivars grown today in the United States lack tannins, which inhibit starch digestion due to their ability to bind to proteins and microbes.

Sorghum has been tested with different processing techniques, such as micronization, dry heat, ensiling, and steam rolling; however, steam flaking yields the greatest improvements in susceptibility to microbial digestion (McCuistion et al., 2019). Usually *in vitro* and *in situ* methods are utilized in the preliminary phase of testing chemical and processing methods on sorghum due to lesser costs of *in vitro* and *in situ* techniques compared to animal growth-

performance trials (Lane et al., 1972; Prasad et al., 1975). This has led to the conclusion that sorghum grain can vary widely in its susceptibility to ruminal and enzymatic digestion. This experiment's objective was to evaluate three processing methods: (i.e., grinding, reconstitution followed by ensiling (RE), and steam-flaking), on 39 commercial sorghum hybrids to estimate their extents and rates of *in vitro* and *in situ* ruminal digestion in order to identify sorghum hybrids with traits that could improve grain utilization in finishing cattle diets.

2.3 Materials and Methods

All procedures were reviewed and approved by the Kansas State University IACUC committee before the initiation of study.

2.3.1 Experimental Design

Sorghum hybrids were grown in a 2-row, 2-fold replicated randomized complete block design at the Kansas State University Agronomy Farm (Manhattan, Kansas) before being submitted to the Kansas State University Pre-Harvest Food Safety Laboratory (Manhattan, Kansas) for analysis. Grow plot was used to designate each replicate grown. At the lab, each grain was mixed to ensure homogeneity before it was split into subsamples for processing by grinding, RE, and steam-flaking.

Ground sorghum was processed using a Udy Cyclone mill (Udy Corporation, CO) equipped with a 1-mm screen. Grains were ground with an Udy Cyclone mill, since particle distribution is more uniform, and these ground samples were used in the NIR analysis. Ground sorghum was then sealed in plastic bags (Whirl-Pak, WI) and kept in plastic containers at 21 ℃ pending analyses.

Reconstituted and ensiled sorghum grain (300 g) from each growth plot was processed in duplicate by cracking in a Wiley Mill (Thomas Scientific, PA) equipped with a 6-mm screen. Cracked sorghum grain was then transferred into polyethylene terephthalate bags, and between

95 and 100 mL of water was added to the cracked sorghum. The amount of water was a function of the dry matter content of the original sample, in which a final moisture content of 30% was achieved. Polyethylene terephthalate bags were inverted three times to mix water with the grain before the bags were vacuum sealed and placed into an insulated chest for 90 days to allow ensiling to take place. After 90 days, polyethylene terephthalate bags were removed from insulated chests and placed in a -20 ℃ freezer to terminate fermentation. Ensiled grains were then ground through a Wiley mill (Thomas Scientific, PA) equipped with a 2-mm screen. In total four samples of each sorghum hybrid were processed with RE processing.

Steam-flaking required at least 500 g of grain to ensure uniform steam conditioning of the grain. Thus, the original growth plots of sorghum grain that were designated for the steam flaking treatment were combined to ensure that there was sufficient sample. Each steam-flaked sorghum hybrid was processed by weighing 800 g of sorghum grain into glass jars. Depending on the grain dry matter content, between 43 and 57 mL of water were combined with sorghum grain to achieve a final moisture content of 13%. The glass jars with grain were then placed on a roller bed (87.6 cm × 55.9 cm), and sorghum grain was mixed for one hour at 60 rpm. Sorghum grain was then transferred to perforated steel baskets and placed into individual chambers (⌀ 16.7 cm, ↧ 21.9 cm) in a customized steam table (121.9 cm × 45.7 cm) and subjected to steam conditioning for 1 h at 99 ℃ at a pressure of 138 kPa. Steamed grain was transferred to a steam flaker (R & R Machine Works; Dalhart, TX) equipped with 46 cm × 91 cm corrugated rolls (6.3 grooves/cm) and rolls were set for flaking grain to a bulk density of 373 g/L. After grains were flaked, grains were allowed to cool for 20 minutes before being transferred to a -20 ℃ freezer. Steam-flaked sorghum grains were ground with a Wiley mill (Thomas Scientific, PA) equipped with a 1-mm screen before being transferred back to a -20 ℃ freezer.

2.3.2 Near-infrared scanning of sorghum hybrid

Each grain was scanned utilizing a Perten DA 7250 (Perten Instruments, Springfield, IL, USA) spectrometer to estimate each grain's protein and starch content. Grains were scanned as whole grains and ground grains. The calibration and statistical models used to estimate each grain's protein and starch content were obtained from previous studies (Peiris et al., 2019; Peiris et al., 2020; Peiris et al., 2021). These were later used to assess correlations between our sorghum grains estimated protein and starch content with our response variables.

2.3.3 *In Vitro* Gas Production

Each processed grain was analyzed using an *in vitro* gas production technique to estimate each sorghum grain's extent and rate of gas production due to microbial fermentation. A randomized complete block design was utilized to compare *in vitro* gas production, volatile fatty acid concentrations from *in vitro* cultures, and *in vitro* dry matter disappearance (IVDMD) of the 39 sorghum hybrids. This was accomplished using the Ankom RF gas production system (Ankom Technology, Macedon, NY). Each processed sorghum grain (1.5 g DM) was first weighed into individual Pyrex® bottles. Ground sorghum *in vitro* incubations included 40 individual Pyrex® bottles representing the 39 ground sorghum hybrids and a blank culture that was devoid of substrate and consisted of just buffered ruminal fluid and would be used later for *in vitro* dry matter disappearance (IVDMD) analysis. *In vitro* incubations of RE sorghum consisted of 41 Pyrex® bottles that included the 39 sorghum hybrids, a blank culture, and a similar processed RE corn sample for that could be used to compare each sorghum hybrids *in vitro* estimates to corns. *In vitro* incubations using steam-flaked grain followed the same setup as RE *in vitro* incubation's except that the corn sample was steam-flaked. On each day of the *in vitro* incubation, ruminal fluid was collected from one fistulated steer to be used as inoculum.

One steers ruminal fluid was used as an inoculum per *in vitro* incubation. The steer's diet consisted of 70% concentrate and 30% roughage, with the primary concentrate being corn. Ruminal fluid was screened through two layers of cheesecloth before being deposited into pre-warmed insulated containers (1L). Strained ruminal fluid was then transferred to the laboratory, where it was sparged with nitrogen gas before being transferred to a separatory funnel. The ruminal fluid was allowed to stratify into three layers over a period of 20 min. The bottom sediment layer was discarded, and the microbe-rich layer was used as inoculant (middle layer). Ruminal fluid inoculum was combined in a 1:4 ratio with McDougall's buffer to make a final buffered ruminal fluid solution (McDougall, 1948). Each Pyrex® bottle was inoculated with 150 mL of buffered ruminal fluid and then immediately flushed with nitrogen gas, and an Ankom RF gas production module (Ankom, Macedon, NY) was secured onto the top of each bottle. *In vitro* cultures were allowed to incubate at 39 ℃ until cumulative *in vitro* gas production reached a plateau. The *in vitro* cultures with ground sorghum grain were analyzed after 28.25 hours, while those with RE and steam-flaked sorghum were analyzed after 30 hours, because the time needed to reach a plateau in cumulative *in vitro* gas production differed between incubations. *In vitro* fermentation was terminated by placing each Pyrex® bottle in ice for 10 min. Ankom modules (Ankom, Macedon, NY) recorded gas measurements every 15 min during fermentation. A 4-mL sample of grain and ruminal fluid solution was collected from each *in vitro* culture and mixed with 1 mL of 25 % (w/v) meta-phosphoric acid for the purpose of quantifying volatile fatty acid concentrations. The remaining solution and residue were transferred from each bottle to individual pans and dried for 48 hours at 105 ℃ oven to calculate IVDMD. The blank that was included in each incubation was dried to calculate the background sources of DM and used to

correct each *in vitro* incubations IVDMD analysis since each *in vitro* culture had used the same inoculum.

2.3.4 *In situ* Dry Matter Disappearance

In situ dry matter disappearance (ISDMD) was performed on ground, and RE sorghum grain samples with 12 replications for each ground grain and 6 replications for RE sorghum grain. Since only 16 hybrids could be analyzed per mesh bag (40.5 cm × 51 cm; n=14/hybrids + corn + blank), an incomplete block design was used for the *in situ* dry matter disappearance (ISDMD) analysis of the 39 sorghum hybrids. Each replication (1.0g; DM basis) was dispensed into an Ankom R510 concentrate bag (5 cm X 10 cm; ANKOM Technology Macedon, NY). Three steers were utilized for the *in situ* analysis. One mesh bag per steer was utilized to hold the Ankom concentrate bags in the rumen, and each mesh bag contained 16 nylon bags representing 14 different sorghum hybrids, a similar processed corn sample, and a blank sample consisting of no additional substrate that was used to account for background DM from the rumen. Each mesh bag was placed inside the rumen of fistulated steers for 16 h. This procedure was repeated over 12 days for ground sorghum and 8 days for RE sorghum. Concentrate bags were removed and placed into plastic containers containing warm water. Concentrate bags were then gently agitated in warm water to remove residue adhering to the exterior of each bag. This washing procedure was repeated twice before concentrate bags were placed on paper towels to remove excess moisture. After five minutes, concentrate bags were transferred to the laboratory and placed in a forced air oven at 100 ℃ for 24 h and then weighed.

2.3.5 Calculation of Gas Parameters, Volatile Fatty Acids Concentrations, and IVDMD

Gas production, volatile fatty acid concentrations, and IVDMD were analyzed following each incubation. Twelve *in vitro* incubations of ground sorghum hybrids were performed; however,

one *in vitro* incubation had to be disregarded due to issues with gas measuring equipment. In addition, only ten replications were performed for IVDMD for ground sorghum due to issues with ruminal fluid corroding the aluminum pans as the solution dried. Twelve *in vitro* incubations of RE sorghum were performed; however, one IVDMD set was discarded from the RE dataset due to the microbial fluid corroding the aluminum pans. Six *in vitro* incubations of steam-flaked sorghum were performed with no incubations having to be removed. Volatile fatty acid concentrations were measured using an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector, and a (DB Wax) capillary column was used with hydrogen as the carrier gas. Acetate, propionate, and butyrate concentrations were measured and expressed as millimoles of VFA. The Formula to calculate IVDMD is below and is expressed as a coefficient.

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

$$\text{Blank} = \text{Dried blank residue, g} - \text{Pan weight, g}$$

Gas measurements from the ANKOM system were recorded as psi. Therefore, gas measurements were converted to milliliters of gas produced using the ideal gas law and Avogadro's law (Ankom Technologies, 2021).

Ideal gas law

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

where, n = amount of gas produced in moles (mol), p = pressure (kPa), V= head space volume in pyrex bottle (L), R = gas constant (8.314472 L·kPa·K-1·mol-1), and T = Temperature in Kelvin (K)

Using Avogadro's law, 1 mole of gas at 0 ℃ (273.5 K) occupies 22.4 L of space. This conversion was used to calculate the total milliliters of gas produced from the moles of gas produced using the following equation.

$$\text{Gas produced in milliliters} = n \times 22.4 \times 1000$$

2.3.6 In Situ Dry Matter Disappearance

In situ dry matter disappearance was calculate similar to IVDMD and is also expressed as a coefficient of the dry matter that disappeared. A blank bag devoid of substrate and a corn sample were used in each *in situ* incubation in order to account for DM from the rumen, and to see how the sorghum hybrids compared to the corn sample. Below is formula used to calculate ISDMD.

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

$$\text{Blank} = \text{Dried blank residue, g} - \text{Nylon bag weight, g}$$

2.3.7 Statistical Analyses

Gas production data were analyzed using the nonlinear regression procedure NLIN of the Statistical Analysis System (SAS, ver. 9.4) for each individual *in vitro* culture. A logistic and a log-logistic model were used to analyze the gas production data.

Logistic model

$$Gas\ production = \frac{K}{1+\exp^{-beta(Time - t_{1/2})}}$$

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),

K- Theoretical maximum cumulative gas production (mL),

Beta- Growth rate of gas production (1/time), and

$t_{1/2}$- Time midpoint of *K* (hours).

Log-logistic model

$$Gas\ production = \frac{K}{1 + (t_{1/2} \div Time)^{r}}$$

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),

K- Theoretical maximum cumulative gas production (mL),

r- Growth rate of gas production (unit-less), and

$t_{1/2}$- Time midpoint of *K* (hours).

These two models estimated each gas production parameter using time (independent variable) and gas production (dependent variable). These gas production parameters were used to estimate the extent of the gas production potential (parameter *K)* and the rate of gas production due to substrate digestion (parameters *beta*, *r*, and $t_{1/2}$). By taking the first derivative of each model and setting the derivative equal to zero, the maximum rate of gas production (*m*) during each *in vitro* incubation was calculated using our response variables as inputs. Below are the functions that were used to calculate *m* in terms *K*, *beta*, *r*, and $t_{1/2}$ from the logistical and log-logical models.

Max slope (*m*) for the logistical model

$$m = beta \times K / 4$$

Max slope (*m*) for the log-logistic model

$$m = \left[\left(1 - {}^{1}/_{r}\right) \times \left(t_{1/2}\right)^{r} / 1 + {}^{1}/_{r}\right]^{-{}^{1}/_{r}} \times \left[rK\left(1 - {}^{1}/_{r^{2}}\right)/4\right]$$

When comparing the two models, those with the lowest mean square error were chosen for further analysis. Consistently across all three processed grain datasets, the log-logistic model provided a superior fit to the data; thus, *K*, *r*, $t_{1/2,}$ and *m* were subjected to further analyses using the Glimmix procedure in SAS. The Glimmix procedure was also utilized to evaluate *in vitro* cultures acetate, propionate, and butyrate concentrations, estimated methane yield, IVDMD, and

ISDMD measurements. Sorghum hybrid was used as the fixed effect, while growth plot and incubation within animal were used as the random effects for *in vitro* analysis of ground sorghum. Since the growth plots were duplicated for RE sorghum, growth plot replicate within growth plot and incubation within animal were used the random effects while sorghum hybrid was maintained as a fixed effect. For steam-flaked sorghum, hybrid was used as a fixed effect and incubation was used the random effect. For the *in situ* data analyses, fixed effects and random effects followed the same format as each respective *in vitro* analysis. Sorghum hybrid means were separated using the lines function. The Corr procedure of SAS was used to perform Person correlations on the estimated protein and starch content of each grain, with their respective least square means for K, $t_{1/2}$, r, m, VFA concentrations from *in vitro* cultures, IVDMD, and ISDMD.

2.4 Results

2.4.1 Protein and Starch Content of Sorghum Hybrids

Sorghum hybrids we evaluated were sourced from a combination of institutional and commercial sources. The estimated protein and starch content varied between sorghum hybrids. Figures 2.1 and 2.2 summarize the variations in protein and starch content respectively. Hybrid names are designated with a specific hybrid number in each figure since hybrid names for the sorghum grains were not disclosed.

2.4.2 Ground Sorghum

2.4.2.1 Gas Production Parameters

Ground sorghum's estimated K, r, and $t_{1/2}$ and m are summarized in Figures 2.3, 2.4, 2.5, and 2.6 respectively. All gas production parameters were affected by hybrid ($P<0.05$). Maximum *in vitro* gas production is an estimate of the extent of *in vitro* gas production from microbial digestion in the *in vitro* cultures. The variation in K values from our *in vitro* cultures indicated

that there is significant variations in the extent of microbial digestion of these ground sorghum hybrids. The rate of gas production is also, an important component in terms of measuring each grain's susceptibility to *in vitro* microbial digestion since the grains would spend a finite amount of time in the rumen, and thus, may not be digested completely. Gas production parameters *r*, $t_{1/2,}$ and *m* can estimate the rate of gas production over time and could improve the selection process of identifying sorghum hybrids with improved susceptibilities to ruminal fermentation. Several of the sorghum hybrids had lower $t_{1/2}$ estimates and would theoretically be digested faster (Figure 2.4). In Figure 2.5, most of our sorghum hybrids had similar values for r, indicating that the overall rate of digestion was similar among the majority of our sorghum hybrids. Figure 2.6 summarizes *m* and significant difference were observed among the ground sorghum hybrids.

2.4.2.2 Concentrations of VFA and Methane Yield from *In Vitro* Cultures

Volatile fatty acids are end products of anaerobic fermentation. Acetate, propionate, and butyrate are the predominant VFAs and will be further discussed. Concentrations of acetate, propionate, and butyrate from *in vitro* cultures are summarized in Figures 2.7, 2.8, and 2.9, respectively. Sorghum hybrid did not affect *in vitro* concentrations of acetate (P=0.692), propionate (P=0.669), or butyrate (P=0.610). Formation of acetate and butyrate by anerobic microbes in the rumen leads to the formation of H_2 in ruminal fluid. Increased ruminal hydrogen concentrations contribute to increased methane production due to the reduction of carbon dioxide to methane by methanogens (Janssen, 2010). Reduced methane production by cattle is desirable as the beef and dairy industries attempt to be more sustainable. Propionate is the only VFA that does not produce hydrogen during formation; thus greater ruminal production of propionate usually leads to decreased methane production (Janssen, 2010). Williams et al. (2019) used volatile fatty acid concentrations to predict *in vivo* methane yield (g/kg DM) with an R^2 between

0.501 and 0.511 for the top three performing models. Therefore, the model Methane Yield (g/kg DM) = 4.08×[Acetate (mol/100 mol)/Propionate (mol/100 mol)] + 7.05 was chosen to estimate methane yields of the ground sorghum hybrid evaluated in our experiment. These are summarized in Figure 2.10 (Williams et al., 2019). Hybrid was observed to affect methane yield (P=0.026), indicating that some could be exploited for their capability to reduce methane formation in cattle.

2.4.2.3 *In Vitro* Dry Matter Disappearance

In Vitro Dry Matter Disappearances is another analysis which can estimate the extent of anaerobic digestion of these grains. Figures 2.11 summarize IVDMD for the ground sorghum hybrids used in our experiment. Hybrid affected IVDMD (P<0.01). This observation corresponds well to the observed responses in *K*, which is also an estimate of the extent of digestion.

2.4.2.4 *In Situ* Dry Matter Disappearance

In situ dry matter disappearance is another assay which can measure the extent of anaerobic digestion of these grains. Figure 2.12 summarizes this for the ground sorghum hybrids used. Hybrid effected ISDMD (P<0.001), with many sorghum hybrids having similar ISDMD values as corn. This observation was also comparable to the observations of *K* and IVDMD in our previous assays, where the difference in the degree of *in vitro* microbial digestion of the sorghum grains was significant.

2.4.2.5 Correlations between Starch and Protein Content and Gas Production Parameters, VFA Concentrations, IVDMD, and ISDMD

Only correlations of $r \geq 0.50$ or $r \leq -0.50$ will be discussed here. Starch content of sorghum hybrids was positively correlated (r = 0.81, P<0.001) with *K* while protein was negatively correlated with *K* (r = -0.68, P<0.001). Protein content was also negatively correlated with *m* (r =

-0.54, P<0.001). Lastly, starch content was also positively correlated to methane yield (r = 0.51, P=0.001).

2.4.3 Reconstituted and Ensiled Sorghum

2.4.3.1 Gas Production Parameters

Gas production parameters for RE sorghum hybrids are summarized in Figures 2.13, 2.14, 2.15, and 2.16. Maximum gas production, $t_{1/2}$, r, and m were all affected by hybrid (P<0.001), which was similar for ground sorghum; however, the variation of the estimated parameters for K, $t_{1/2}$, r, and m was less for RE sorghum compared to ground sorghum. A RE corn sample was added in each of these *in vitro* incubations to compare differences in gas production parameters between corn and the sorghum hybrids under study. Many RE sorghum hybrids had similarities to corn in terms of their K, $t_{1/2}$, r, and m (P>0.05) estimates. This is not unsurprising since the ensiling process can make the starch in sorghum more accessible for digestion (Hill et al., 1991).

2.4.3.2 Concentrations of VFA and Methane Yield from *In Vitro* Cultures

Concentrations of acetate, propionate, and butyrate from *in vitro* cultures are summarized in Figures 2.17, 2.18, and 2.19 respectively. No effects of hybrid were observed for acetate (P=0.904), propionate (P=0.499) or butyrate (P=0.717). This observation may be explained by similar VFA yields among the ensiled grain due to the fermentation process. This condition may have overwhelmed any differences in the VFA concentration that occurred with other processing methods. *In vitro* concentrations of VFA from corn were similar to the majority of sorghum hybrids (P>0.05), lending credence to this idea. Methane yield is summarized in Figure 2.20 and was affected by hybrid (P<0.001). As was previously observed VFA's concentrations *from in vitro* cultures, RE corn produced similar amount of methane to the majority of the RE sorghum used as substrate (P>0.05).

2.4.3.3 *In Vitro* Dry Matter Disappearance

Figure 2.21 summarizes IVDMD for RE sorghum. In this analysis, hybrid affected IVDMD ($P<0.05$). Likewise, corn had similar IVDMD to the majority of the sorghum hybrids ($P>0.05$) tested. This observation is similar to the effects that sorghum hybrid had on *K* from our *in vitro* cultures using RE sorghum as substrate.

2.4.3.4 *In Situ* Dry Matter Disappearance

In situ dry matter disappearance for RE sorghum is summarized in Figure 2.22. As was observed previously in relation to IVDMD, ISDMD was also influenced by sorghum hybrid. Corn again had similar ISDMD as to the majority of the sorghum hybrids tested which is a similar observation that was seen with *K* and IVDMD.

2.4.3.5 Correlations between Starch and Protein Content and Gas Production Parameters and ISDMD

There were not as many noteworthy correlations between protein and starch content with our response variables from RE sorghum. There was a positive correlation for starch content and *m* ($r = 0.54$, $P<0.001$) and a negative correlation for protein content and *m* ($r = -0.54$, $P<0.001$). Starch content was also observed to have a positive correlation with ISDMD ($r = 0.58$, $P<0.001$), while protein content was observed to have a negative correlation with ISDMD ($r = -0.54$, $P<0.001$).

2.4.4 Steam-Flaked Sorghum

2.4.4.1 Gas Production Parameters

Figures 2.23, 2.24, 2.25, and 2.26 summarize the gas production parameters *K*, $t_{1/2}$, *r*, and *m* for steam-flaked sorghum. Sorghum hybrid affected *K*, *r*, and *m* ($P<0.05$) in the steam-flaked sorghum *in vitro* cultures; however, $t_{1/2}$ was not affected by hybrid ($P=0.117$). Steam-flaked corn

was also observed to have similar gas production parameters for *K*, *r*, and *m* to many of the sorghum hybrids analyzed (P>0.05). Variation among the gas production parameters for our steam-flaked sorghum hybrids was reduced when comparing these results to the results of the other processing methods.

2.4.4.1 Concentrations of VFA and Methane Yield from *In Vitro* Cultures

Figures 2.27, 2.28, and 2.29 summarize concentrations of VFA from *in vitro* cultures. Sorghum hybrid influenced acetate (P=0.033), propionate (P<0.001), and butyrate concentrations (P=0.012) resulting from *in vitro* microbial digestion. *In vitro* digestion of corn again produced similar *in vitro* VFA concentrations with many of the sorghum hybrids tested (P>0.05).

Methane yield is summarized in Figure 2.30 and was affected by sorghum hybrid (P<0.01). Steam-flaked corn also had a similar methane yield to the majority of our sorghum hybrids (P>0.05).

2.4.4.2 *In Vitro* Dry Matter Disappearance

In vitro dry matter disappearance for steam-flaked sorghum grain is presented in Figure 2.31. There was no effect of hybrid on IVDMD (P=0.375). This is not an unexpected result due to improvements that steam flaking has on sorghum starch's susceptibility to ruminal digestion (Richards and Hicks, 2007). Since no differences were observed between sorghum hybrids in terms of IVDMD, ISDMD was not performed on steam-flaked samples.

2.4.4.3 Correlations between Starch and Protein Content and Gas Production Parameters

There was a positive correlation between starch content and *K* (r = 0.62, P<0.001) and a negative correlation between protein content and *K* (r = -0.65, P<0.001). Other gas production parameters, and VFA concentrations from *in vitro* cultures were not noteworthy between the protein and starch content of our grains and our response variables.

2.5 Discussion

2.5.1 Ground Sorghum

Based on our *in vitro* analyses, there was significant variation among our ground sorghum hybrids susceptibility to *in vitro* digestion. When observing the differences in our response variables among our ground sorghum hybrids, the starch and protein content of the grain were correlated with *K*, and *m*. With *K* measuring the extent of *in vitro* gas production, the starch and protein content is essentially inverse of one another since protein and starch are the major components of the grain. Proteins in sorghum though, have been observed to have reduced digestibility compared to protein from wheat or maize when the grain is cooked (Duodu et al., 2003). Decreases in sorghum protein digestibility is caused by a multitude of factors but protein crosslinking seems to have the greatest impact (Duodu et al., 2003). Since the protein in our sorghum hybrids could be less digestible, this could explain the negative correlations of protein content with *K* and *m* in our samples. Methane yield being positively correlated to starch, was likely due to methane being a component of the total gas production from microbial digestion in the *in vitro* cultures.

2.5.2 Reconstituted and Ensiled Sorghum

The variation in susceptibilities to *in vitro* digestion among RE sorghum hybrids was reduced when comparing these results to ground sorghum. More extensive processing methods alter the protein-starch matrix in sorghum, which could allow microbial enzymes improved access to the starch and protein inside the grain (Hill et al., 1991; Richards and Hicks, 2007; Silva et al., 2020). The observations of reduced impact of the protein content of RE sorghum and its lack of a relationship with parameter *K* could be explained by the improved microbial access to the starch and proteins of the RE sorghum grain. This suggests that RE sorghum is not as negatively

affected in terms of microbial digestion by increasing protein content of the grain as ground sorghum was.

2.5.3 Steam-flaked Sorghum

The differences among steam-flaked sorghum in terms of susceptibility to *in vitro* digestion were also reduced. The only noteworthy correlation between the protein or starch content of the grains was with *K* using steam-flaked sorghum. Protein content still could be having a negative effect on the extent of microbial digestion of our steam-flaked grains since *K* was negatively correlated with protein. This is probably due to the steam-flaking process increasing the rumen undegradable protein fraction of the grains as well as the protein crosslinking that could happen when these grains are steamed (Duodu et al., 2002; Maria et al., 2018). Parameter *m* was not correlated with the protein content of the grain, which is unlike the observations seen in ground or RE sorghum. This could indicate that the rate of microbial digestion of these steam-flaked grains is not as negatively impacted by the protein content of the grains.

2.6 Conclusion

Wester et al. (1992) and Pedersen et al. (2000) have reported that sorghum grains exhibit a wide range of susceptibility to *in vitro* ruminal digestion. Our research further supports this and indicates that variations in sorghum protein and starch content among varieties could influence the extent and rate of *in vitro* digestion of ground sorghum, and to a lesser degree, the extent of *in vitro* digestion of steam-flaked sorghum. Our observations revealed there were noteworthy variations in the extent and rates of *in vitro* gas production in our ground sorghum hybrids. Our results also suggest that many of our sorghum hybrids processed with RE, or steam-flaking have reduced variations in terms of different *in vitro* gas production parameters, VFA concentrations from *in vitro* cultures, IVDMD, and ISDMD as similarly processed corn. These findings suggest

that some of our sorghum hybrids with better responses to *in vitro* digestion could be substituted for corn in diets for finishing cattle.

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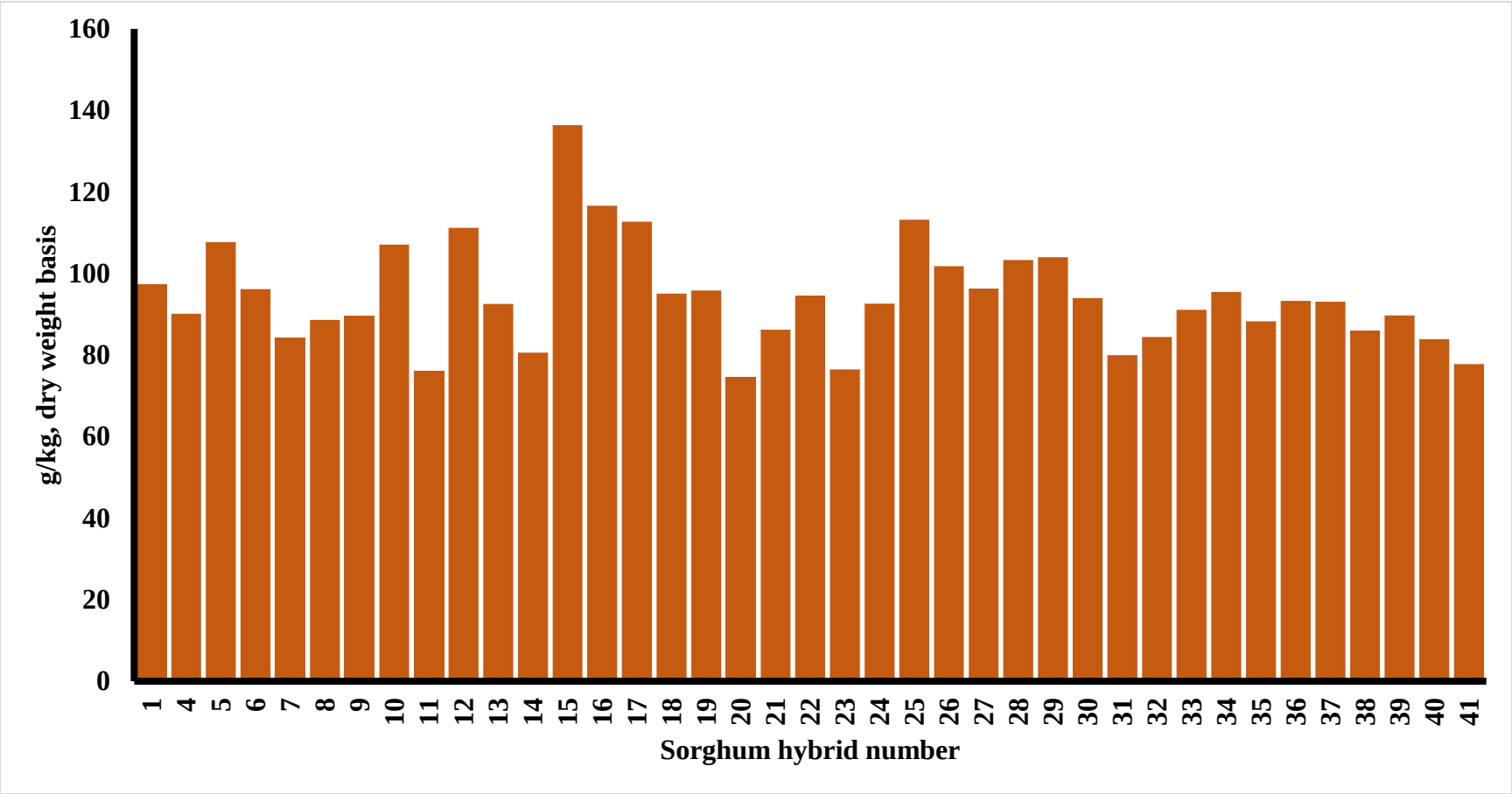

Figure 2.1 Near infrared spectroscopy (NIR) estimation of protein content of each sorghum hybrid.
A Perten DA 7250 spectrometer (Perten Instruments, Springfield, IL, USA) was used to scan each grain to estimate protein content. Calibration and statistical models used to estimate starch content were developed from previous work with sorghum cultivars (Peiris et al., 2019; Peiris et al., 2020).

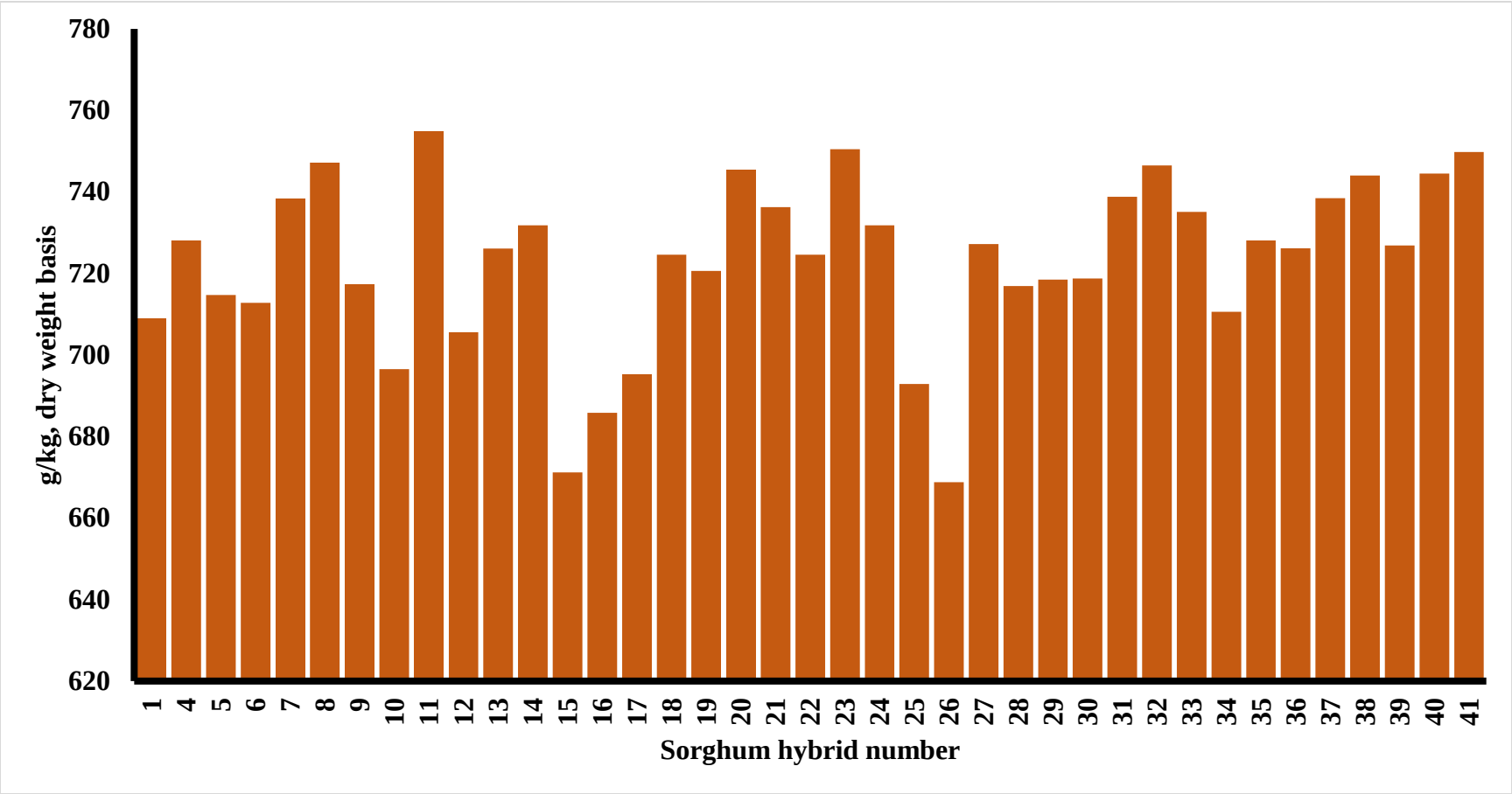

Figure 2.2 Near infrared spectroscopy (NIR) estimation of starch content of each sorghum hybrid.
A Perten DA 7250 spectrometer (Perten Instruments, Springfield, IL, USA) was used to scan each grain to estimate starch content. Calibration and statistical models used to estimate starch content were developed from previous work with sorghum cultivars (Peiris et al., 2021).

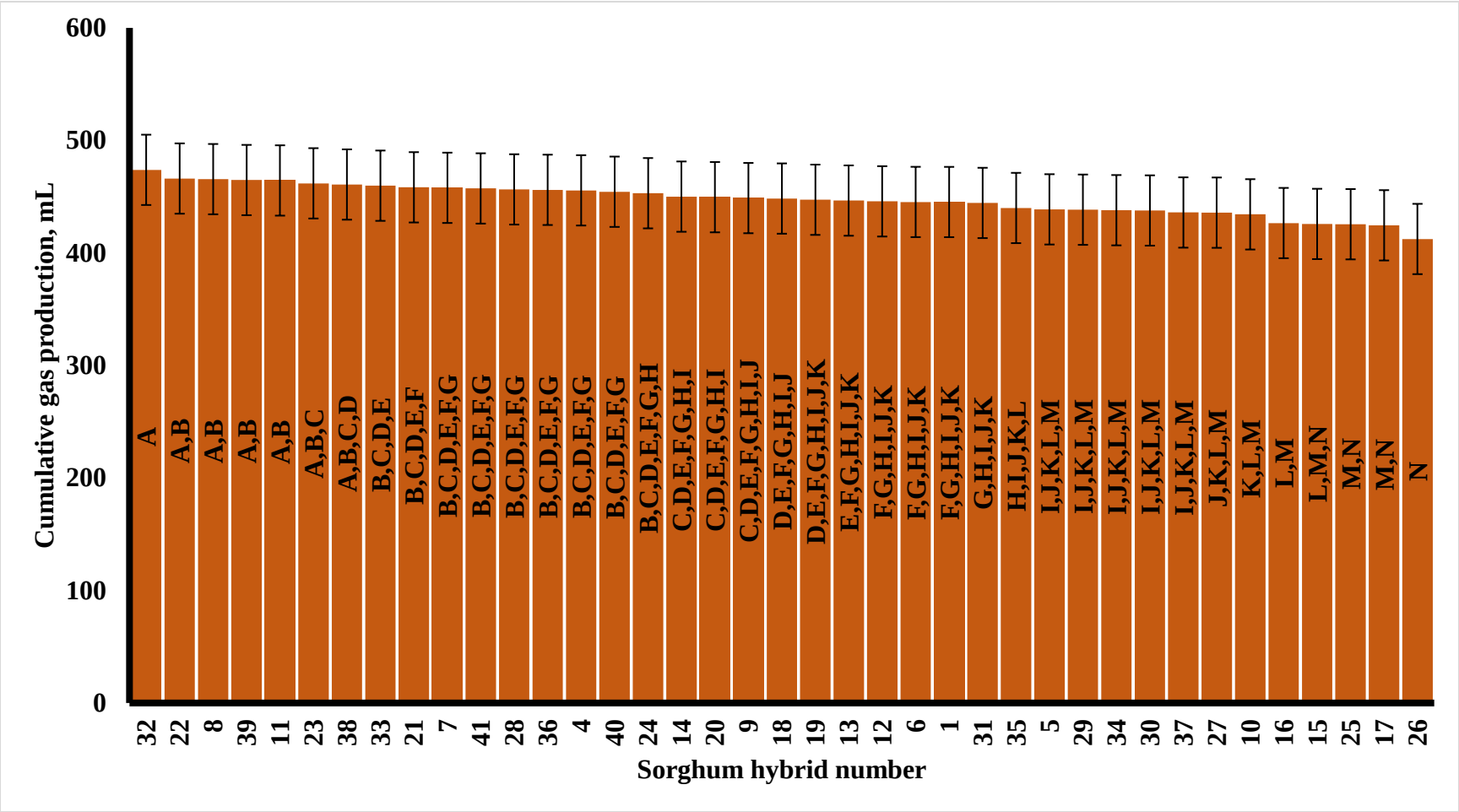

Figure 2.3 Maximum *in vitro* gas production (*K*) for *in vitro* cultures of mixed ruminal microorganisms with different ground sorghum hybrids as substrate.

Values are least-square means ± standard error of the means. Hybrid affected *K* (P<0.001). Sorghum hybrids were grown in duplicate, and 11 *in vitro* analyses were utilized to estimate *K* which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different (P<0.05).

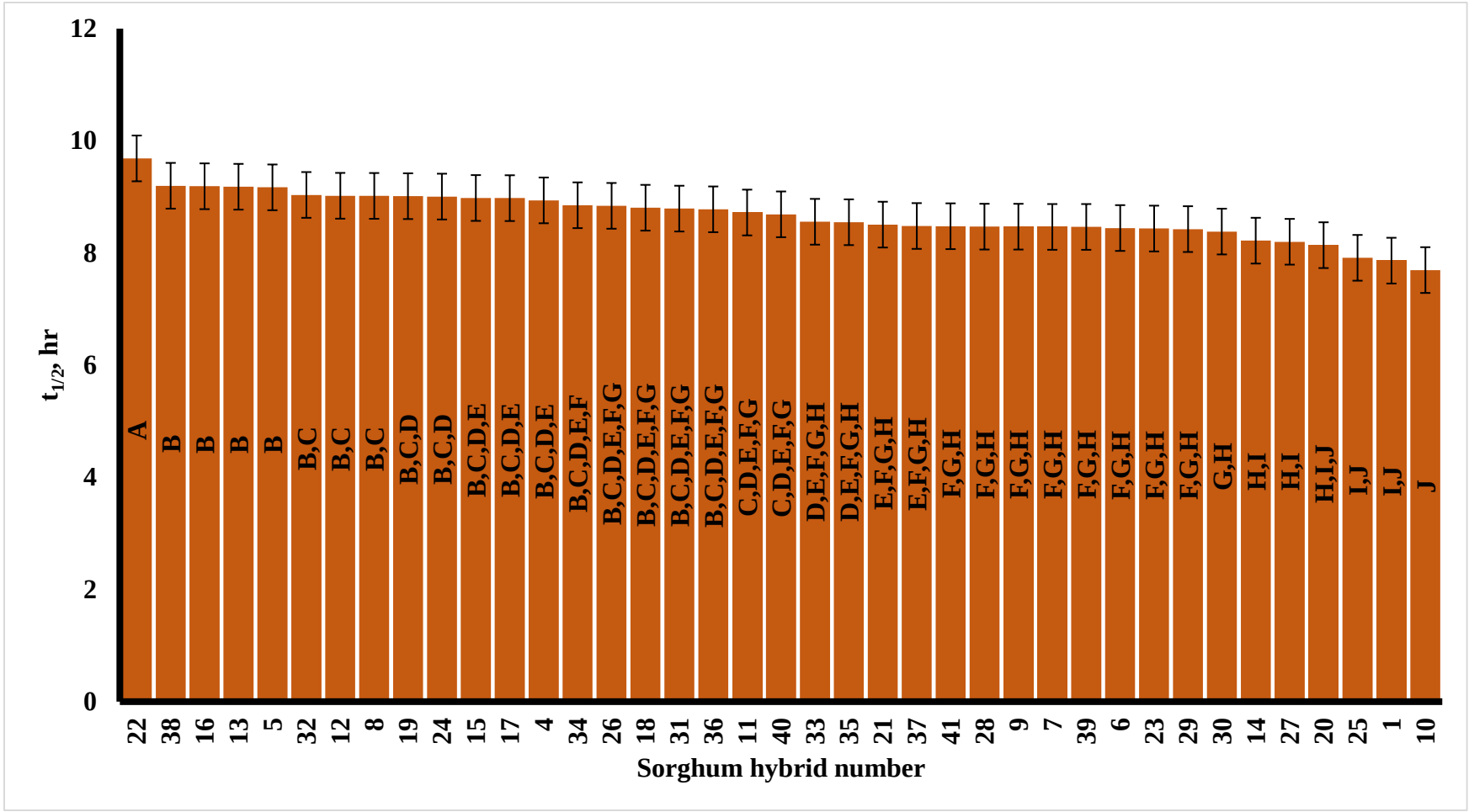

Figure 2.4 Time to reach half maximum gas production ($t_{1/2}$) for *in vitro* cultures of mixed ruminal microorganisms with different ground sorghum hybrids as substrate.

Values are least-square means ± standard error of the means. Hybrid affected $t_{1/2}$ ($P<0.001$). Decreased $t_{1/2}$ values indicate faster rates of digestion. Sorghum hybrids were grown in duplicate, and 11 *in vitro* analyses were utilized to estimate $t_{1/2}$ which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different ($P<0.05$).

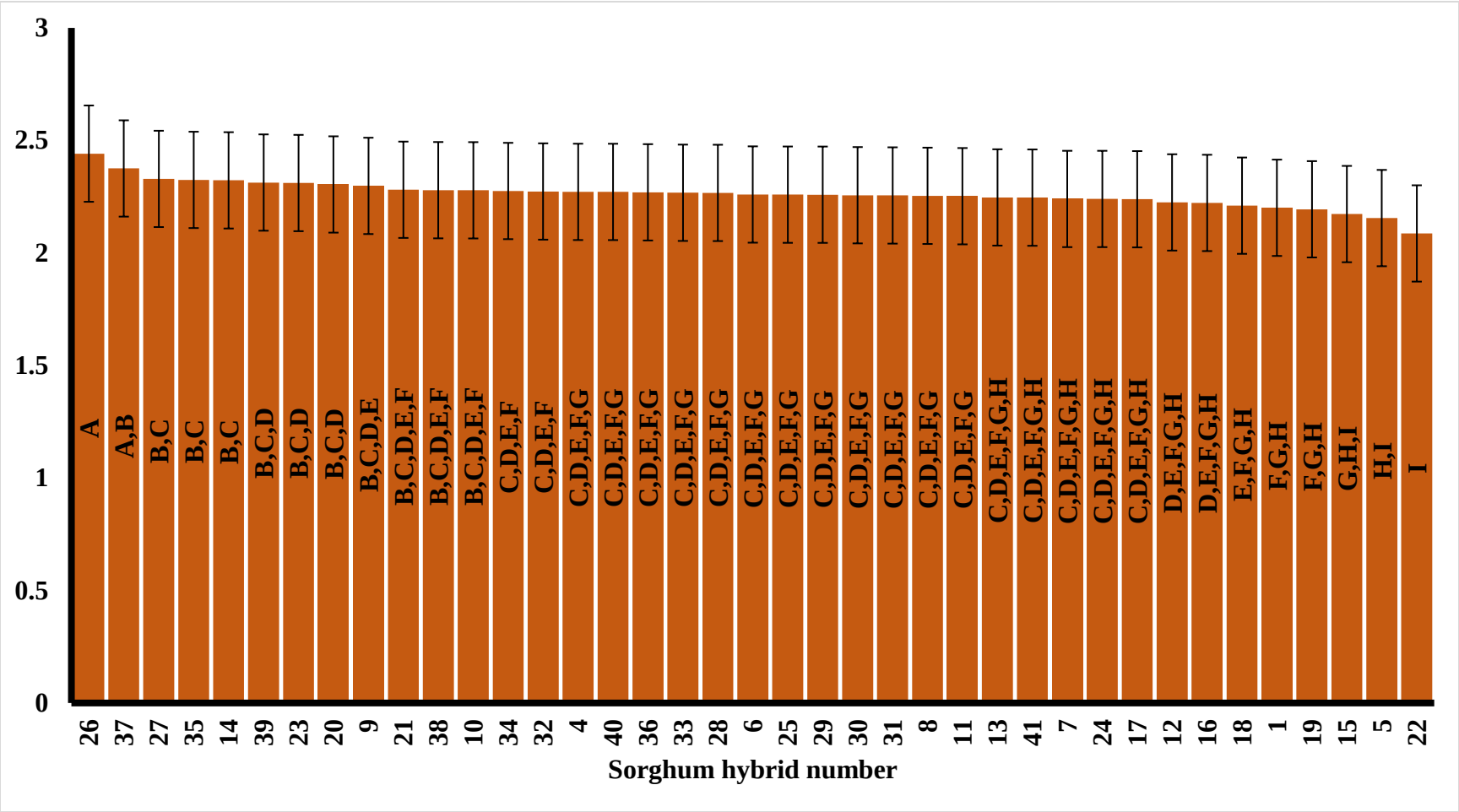

Figure 2.5 Rate of gas production (*r*) for *in vitro* cultures of mixed ruminal microorganisms with different ground sorghum hybrids as substrate.

Values are least-square means ± standard error of the means. Hybrid affected *r* ($P<0.001$). Greater *r* values indicate faster rates of digestion. Sorghum hybrids were grown in duplicate, and 11 *in vitro* analyses were utilized to estimate *r* which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different ($P<0.05$).

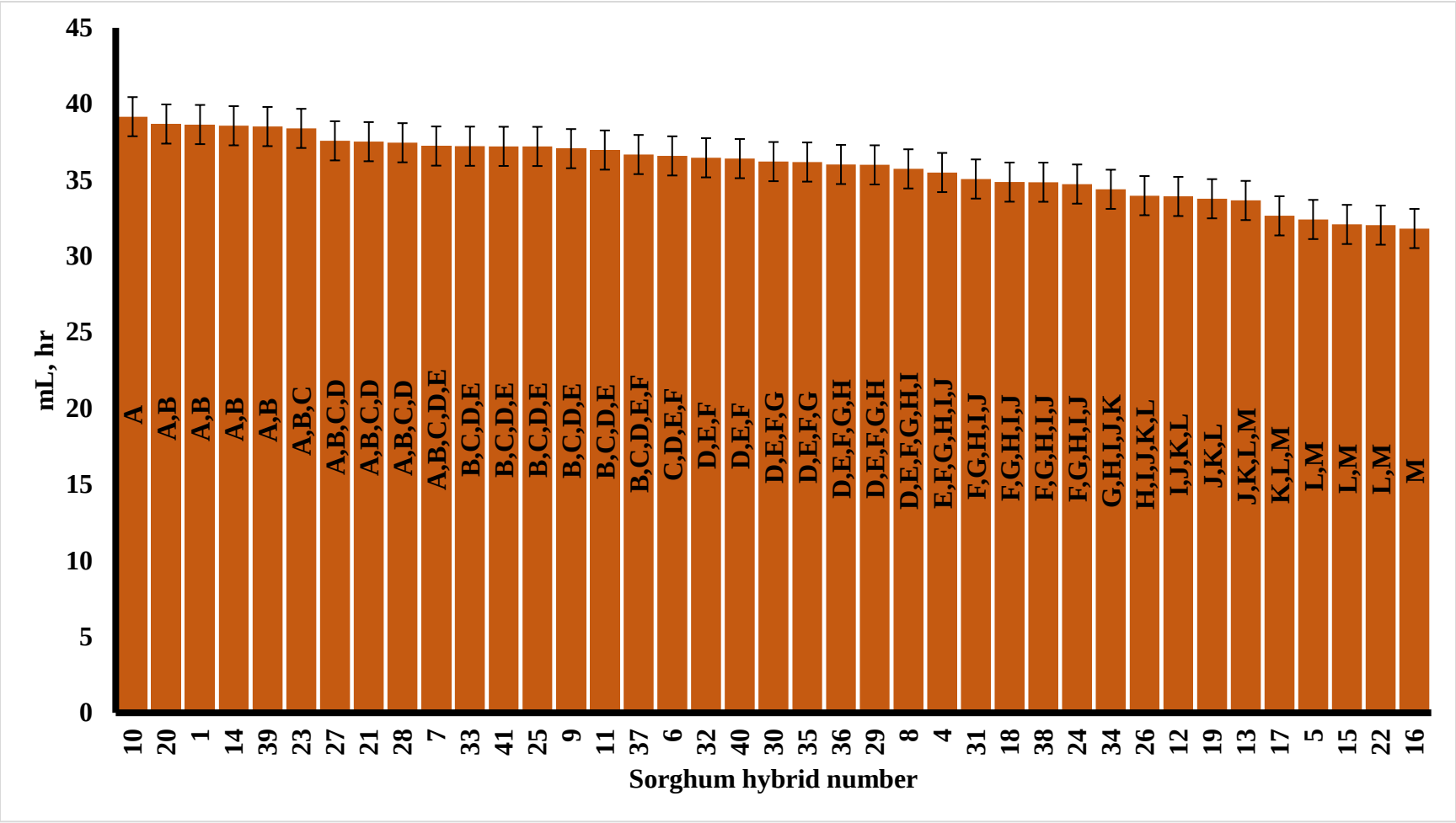

Figure 2.6 Maximum rate of gas production (*m*) for *in vitro* cultures of mixed ruminal microorganisms with different ground sorghum hybrids as substrate.

Values are least-square means ± standard error of the means. Hybrid affected *m* ($P<0.001$). Greater *m* values indicate faster rates of digestion. Sorghum hybrids were grown in duplicate, and 11 *in vitro* analyses were utilized to estimate *m* which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different ($P<0.05$).

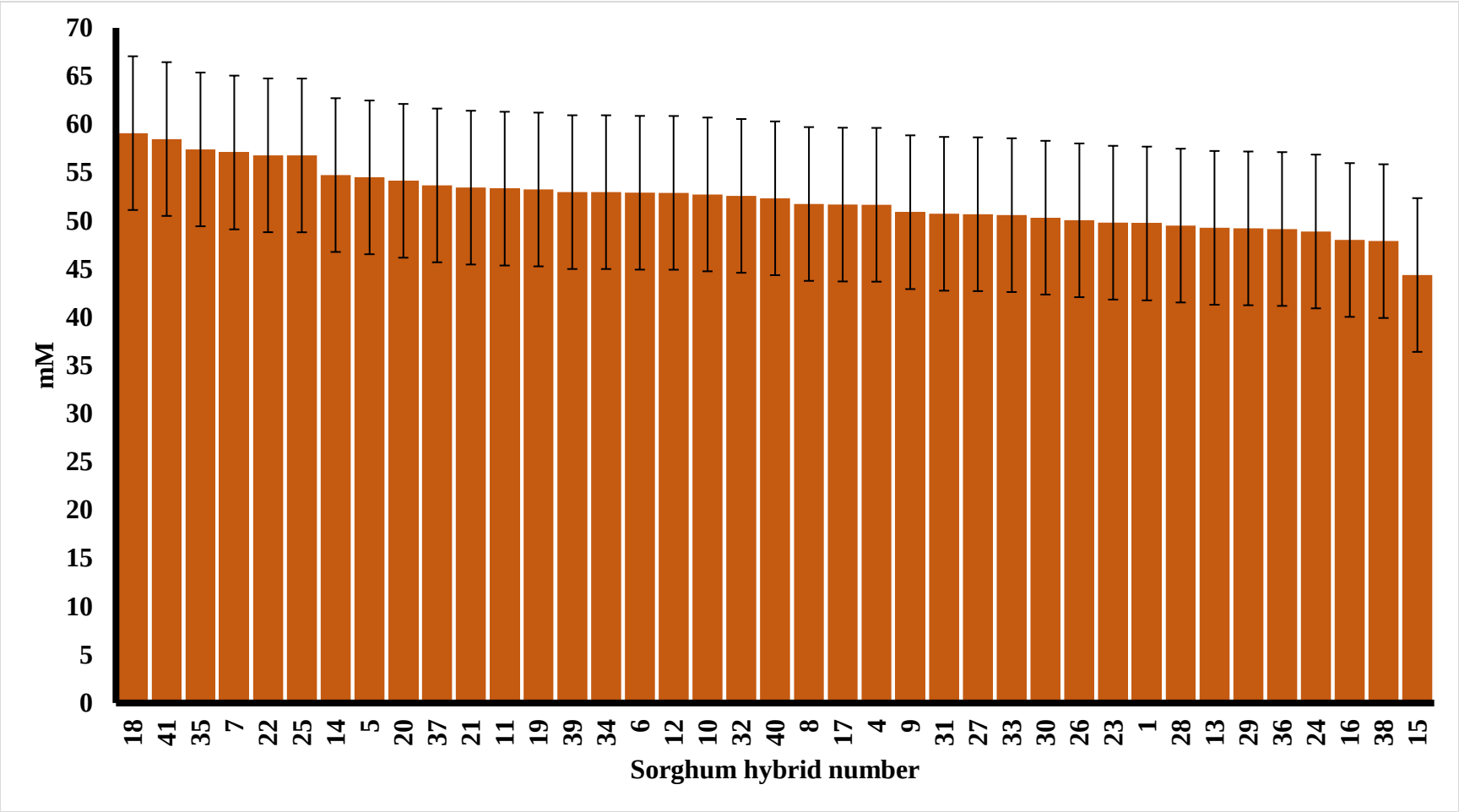

Figure 2.7 Acetate concentrations from *in vitro* cultures of mixed ruminal microorganisms with different ground sorghum hybrids as substrate.

Values are least-square means ± standard error of the means. Hybrid did not affect acetate concentrations (P=0.692). Sorghum hybrids were grown in duplicate, and 11 *in vitro* analyses were utilized to estimate acetate concentration from *in vitro* cultures.

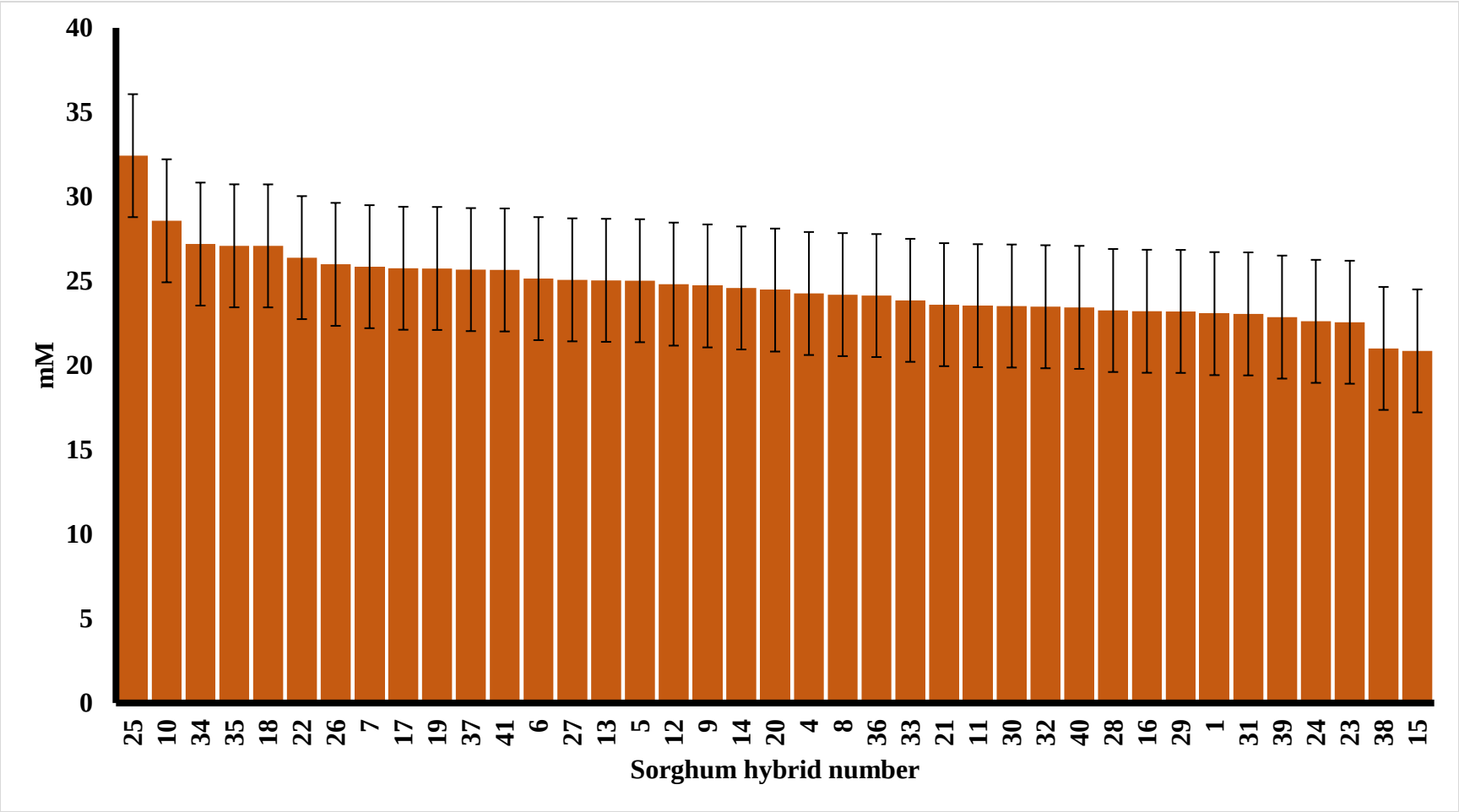

Figure 2.8 Propionate concentrations from *in vitro* cultures of mixed ruminal microorganisms with different ground sorghum hybrids as substrate.

Values are least-square means ± standard error of the means. Hybrid did not affect propionate concentrations (P=0.669). Sorghum hybrids were grown in duplicate, and 11 *in vitro* analyses were utilized to estimate propionate concentration from *in vitro* cultures.

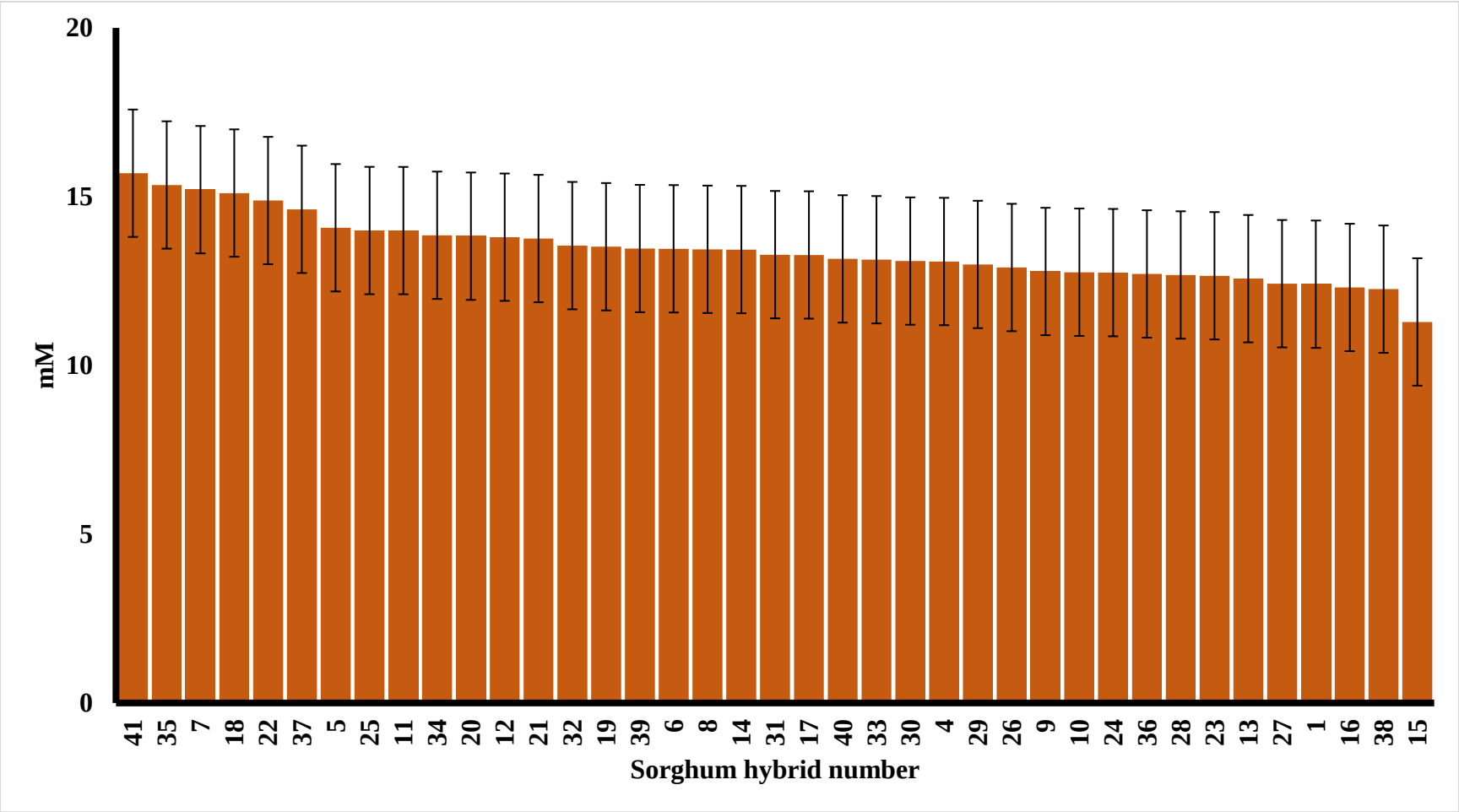

Figure 2.9 Butyrate concentrations from *in vitro* cultures of mixed ruminal microorganisms with different ground sorghum hybrids as substrate.

Values are least-square means ± standard error of the means. Hybrid did not affect butyrate concentrations (P=0.610). Sorghum hybrids were grown in duplicate, and 11 *in vitro* analyses were utilized to estimate butyrate concentration *in vitro* cultures.

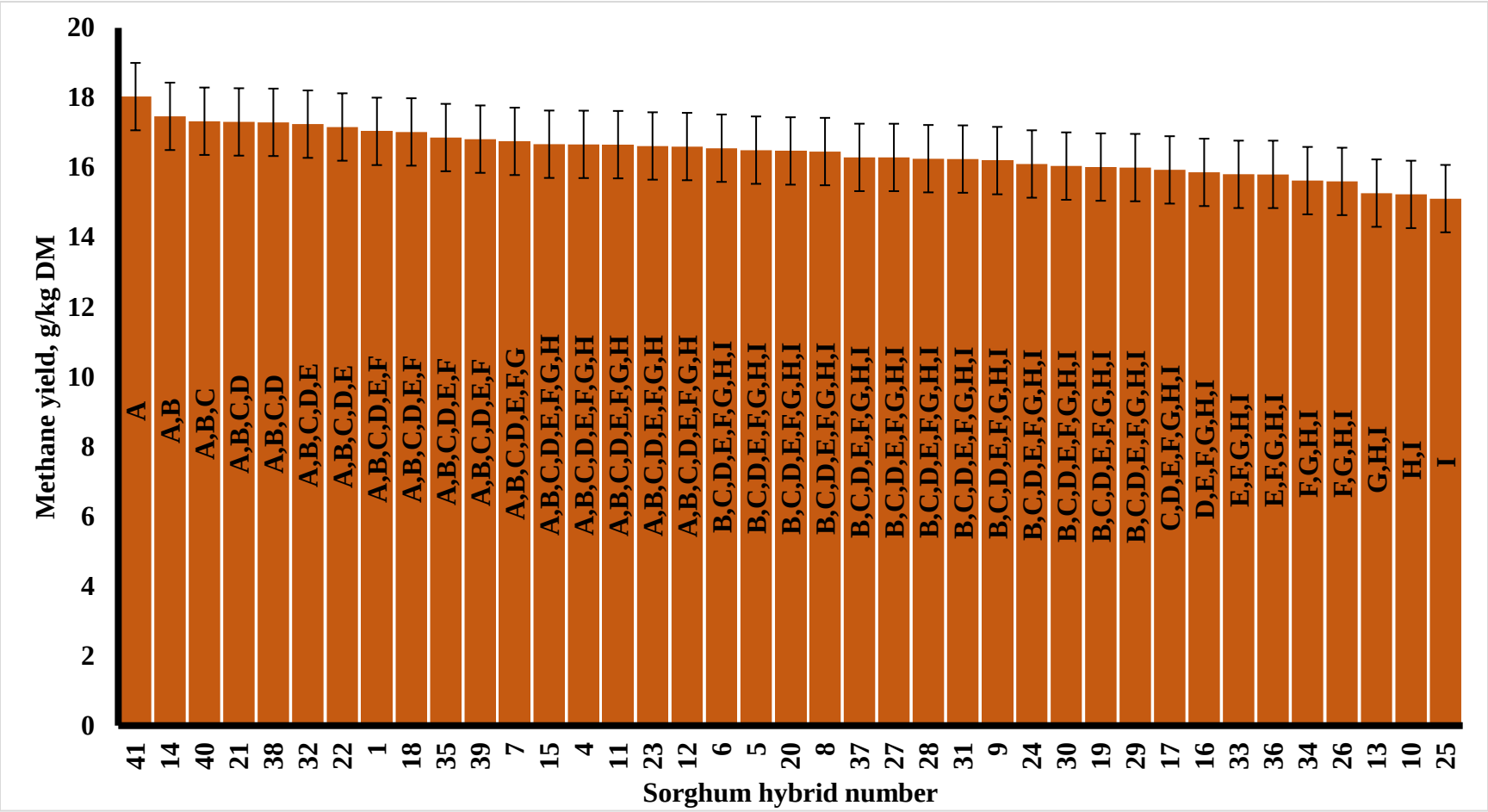

Figure 2.10 Methane yield from *in vitro* cultures of mixed ruminal microorganisms with different ground sorghum hybrids as substrate.

Values are least-square means ± standard error of the means. Hybrid affected methane yield (P=0.026). Methane yield was estimated using the formula "Methane yield = 4.08×(A/P) + 7.05" (Williams et al., 2019). Sorghum hybrids were grown in duplicate, and 11 *in vitro* analyses were utilized to estimate methane yield which was derived from the *in vitro* digestion of each hybrid. Bars without a common letter are different (P<0.05).

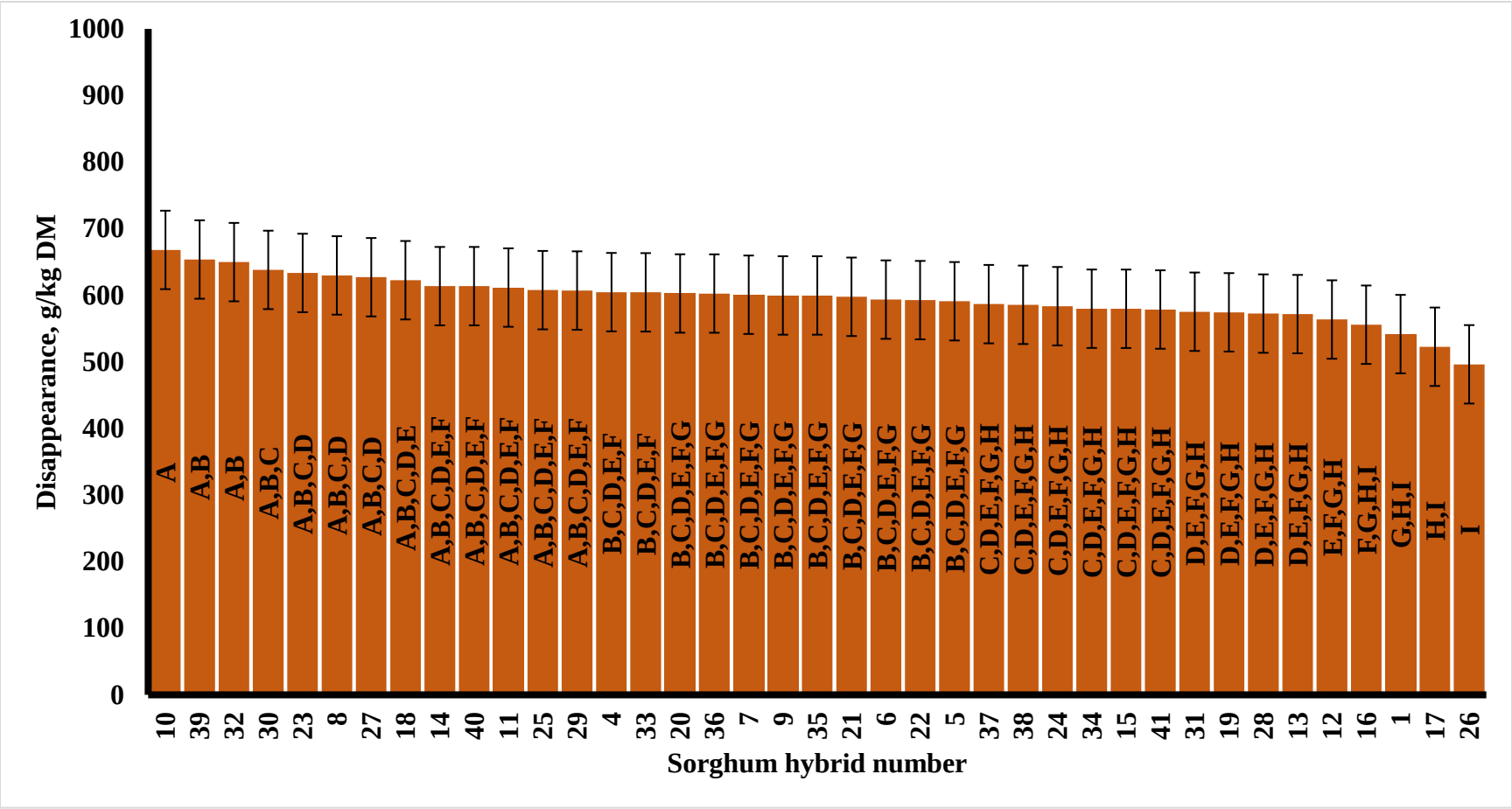

Figure 2.11 *In vitro* dry matter disappearance (IVDMD) from cultures of mixed ruminal microorganisms with different ground sorghum hybrids as substrate.

Values are least-square means ± standard error of the means of substrate dry matter that disappeared on g/kg basis. Hybrid affected IVDMD ($P<0.001$). Greater IVDMD indicates a greater extent of *in vitro* microbial digestion of sorghum hybrids. Sorghum hybrids were grown in duplicate, and 10 *in vitro* analyses were utilized to estimate IVDMD. Bars without a common letter are different ($P<0.05$).

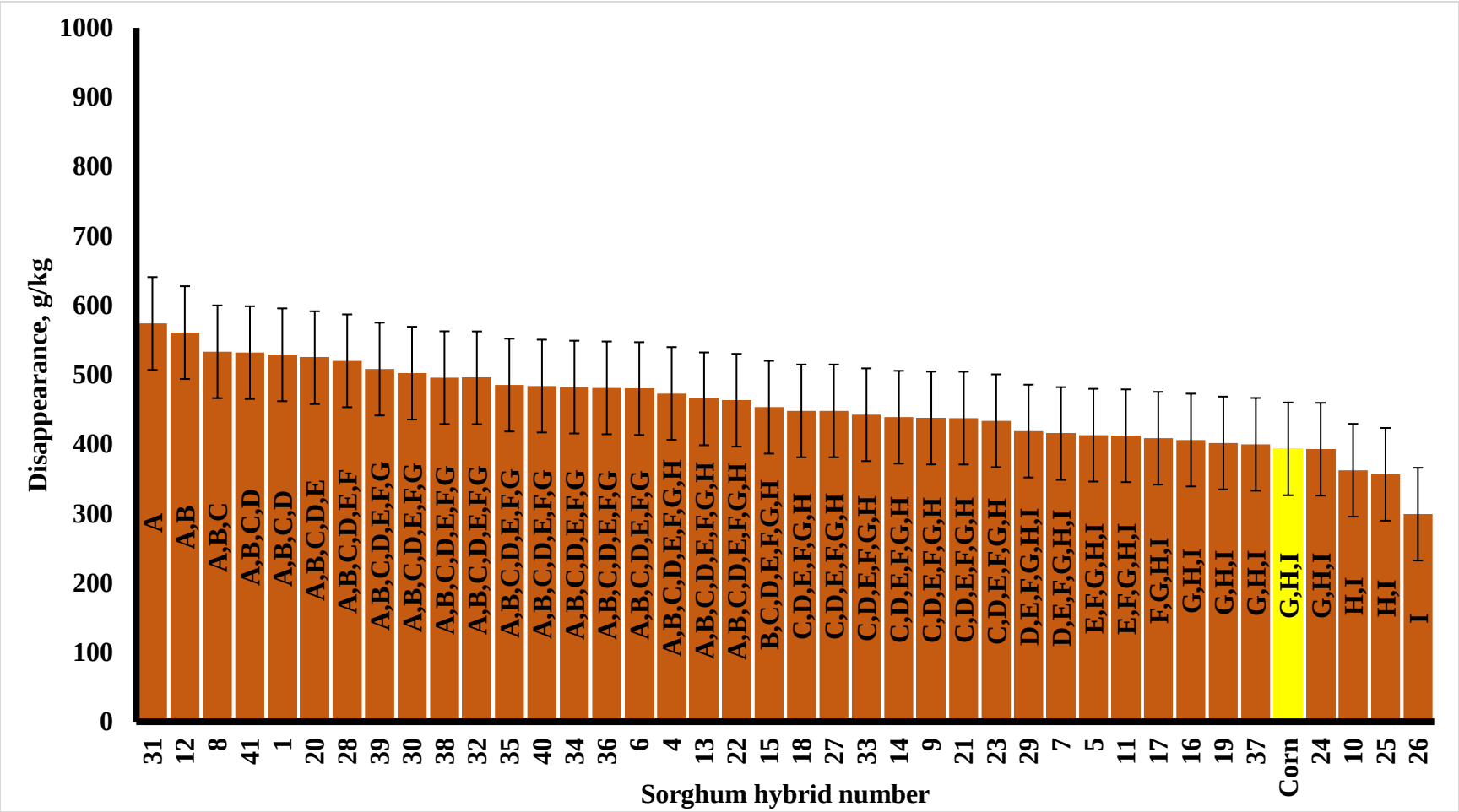

Figure 2.12 *In situ* dry matter disappearance (ISDMD) of ground sorghum and corn from incubation of grains in Ankom R510 concentrate bags in 3 ruminally fistulated steers for 16 h.

Values are least-square means ± standard error of the means of substrate dry matter that disappeared while incubated in the rumen on a g/kg basis. Hybrid affected ISDMD ($P<0.001$). Greater ISDMD indicates a greater extent of microbial digestion of sorghum hybrids. Sorghum hybrids were grown in duplicate, and 12 replications were performed to estimate ISDMD. Bars without a common letter are different ($P<0.05$).

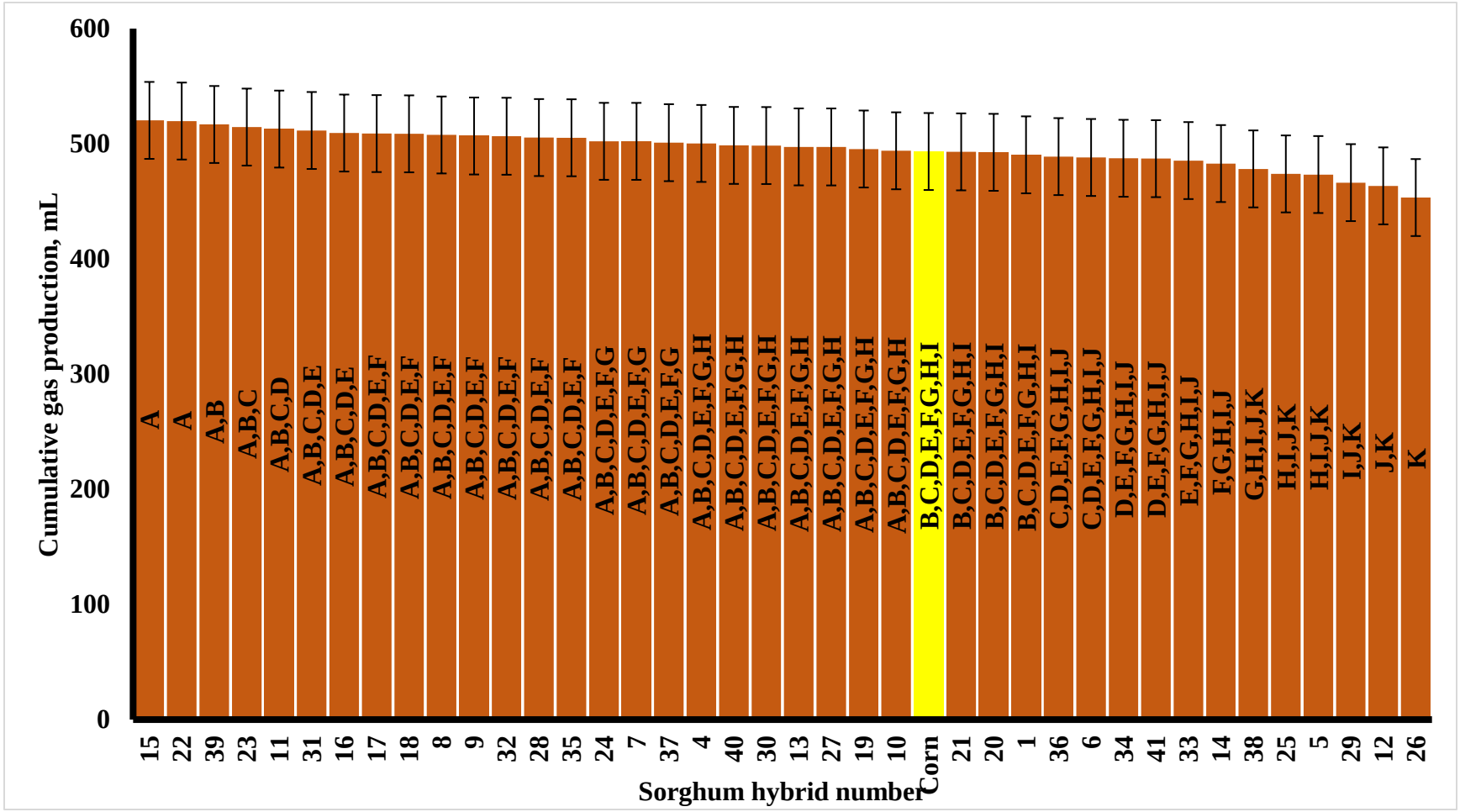

Figure 2.13 Maximum *in vitro* gas production (*K*) for *in vitro* cultures of mixed ruminal microorganisms with different reconstituted and ensiled sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected *K* ($P<0.001$). Sorghum hybrids were grown in duplicate, and 12 *in vitro* analyses were utilized to estimate *K* which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different ($P<0.05$).

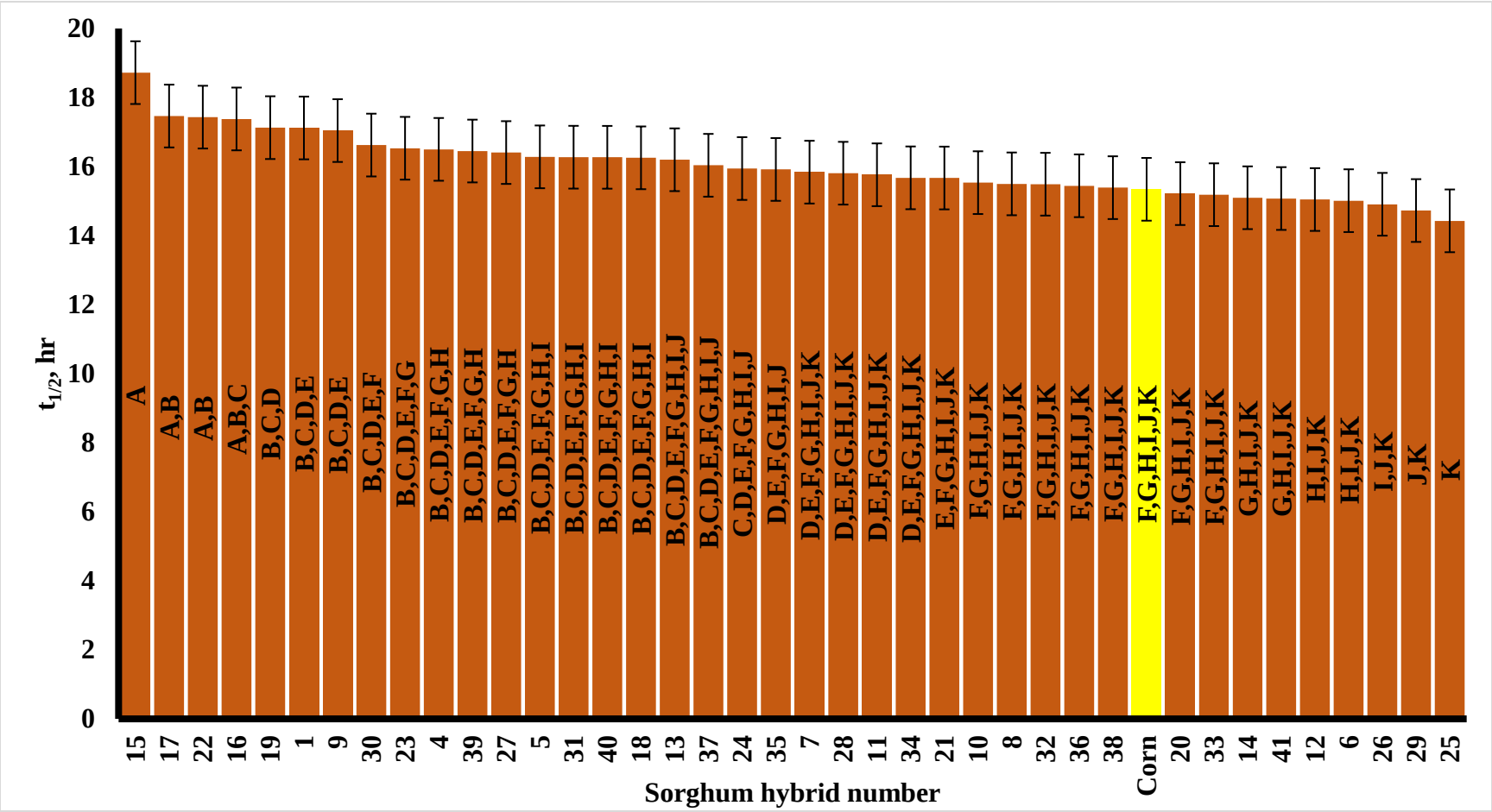

Figure 2.14 Time to reach half maximum gas production ($t_{1/2}$) for *in vitro* cultures of mixed ruminal microorganisms with different reconstituted and ensiled sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected $t_{1/2}$ ($P<0.001$). Sorghum hybrids were grown in duplicate, and 12 *in vitro* analyses were utilized to estimate $t_{1/2}$ which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different ($P<0.05$).

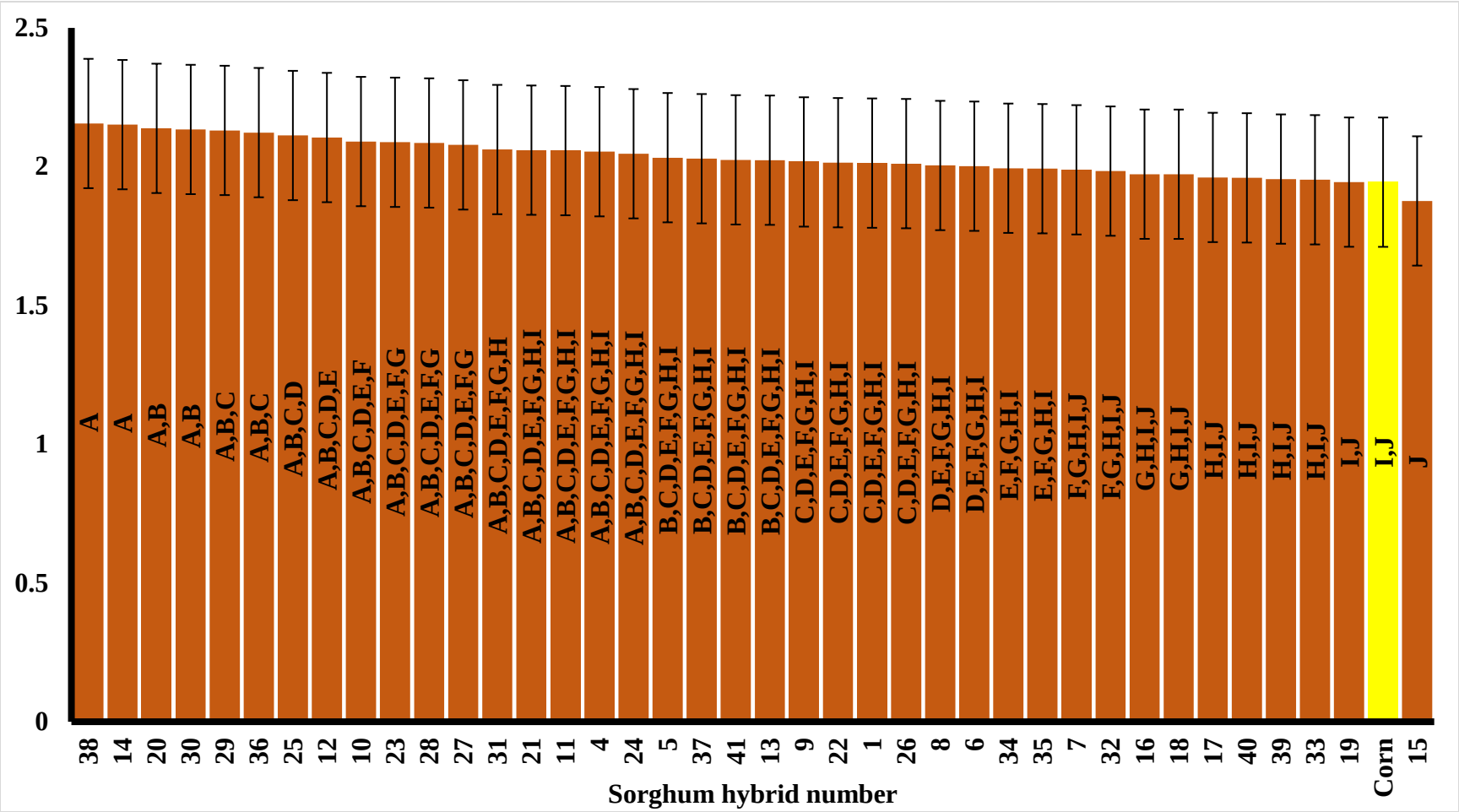

Figure 2.15 Rate of gas production (*r*) for *in vitro* cultures of mixed ruminal microorganisms with different reconstituted and ensiled sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected *r* (P<0.001). Greater *r* values indicate faster rates of digestion. Sorghum hybrids were grown in duplicate, and 12 *in vitro* analyses were utilized to estimate *r* which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different (P<0.05).

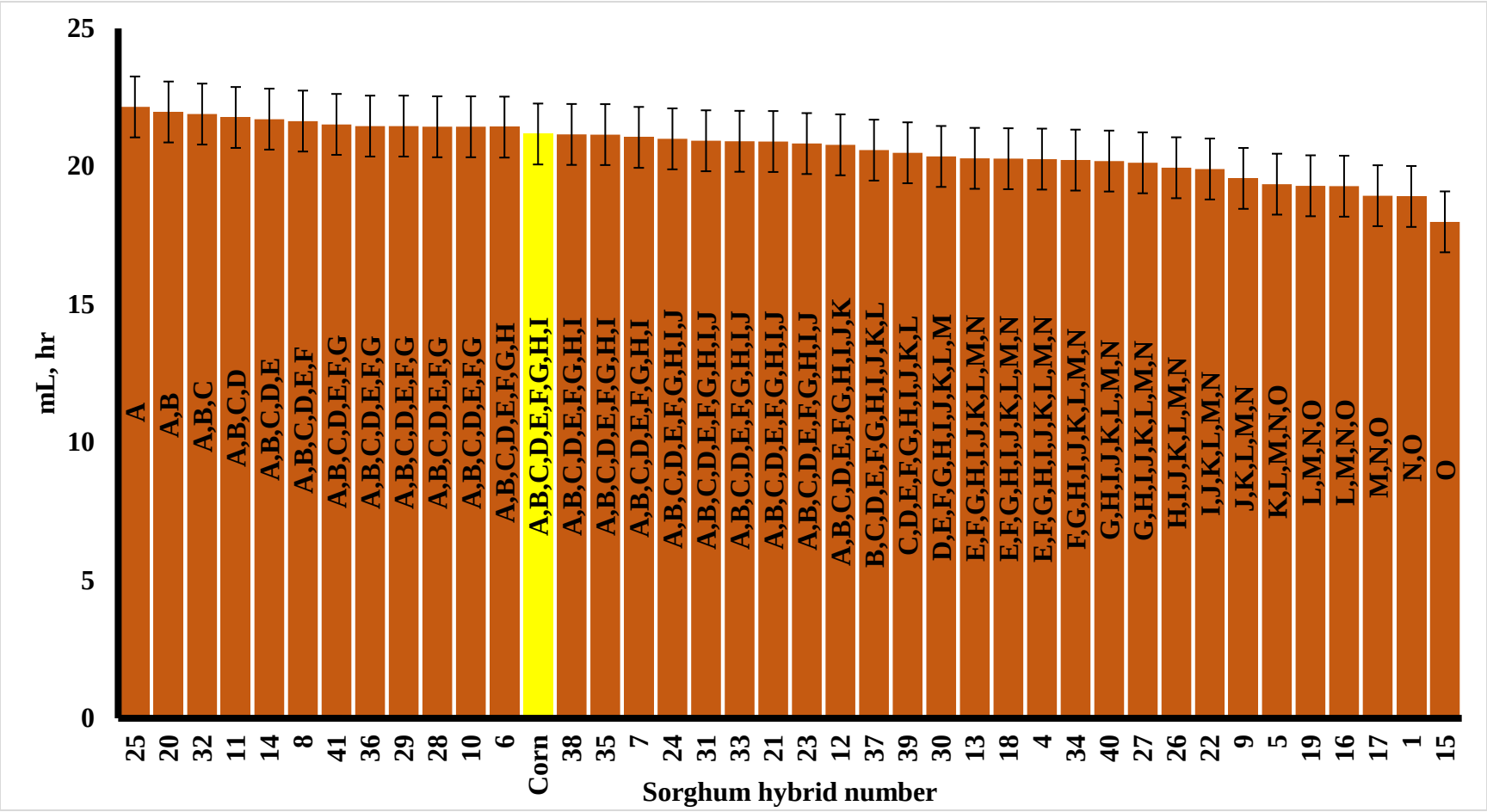

Figure 2.16 Maximum rate of gas production (*m*) for *in vitro* cultures of mixed ruminal microorganisms with different reconstituted and ensiled sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected *m* ($P<0.001$). Greater *m* values indicate faster rates of digestion. Sorghum hybrids were grown in duplicate, and 12 *in vitro* analyses were utilized to estimate *m* which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different ($P<0.05$).

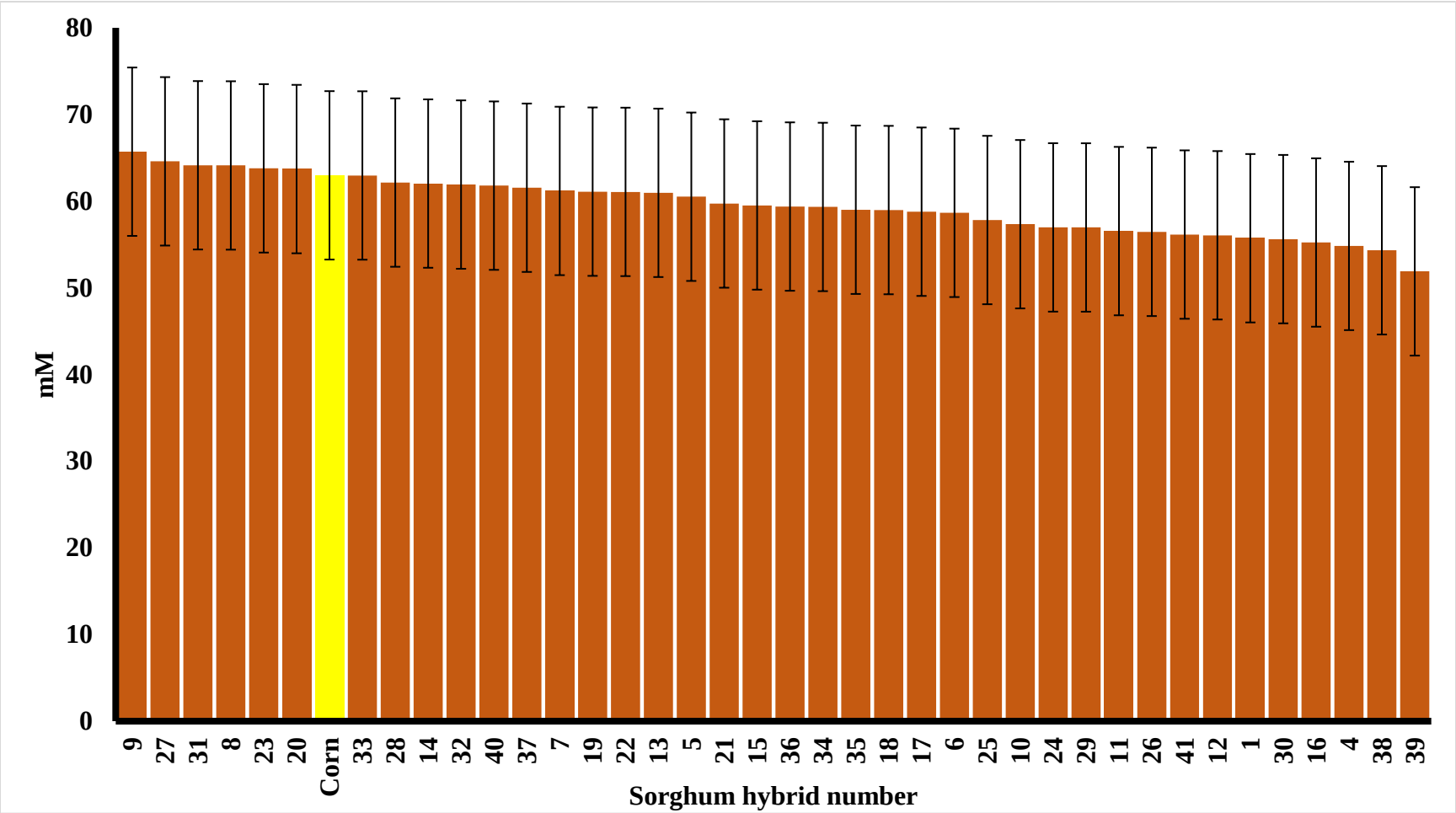

Figure 2.17 Acetate concentrations from *in vitro* cultures of mixed ruminal microorganisms with different reconstituted and ensiled sorghum hybrids and corn.

Values are least-square means ± standard error of the means. Hybrid did not affect acetate concentrations (P=0.904). Sorghum hybrids were grown in duplicate, and 12 *in vitro* analyses were utilized to estimate acetate concentration from *in vitro* cultures.

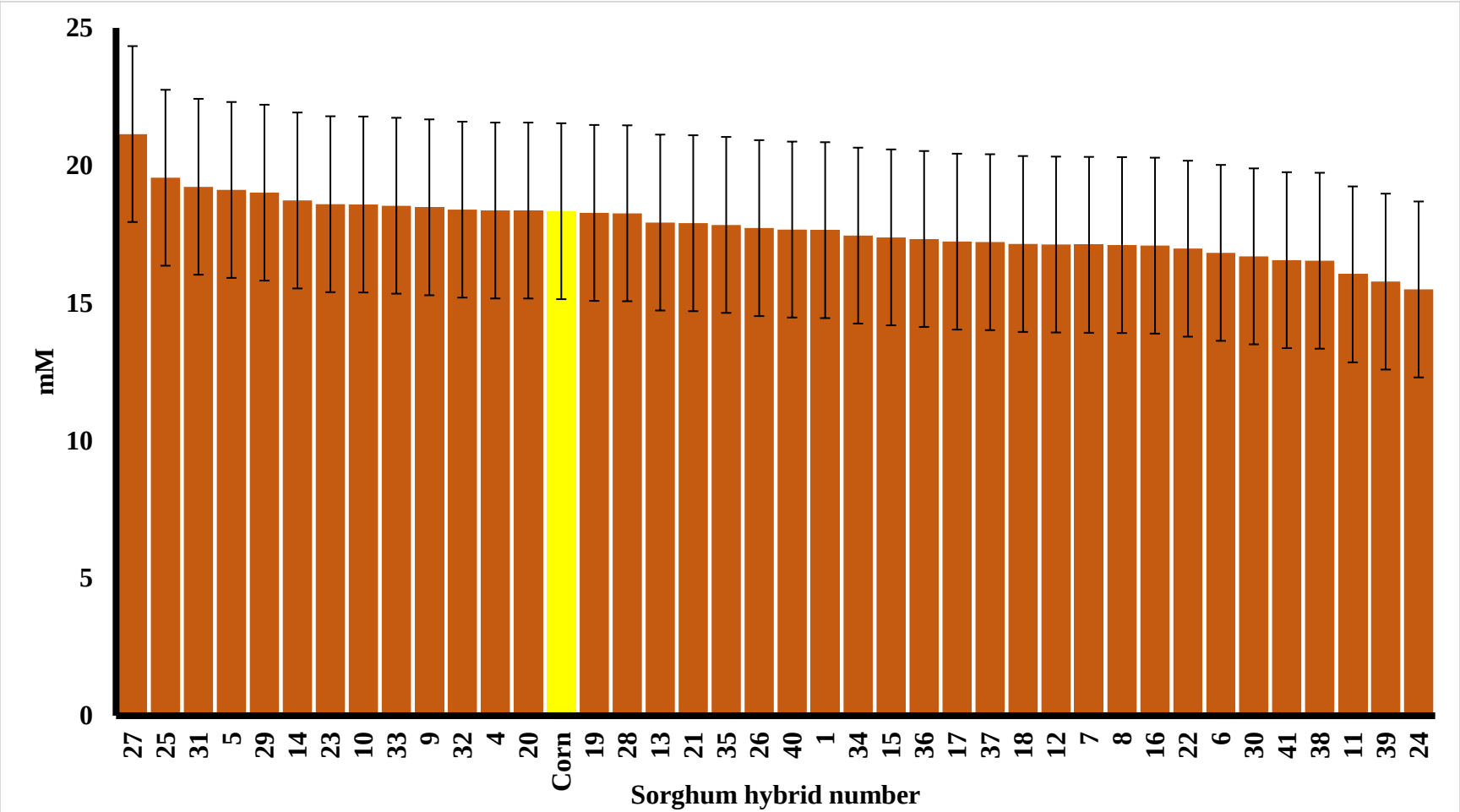

Figure 2.18 Propionate concentrations from *in vitro* cultures of mixed ruminal microorganisms with different reconstituted and ensiled sorghum hybrids and corn.

Values are least-square means ± standard error of the means. Hybrid did not affect propionate concentrations (P=0.499). Sorghum hybrids were grown in duplicate, and 12 *in vitro* analyses were utilized to estimate propionate concentration *in vitro* cultures.

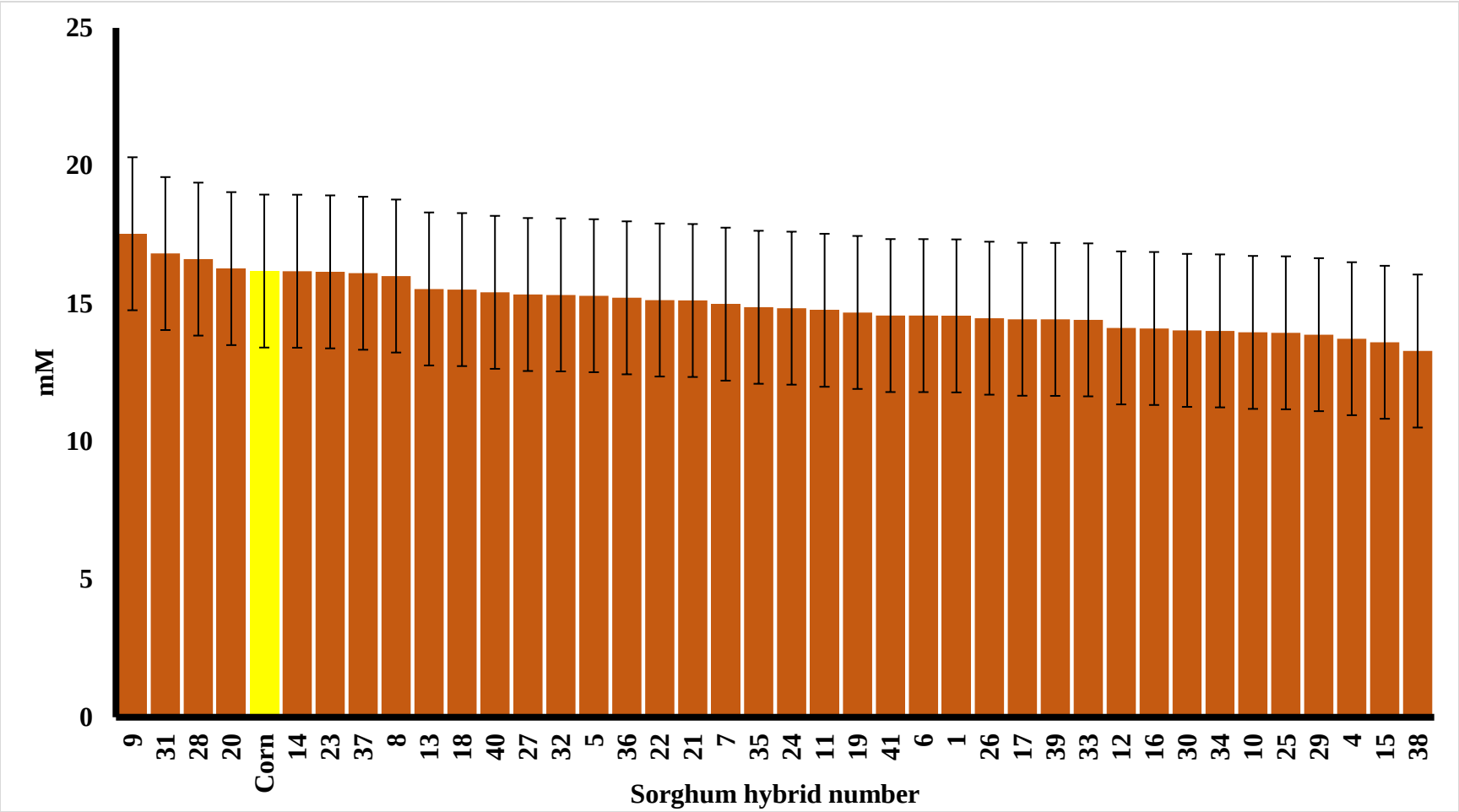

Figure 2.19 Butyrate concentrations from *in vitro* cultures of mixed ruminal microorganisms with different reconstituted and ensiled sorghum hybrids and corn.

Values are least-square means ± standard error of the means. Hybrid did not affect butyrate concentrations (P=0.717). Sorghum hybrids were grown in duplicate, and 12 *in vitro* analyses were utilized to estimate butyrate concentration *in vitro* cultures.

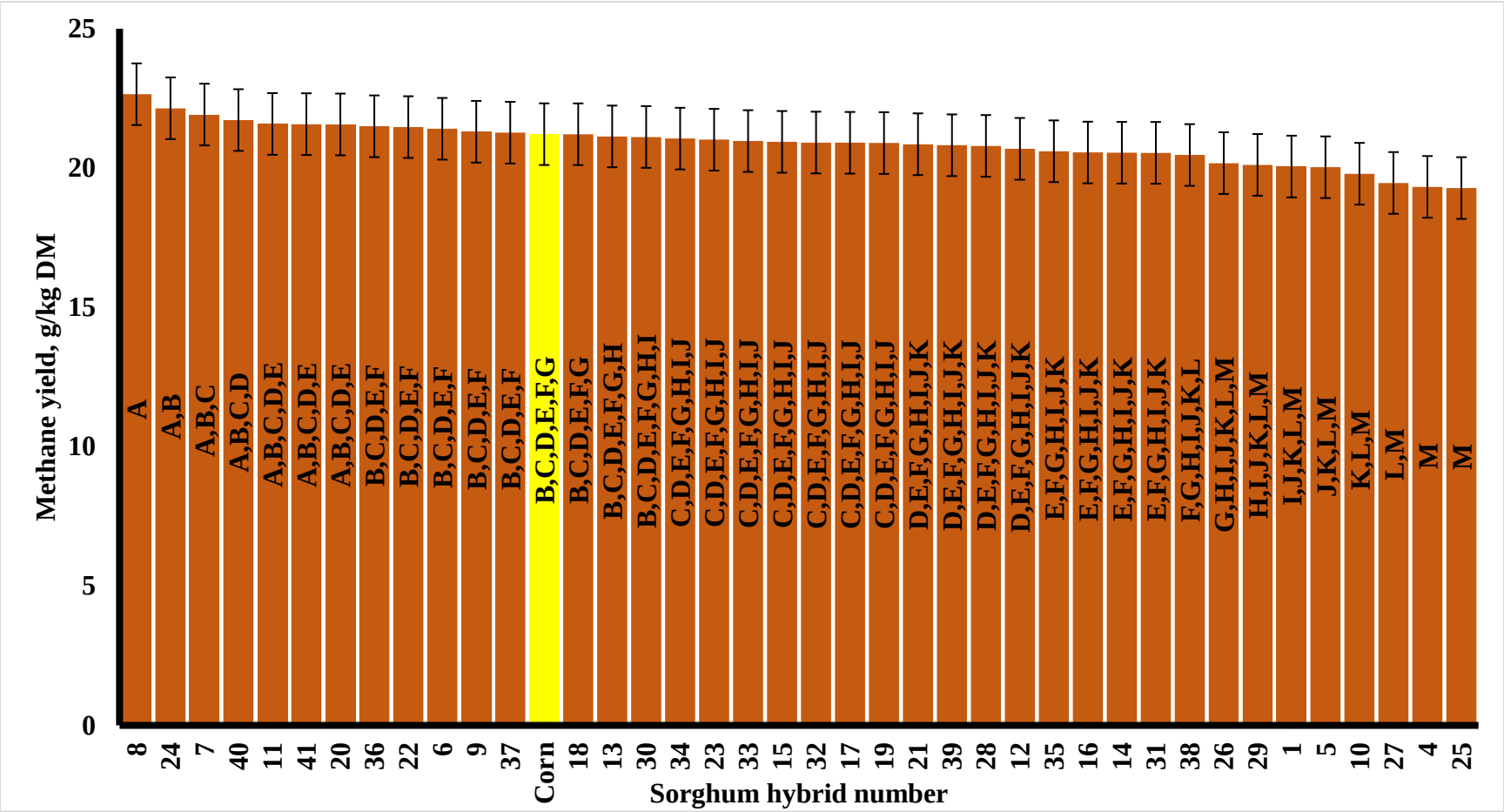

Figure 2.20 Methane yield from *in vitro* cultures of mixed ruminal microorganisms with reconstituted and ensiled sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected methane yield ($P<0.001$). Methane yield was estimated using the formula "methane yield = 4.08×(A/P) + 7.05" (Williams et al., 2019). Sorghum hybrids were grown in duplicate, and 12 *in vitro* analyses were utilized to estimate methane yield which was derived from the *in vitro* digestion of each hybrid. Bars without a common letter are different ($P<0.05$).

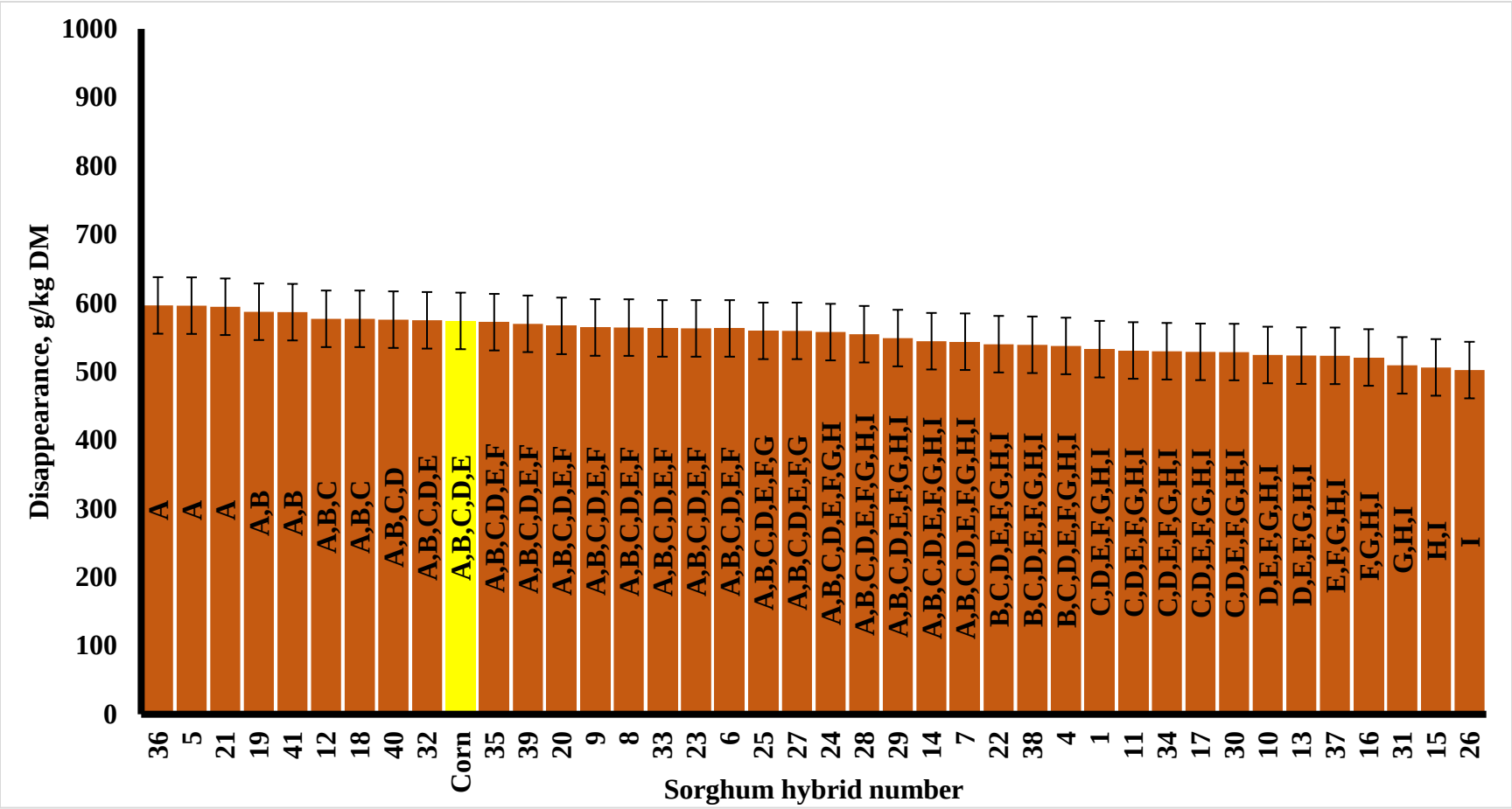

Figure 2.21 *In vitro* dry matter disappearance (IVDMD) from cultures of mixed ruminal microorganisms with different reconstituted and ensiled sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means of substrate dry matter that disappeared while incubated in the rumen on a g/kg basis. Hybrid affected IVDMD (P=0.021). Greater IVDMD indicates a greater extent of *in vitro* microbial digestion of sorghum hybrids. Sorghum hybrids were grown in duplicate, and 11 *in vitro* analyses were utilized to estimate IVDMD. Bars without a common letter are different (P<0.05).

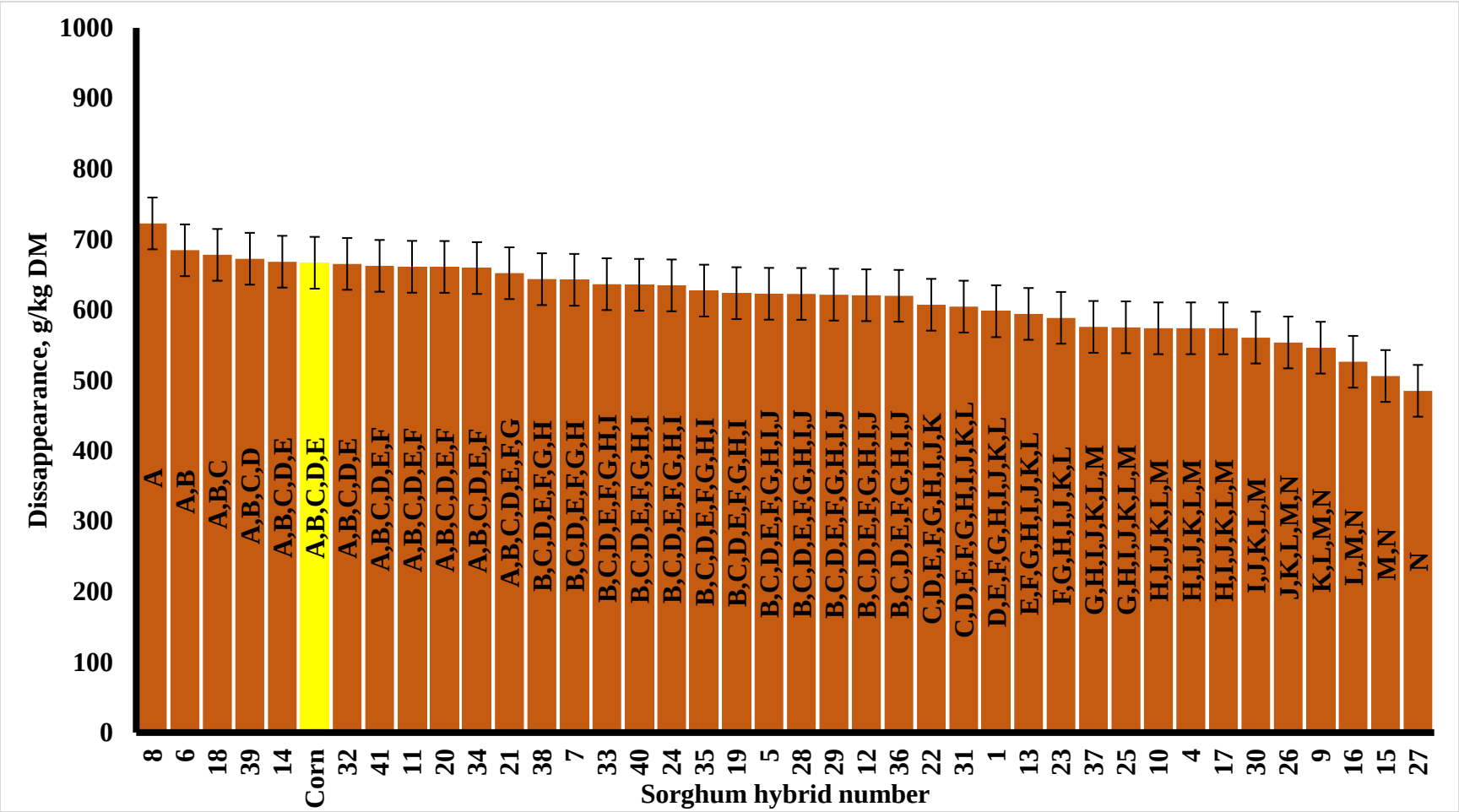

Figure 2.22 *In situ* dry matter disappearance (ISDMD) of reconstituted and ensiled sorghum and corn from incubation of grains in Ankom R510 concentrate bags in 3 ruminally fistulated steers for 16 h.

Values are least-square means ± standard error of the means of substrate dry matter that disappeared on g/kg basis. Hybrid affected ISDMD ($P<0.001$). Greater ISDMD indicates a greater extent of microbial digestion of sorghum hybrids. Sorghum hybrids were grown in duplicate, and 6 replications were performed to estimate ISDMD. Bars without a common letter are different ($P<0.05$).

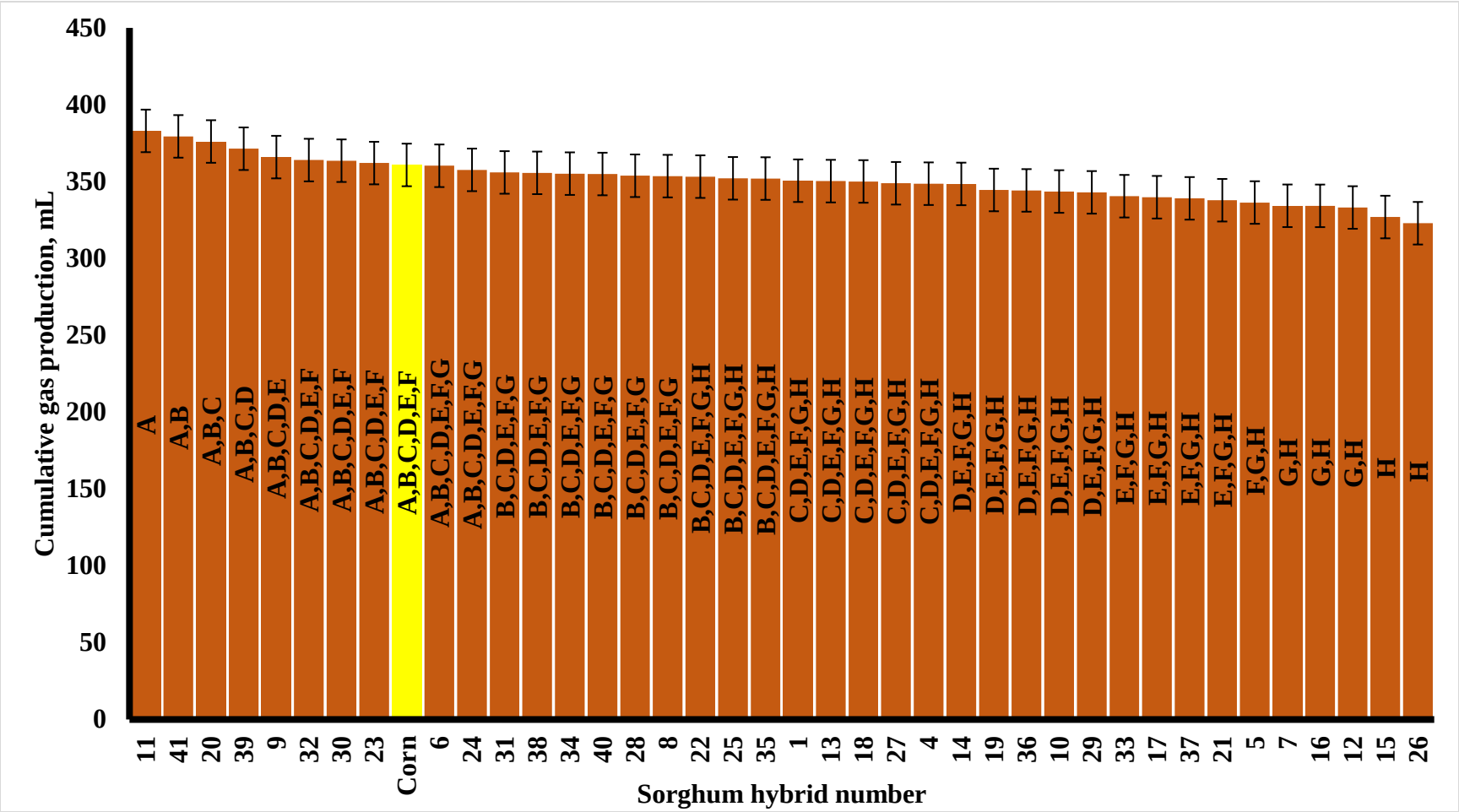

Figure 2.23 Maximum *in vitro* gas production (*K*) from *in vitro* cultures of mixed ruminal microorganisms with steam-flaked sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected *K* ($P<0.001$). Sorghum hybrids growth plots were combined, and 6 *in vitro* analyses were utilized to estimate *K* which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different ($P<0.05$).

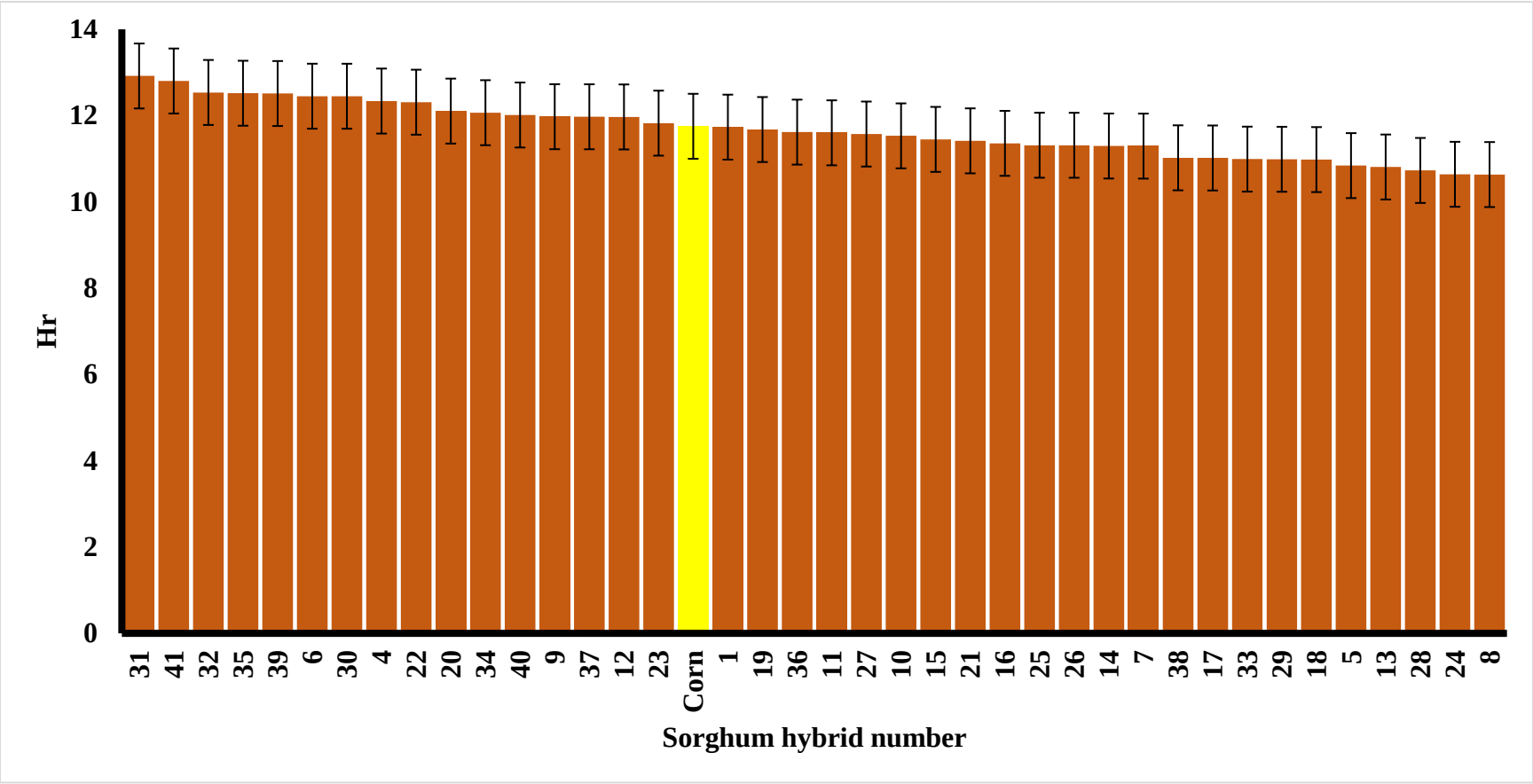

Figure 2.24 Time to reach half maximum gas production ($t_{1/2}$) from *in vitro* cultures of mixed ruminal microorganisms with steam-flaked sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid did not affect $t_{1/2}$ (P=0.117). Sorghum hybrids growth plots were combined, and 6 *in vitro* analyses were utilized to estimate $t_{1/2}$ which was derived from the *in vitro* microbial digestion of each hybrid.

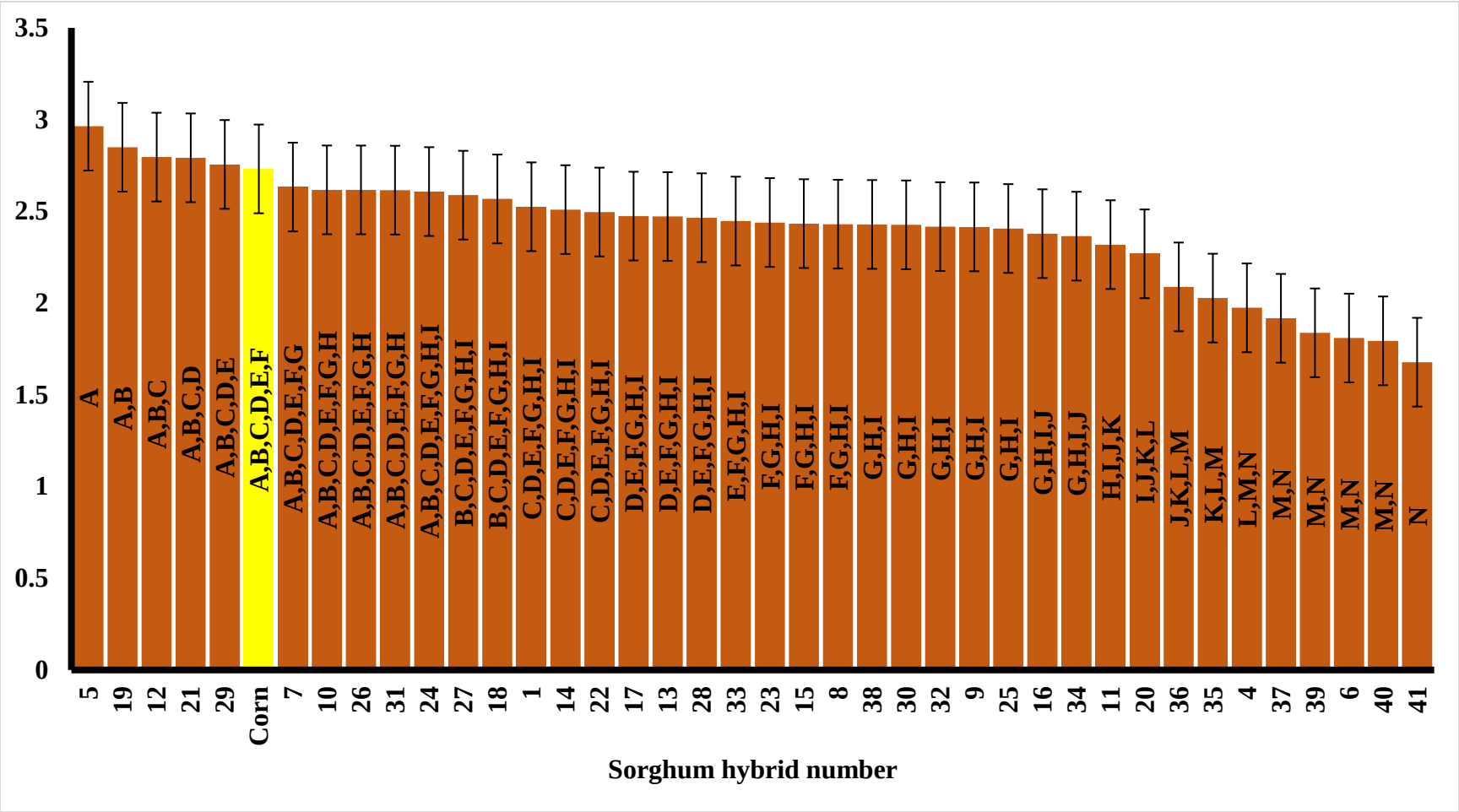

Figure 2.25 Rate of gas production (*r*) for *in vitro* cultures of mixed ruminal microorganisms with different steam-flaked sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected *r* (P<0.001). Greater *r* values indicate faster rates of digestion. Sorghum hybrids growth plots were combined, and 6 *in vitro* analyses were utilized to estimate *r* which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different (P<0.05).

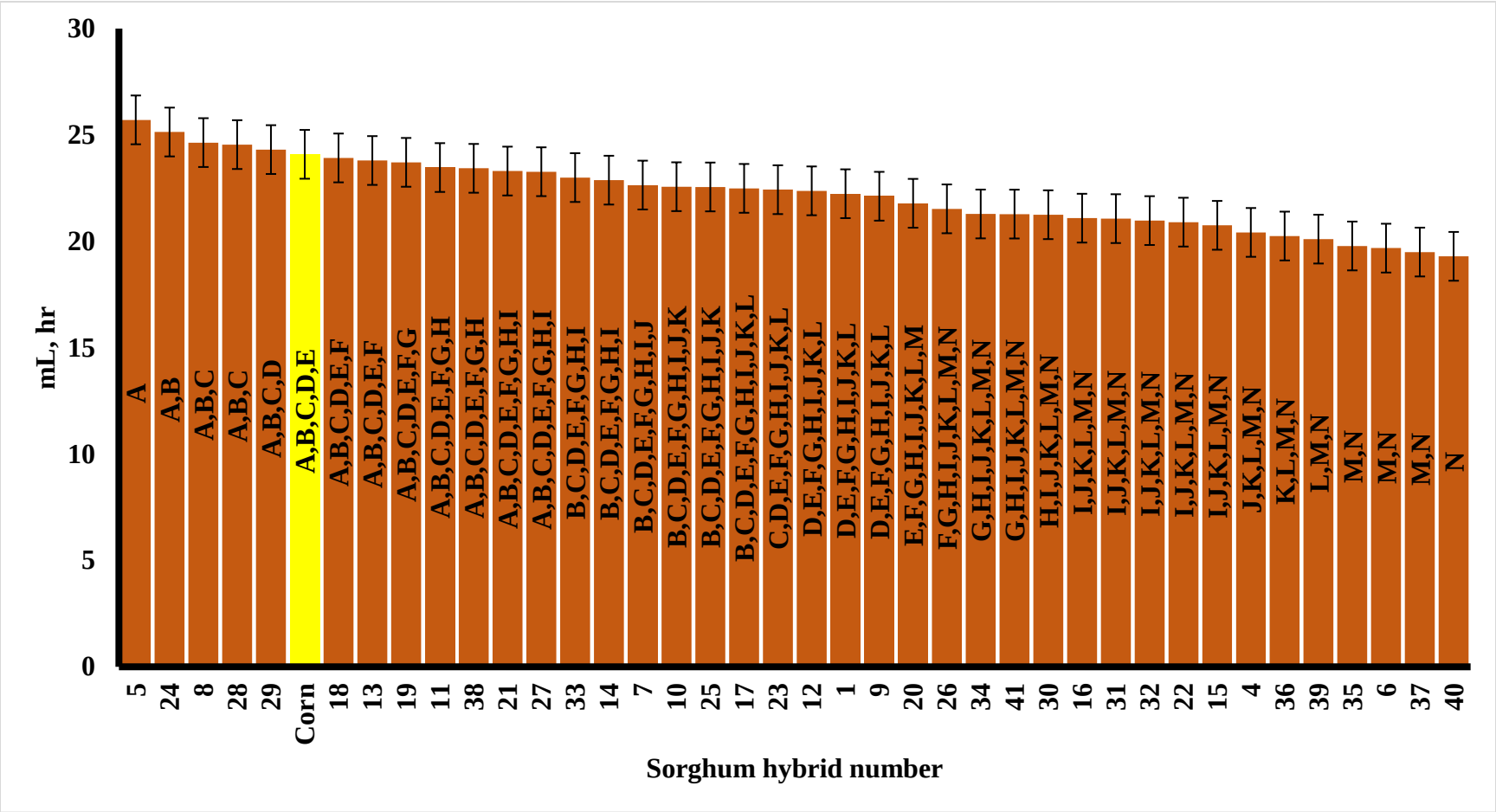

Figure 2.26 Maximum rate of gas production (*m*) for *in vitro* cultures of mixed ruminal microorganisms with different steam-flaked sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected *m* ($P<0.001$). Greater *m* values indicate faster rates of digestion. Sorghum hybrids growth plots were combined, and 6 *in vitro* analyses were utilized to estimate *m* which was derived from the *in vitro* microbial digestion of each hybrid. Bars without a common letter are different ($P<0.05$).

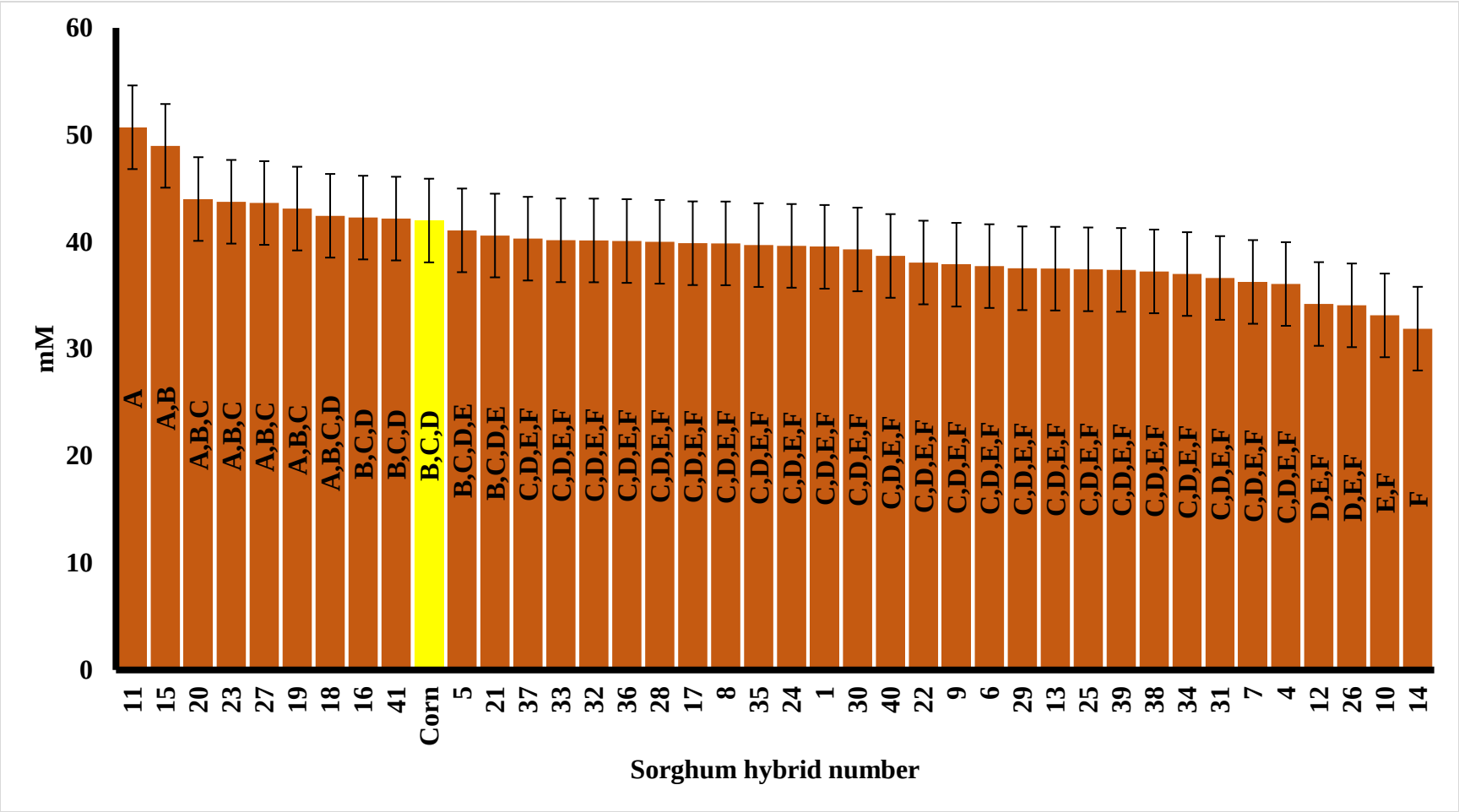

Figure 2.27 Acetate concentrations from *in vitro* cultures of mixed ruminal microorganisms with different steam-flaked sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected acetate concentrations (P=0.033). Sorghum hybrids growth plots were combined, and 6 *in vitro* analyses were utilized to estimate acetate concentrations *in vitro* cultures. Bars without a common letter are different (P<0.05).

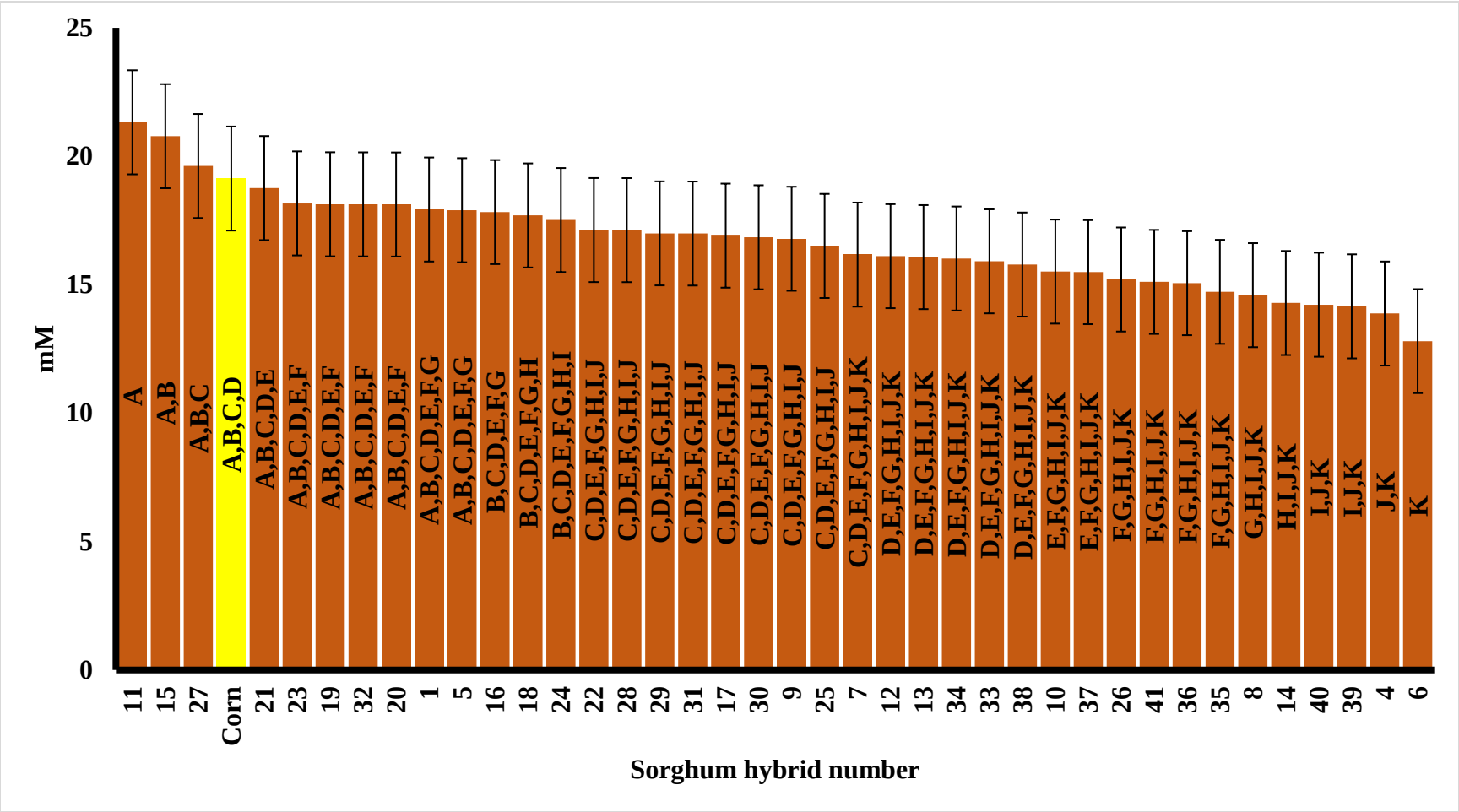

Figure 2.28 Propionate concentrations from *in vitro* cultures of mixed ruminal microorganisms with different steam-flaked sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected propionate concentrations ($P<0.001$). Sorghum hybrids growth plots were combined, and 6 *in vitro* analyses were utilized to estimate propionate concentrations in *in vitro* cultures. Bars without a common letter are different ($P<0.05$).

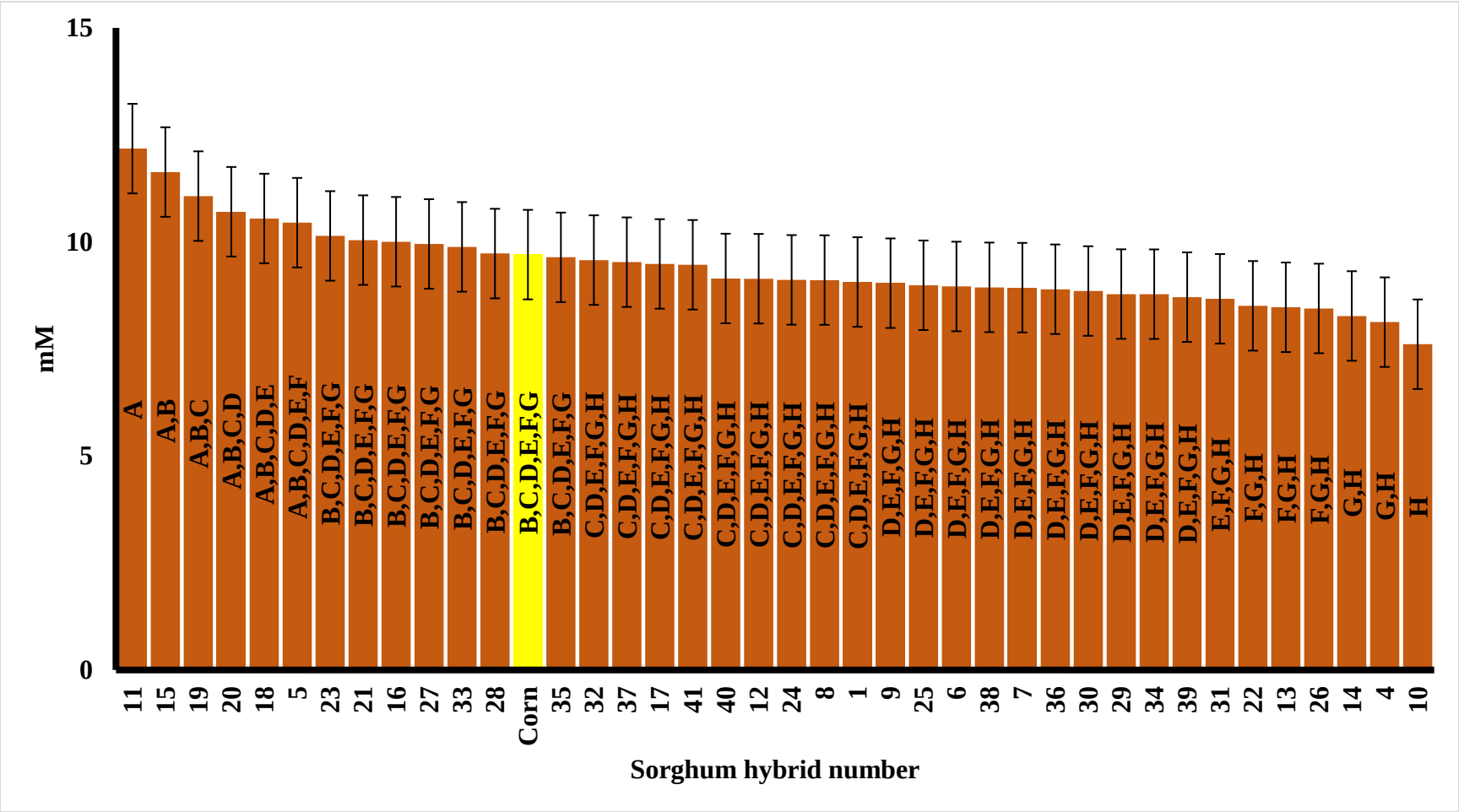

Figure 2.29 Butyrate concentrations from *in vitro* cultures of mixed ruminal microorganisms with different steam-flaked sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected butyrate concentrations (P=0.012). Sorghum hybrids growth plots were combined, and 6 *in vitro* analyses were utilized to estimate butyrate concentrations from *in vitro* cultures. Bars without a common letter are different (P<0.05).

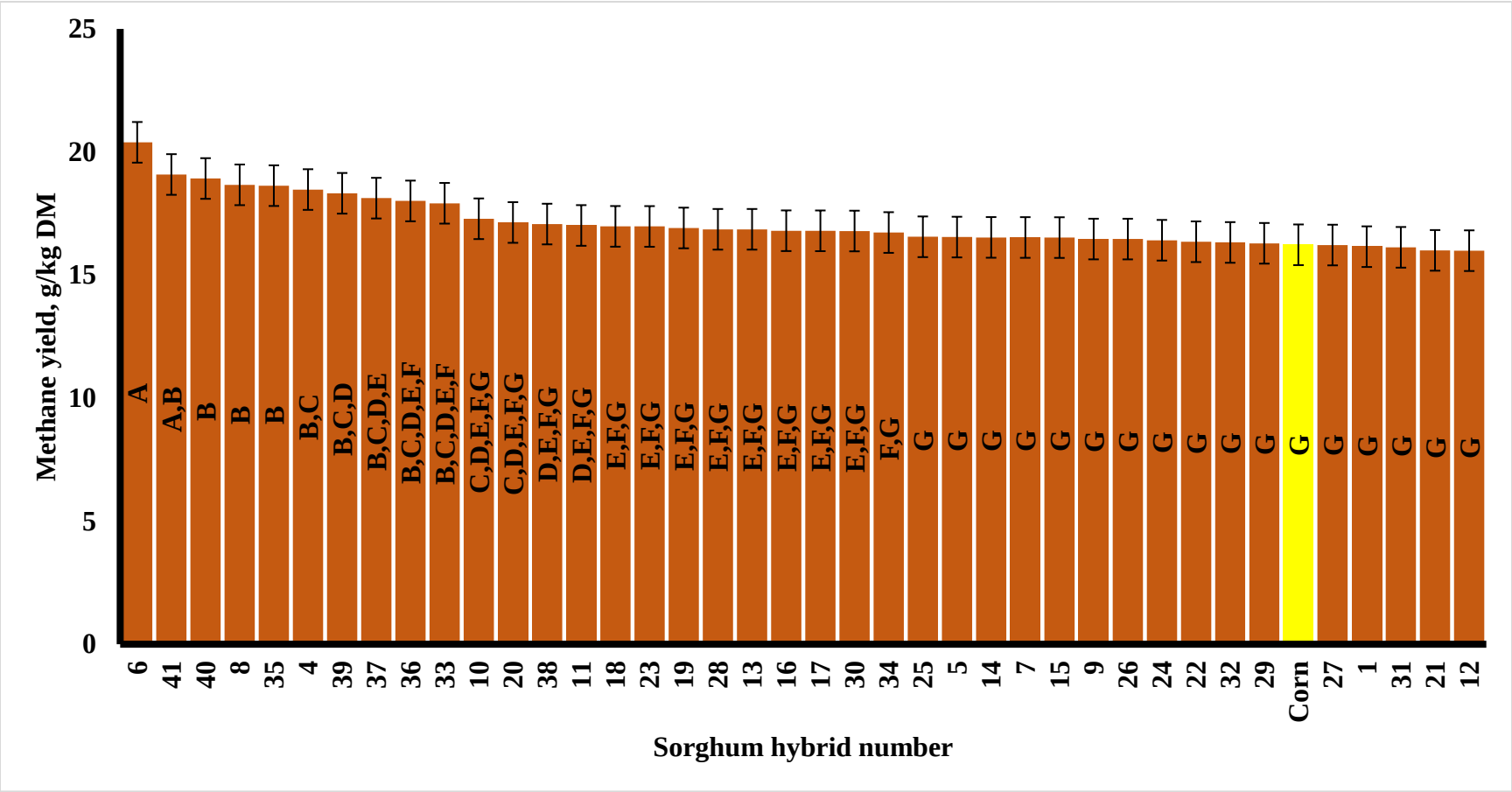

Figure 2.30 Methane yield from *in vitro* cultures of mixed ruminal microorganisms with steam-flaked sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means. Hybrid affected methane yield ($P<0.001$). Methane yield was estimated using the formula "Methane yield = 4.08 × (A/P) + 7.05" (Williams et al., 2019). Sorghum hybrids growth plots were combined, and 6 *in vitro* analyses were utilized to estimate butyrate concentrations from *in vitro* cultures. Bars without a common letter are different ($P<0.05$).

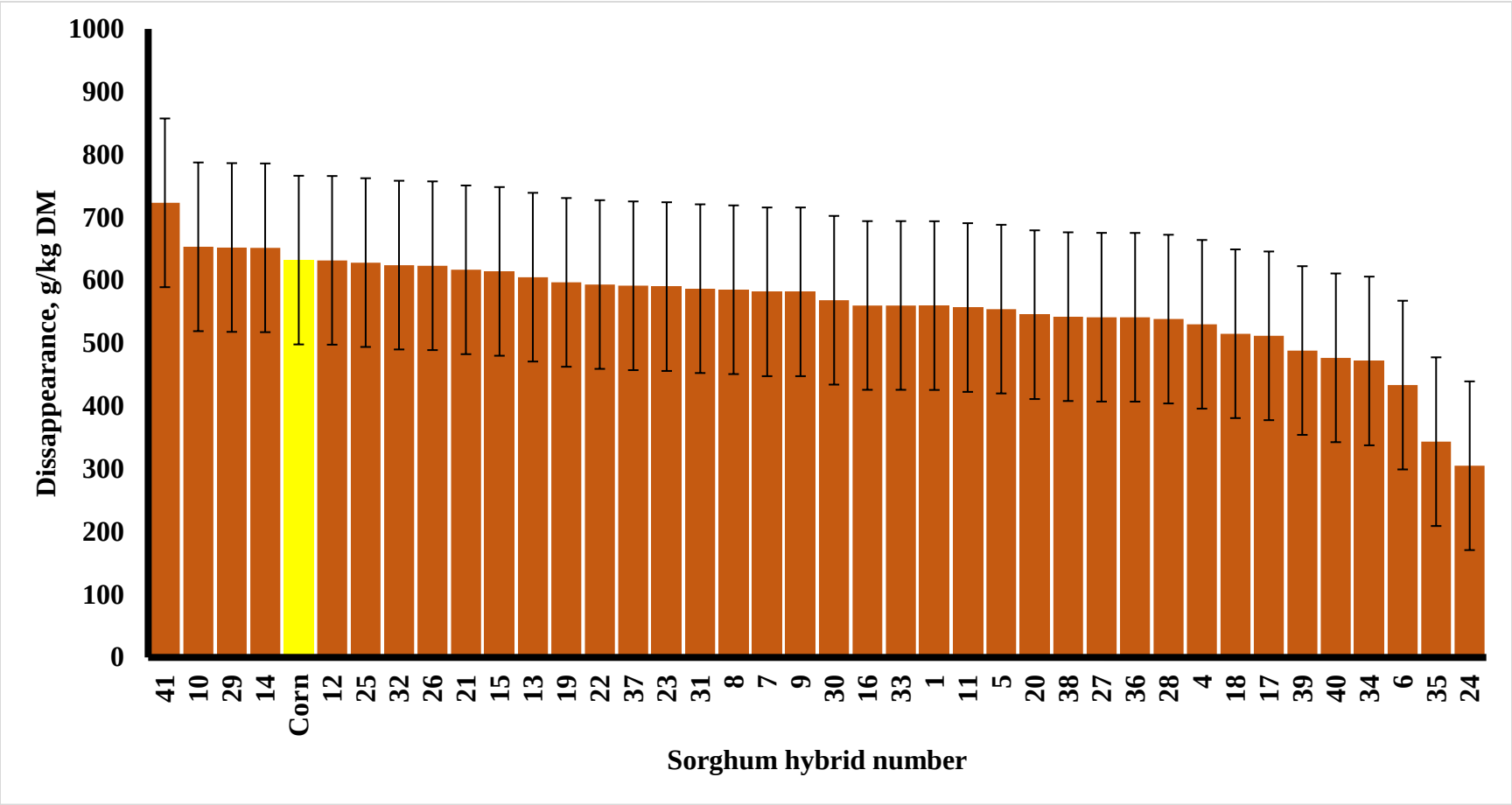

Figure 2.31 *In vitro* dry matter disappearance (IVDMD) from cultures of mixed ruminal microorganisms with different steam-flaked sorghum hybrids and corn as substrate.

Values are least-square means ± standard error of the means of substrate dry matter that disappeared while incubated in the rumen on a g/kg basis. Hybrid did not affected IVDMD (P=0.375). Sorghum hybrids growth plots were combined, and 6 *in vitro* analyses were utilized to estimate IVDMD which was derived from the *in vitro* microbial digestion of each hybrid.

3 Use of an extruded microalgae and flaxseed blend product and its effects on ruminal fermentation and nutrient disappearance

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[1]Principal investigator: jdrouill@ksu.edu

3.1 Abstract

Omega-3 fatty acid supplementation has been researched in both the dairy and beef cattle sectors since these fatty acids are considered essential to animal health. Naturally Better Omega-3 Technologies (Manhattan, Kansas) has developed a supplement consisting of an extruded blend of flaxseed and microalgae (FAB; *greatOplus*) that is fed to livestock to supplement omega-3 fatty acids. Our objective was to evaluate how ruminal microbes alter composition of the fatty acids in the FAB, and their post ruminal disappearance.

Eleven steers fitted with ruminal and duodenal cannulas were housed in a facility equipped with the Insentec feed and water monitoring system (Hokofarm, Emmeloord the Netherlands). A cross over design was utilized and treatments included a control diet without omega-3 supplementation and a treatment diet with supplementation of the FAB at 10% of the diet dry matter. Duodenal flow of total fatty acids (g) was greater (P=0.002) for cattle supplemented with FAB than for non-supplemented cattle. In particular, duodenal flow of α-linolenic acid (ALA; g) was observed to be four times greater (6.3 g/d vs 1.6 g/d; P=0.001) for steers fed the FAB supplement compared to steers fed the control diet. The coefficient of apparent ruminal fatty acid disappearance of C18:0 was negative (-12.92 vs -11.12; P=0.960) in both treatments, indicating substantial biohydrogenation of the FAB occurred in the rumen. The coefficient of apparent intestinal disappearance of total fatty acids did not differ between treatments (0.74 vs 0.68; P=0.128) indicating that the FAB did not affect the total disappearance of all the fatty acids leaving the rumen. Lastly the coefficient of apparent total tract disappearance of organic matter did not differ between treatments (0.78 vs 0.77; P>0.1)

Keywords: biohydrogenation, *greatOplus*, Omega-3 fatty acids, α-Linolenic acid

Abbreviations FAB, extruded flax-algae blend; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; TiO_2, titanium dioxide, aNDF, neutral detergent fiber

inclusive of residual ash; VFA, volatile fatty acid; SAS, statistical analysis system; DMI, dry matter intake; MY, methane yield; A/P, acetate concentration/ propionate concentration; NDF, neutral detergent fiber; OM, organic matter; Cr_2O_3, chromium oxide; SEM, standard error of means.

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3.2 Introduction

Omega-3 fatty acids are polyunsaturated and essential in humans as these fatty acids are used as components of hormones and cells. Of particular importance are α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Plant species, such as flaxseed and chia seeds, are rich in ALA, whereas algae species are the primary producers of EPA and DHA. Marine life consume algae and deposit these fatty acids in their tissue, making fish the primary dietary source of EPA and DHA fats for humans (Doughman et al., 2007). A balanced amount of omega-3 and omega-6 fats in human diets is considered essential since an imbalance could have negative consequences on human health. The omega-3 to omega-6 fatty acid ratio of today's human diets are commonly greater then 1:10 of omega-3 to omega-6 fatty acid which is considered unhealthy (Doughman et al., 2007). Excessive consumption of omega-6 fatty acids has been observed to increase inflammation and the of development of autoimmune disease in humans (Chaves et al., 2019).

Finishing cattle diets normally include substantial amounts of cereal grains and cereal grains by products, and thus contain a larger proportion of omega-6 fatty acids compared to omega-3 fatty acids. Cattle can be supplemented with different products, such as flaxseed or algae, to improve the balance of omega-3 and omega-6 fatty acids in their diets. Flaxseed is rich in ALA and, when consumed, can increase concentrations of omega-3 fatty acids in the bloodstream (Weiss et al., 2014). Many of the unsaturated fatty acids consumed by ruminants are transformed in the rumen through biohydrogenation, yielding saturated fats. Based on a meta-analysis of ruminal flow studies by Jenkins and Bridges (2007), 86% of unprotected linolenic and 82% of unprotected linoleic acid are biohydrogenated in the rumen. Current strategies for reducing biohydrogenation of unsaturated fatty acids as they make their way through the rumen include

encapsulation of the unsaturated fats or by reacting the carboxyl end of fatty acids with either calcium salts or amides (Jenkins and Bridges, 2007).

Naturally Better Omega-3 Technologies (NBO3 Technologies; Manhattan, Kansas) has developed an omega-3 supplement produced through an extrusion process using *Nannochloropsis oculata* microalgae and flaxseed as sources of omega-3 fatty acids (*greatOplus*). This flax-algae blend (FAB) is hypothesized to increase supply of omega-3 fatty acids to the small intestines. Therefore, the objective of this experiment was to observe how the FAB supplement was altered by ruminal microbes and the post-ruminal disappearance of the fatty acids in cannulated Holstein steers.

3.3 Materials and methods

3.3.1 Experimental Design

This study was approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC). This study utilized 11 Holstein steers (initial body weight 586.4 ± 49.7 kg) with ruminal and duodenal cannulas. Steers were housed in a partially covered feeding area consisting of earthen-surfaced pens equipped with a 15-m concrete apron at the Kansas State University Intake facility. This facility was also equipped with an Insentec Roughage Intake Control (RIC) system (Hokofarm, Emmeloord the Netherlands), which measures individual animal feed and water consumption. Treatments were a control diet without FAB supplementation and a treatment diet consisting of the FAB supplement which was included at 10% of the diet dry matter. Each steer was assigned to an individual bunk and waterers were shared among all steers. A cross-over design was utilized for this experiment, where each cannulated steer was assigned to each of the experimental diets during one of two periods. Two sequential 19-d periods were used: a 15-d adaptation interval followed by a 4-d collection interval. Titanium dioxide (TiO_2) was used as an indigestible marker, and 15 g/d of TiO_2 was

dispensed directly into the rumen from d 10 through 19 of each collection period. On day 16, ruminal, duodenal, and fecal contents were collected at starting at 0 h post-feeding. Subsequent collections were performed every 8 hours until d 19 to obtain digesta or fecal samples representing 2 h intervals post-feeding for 24 h. Duodenal and fecal samples were immediately frozen at -20 ℃ until further processing. Whole ruminal contents were collected at each collection time and squeezed through four layers of cheesecloth. A 4-mL sample of ruminal fluid was collected and combined with one mL of 25 % w/v meta-phosphoric acid and then frozen for at least 24 h. Ruminal fluid pH from each steer was measured using an Thermo Scientific Orion Star™ A121 portable pH meter equipped with an Orion™ Ross Ultra™ Refillable pH/ATC Triode™ combination electrodes probe (Thermofisher, Waltham, MA). Strained ruminal fluid was placed in plastic cups at each collection time to facilitate pH measurements.

3.3.2 Calculations for Fatty Acid, OM, NDF Disappearance, and Predicted Methane Yield

Titanium dioxide was used as an indigestible marker to calculate apparent duodenal flow (g) and fecal excretion (g) of nutrients. Total duodenal flow and fecal excretion were calculated by dividing the TiO_2 dosed to the steer (g/d) by the TiO_2 concentration in duodenal and fecal samples (g/g DM). The coefficient of apparent ruminal appearance and disappearance was assumed to be equal to the coefficient of fatty acids, OM and NDF that disappeared in the rumen; the calculation is in formula 1 below.

The coefficient of apparent intestinal disappearance was assumed to be equal to the coefficient of each fatty acid that disappeared in the small and large intestines and was calculated using formula 2 below. The coefficient of apparent total tract disappearance of OM and NDF was assumed to be

equal to the coefficient of OM and NDF that disappeared throughout the entire digestive tract; the calculation is in formula 3 below.

Formula 1 for calculating the coefficient of ruminal disappearance of components

$$1 - \frac{\text{Amount of nutrient in duodenal sample (g)}}{\text{Amount of nutrient consumed (g)}}$$

Formula 2 for calculating the coefficient of intestinal disappearance of components

$$1 - \frac{\text{Amount of nutrient in fecal sample (g)}}{\text{Amount of nutrient in duodenal sample (g)}}$$

Formula 3 for calculating the coefficient of total tract disappearance of components

$$1 - \frac{\text{Amount of nutrient in fecal sample (g)}}{\text{Amount of nutrient consumed (g)}}$$

Digesta and fecal samples from each steer were composited by steer, and period before further processing. Freeze drying was used to preserve the nutrient composition of each composited digesta and fecal sample (SP Scientific Genesis 35 SQ EL Freeze Dryer, Warminster, PA). Freeze dried samples were ground using a Wiley Mill (Thomas Scientific, Chadds Ford Township, PA) equipped with a 1-mm screen and samples were placed in resealable plastic bags for storage before analysis.

Water intake was recorded in kilograms/d and was assumed to be a 1:1 ratio of kilograms to liters of water. Titanium dioxide concentrations were determined using a BioTek PowerWave XS Microplate Reader (BioTek, Winooski, VT) based on the methods described by Short et al. (1996) with modifications to allow analysis in a well plate. Modifications included ashing 0.3 g of duodenal and fecal samples at 450 ℃ for 12 h. Ashed samples were digested in 18M sulfuric acid with 1.0 g of sodium sulfate at 280 ℃ until TiO_2 was fully dissolved, which required approximately 25 to 35 minutes. Digested samples were transferred to pre-weighed conical tubes, and final weight of the solution was brought up to 50 g with water. Samples (750 μL) were

to a 48 well-plate and 90 μL of 30% hydrogen peroxide was added and held for 15 min before measurement.

Neutral detergent fiber inclusive of residual ash (aNDF) was performed using an Ankom 200 Fiber Analyzer (Ankom, NY) based on modifications to Van Soest et al. (1991) using Ankom #F57 filter bags. This procedure was described in the Ankom 200 Fiber Analyzer operator's manual (Ankom Technologies, 2023). Fatty acid measurement was performed using methods developed by (Sukhija and Palmquist, 1988). The internal standard used to calculate fatty acid percentages in the duodenal and fecal samples was tridecanoic acid (C13:0).

Ruminal ammonia concentrations were analyzed using the method described by Broderick and Kang (1980) using a BioTek PowerWave XS Microplate Reader (BioTek, Winooski, VT). Volatile fatty acid (VFA) concentrations were measured using de-proteinized ruminal fluid samples that were thawed, vortexed, and centrifuged at 30,000 x g for 10 min. A 0.4-mL sample supernatant was taken from each processed ruminal fluid sample and combined with 1.2 mL of 4.0 mM solution of pivalic acid. These samples were then analyzed using an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector and a capillary column (DB Fatwax-UI, 20m X 180 μm; Agilent Technologies, Palo Alto, CA). Hydrogen was used the carrier gas with a flow rate of 43.3 mL/min. The injector was operated in split mode with a split ratio of 50:1. The oven temperature was initially 50 ℃ and then increased up at a rate of 20 ℃/min to a final temperature of 240 ℃ and which was maintained for 2 min for each injection.

Methane yield (g/kg of DM) was estimated for each steer using ruminal VFA concentrations and the predictive equation "Methane Yield = 4.08×[acetate (mol/100 mol) /propionate (mol/100 mol)] +7.05" proposed by Williams et al. (2019).

3.3.3 Statistical Analysis

Results were analyzed using the Mixed Procedure of the Statistical Analysis System (SAS, ver. 9.4). A variable called sequence was created in order to organize the data and consisted of a value of one or two. Steers fed the control diet in period one and *greatOplu*s in period two were assigned a value of one and vice versa for the other group of steers who were then assigned a value of two. Dry matter intake and water intake were used as fixed effects with day as the repeated measure and the subject consisting of steer and sequence. Steer was considered a random effect. Volatile fatty acid concentrations, ammonia concentrations, and pH data were analyzed as fixed effects with repeated measure of hour, and steer as a random effect with the subject consisting of steer and sequence. Fatty acid, OM, and NDF data used the Mixed Procedure of SAS as well. Fixed effects of the model consisted of diet, and the random effect consisted of steer within sequence. Significant effects for treatments were declared when P values were ≤ 0.05 and tendencies for an effect if $0.05<P<0.10$.

3.4 Results

3.4.1 Feed and Water Intake

There were no main effects (P=0.403) for DMI; however, there was a treatment-by-day interaction for DMI over the entire experiment (P<0.001). Steers fed the FAB supplement, consume more feed (P<0.05) than non-supplemented steers on days 2, 6, and 7. Steers fed the control diet, tended to consume more feed (P=0.088) on day 1 compared to steers fed the FAB supplement. Water intake did not differ (P>0.10) between the two treatments over the entire experiment (P>0.10). There was a treatment-by-hour interaction (P<0.01) for DMI over the 4-d sample collection interval (P<0.01), where FAB steers consumed more feed at h 8 (P=0.008) and 10 (P=0.015) and tended to consume more feed at h 4 (P=0.094) and 6 (P=0.060).

3.4.2 Volatile Fatty Acid Concentration, Predicted Methane Yield, Ammonia Concentration, and pH of Ruminal Fluid

Volatile fatty acid concentrations are summarized in Figures 3.4, 3.5, and 3.6. Steers supplemented with FAB had no differences (P>0.10) in ruminal acetate concentrations. There was a treatment-by-hour interaction (P<0.05) with greater (P=0.013) ruminal propionate at h 8 and a tendency (P=0.064) for greater ruminal propionate concentrations at hour 0 for steers consuming the FAB. Steer fed the control diet tended (P=0.053) to have greater ruminal propionate concentration at hour 18 compared to steers fed the FAB. There was also a treatment-by-hour interaction (P=0.036), for ruminal butyrate concentrations with steers consuming the FAB tending to have greater ruminal butyrate concentrations at h 8 (P=0.086) and 10 (P=0.065). Conversely, non-supplemented steers tended to have greater ruminal butyrate concentrations at hours 18 (P=0.080) and 24 (P=0.071). There was a treatment-by-hour interaction for methane yield (P=0.001), with steers consuming the FAB, emitting less methane at 0, 6, 8, 14, and 22 h post-feeding (P<0.05) compared to steers consuming the control diet. Ruminal fluid pH and ammonia concentrations were not different (P>0.1) for steers consuming the FAB compared to steers fed the control diet.

3.4.3 Intake, and Duodenal Flow, and Fecal Excretion of Fatty Acids, OM, and NDF

Intake, ruminal flow, and fecal excretion data are summarized in Table 3.2. Greater concentrations (P<0.01) of fatty acids were present in the duodenum of steers consuming FAB compared to steers fed the control diet. In particular duodenal flow of α-linolenic was greater (6.3 g/d vs 1.6 g/d; P=0.001) for steers consuming the FAB. Steers consuming the FAB had greater NDF consumption (P=0.009) and tended (P=0.077) to consume more OM when

compared to steers fed the control diet. Steers supplemented the FAB had greater fecal excretion ($P<0.05$) of NDF and OM when compared to steers fed the control diet.

3.4.4 Coefficient of Apparent Ruminal Appearance and Disappearance of Fatty Acids

Apparent coefficient of ruminal appearance and disappearance represents the proportion of fatty acids that were altered through ruminal fermentation. A negative coefficient represents apparent microbial production of a particular fatty acid. This indicates that microbial biohydrogenation of a particular unsaturated fatty acid occurred. These results are presented in Table 3.1.

In particular the coefficient for C18:0 was much less than 0 (-12.92 vs 11.12; $P=0.243$). When comparing the coefficients for the polyunsaturated fatty acids with those of C16:0 and C18:0, many of them were greater than 0. When taken together, this indicates that many of the polyunsaturated fatty acids were biohydrogenated in the rumen. Overall, the coefficient for total fatty acids did not differ between treatments (0.30 vs 0.33; $P=0.605$).

3.4.5 Coefficient of Apparent Intestinal Disappearance and Total Tract Disappearance of Fatty Acids, OM and NDF

Intestinal disappearance of each individual fatty acid is presented in Table 3.4. Overall the apparent intestinal disappearances for the majority of fatty acids were not different ($P>0.05$) between treatments. Only the intestinal disappearance of ALA was significant with steers consuming the FAB having a smaller coefficient (0.41 vs 0.64; $P=0.039$) than steers fed the control diet. The coefficients of apparent total tract disappearance of OM and NDF did not differ ($P>0.10$) between treatments and is presented in Table 3.5.

3.5 Discussion

3.5.1 Dry Matter Intake

Fat supplementation has been used to increase energy density of diets fed to beef and dairy cattle. Usually, the more unsaturated the fat source, the more likely it is to decrease DMI due to toxic effects of unsaturated fats on ruminal microbes (National Academies of Sciences, Engineering, and Medicine, 2016). Extruded flaxseed has been utilized in some dairy studies to supplement cows with omega-3 fatty acids. Feeding extruded flaxseed has yielded mixed results in terms effects on DMI. Zachut et al. (2010) fed extruded flaxseed to transition dairy cows and observed DMI increased for cows during the first 100 days of lactation ($P<0.01$). Gonthier et al. (2005) fed micronized and extruded flaxseed to dairy cows and observed that processed flaxseed, either with micronization or extrusion, did not affect DMI ($P>0.1$). Likewise, Swanepoel and Robinson (2019) found that DMI was not affected when an extruded blend of flaxseed, alfalfa hay, and pea grain product was fed to dairy cows. Also, the reduction of steam-flaked corn in the FAB diet, could have affected the variation of DMI intake since this diet had less concentrate compared to the control diet

3.5.2 Volatile Fatty Acid Concentrations, and pH

Ruminal microorganisms cannot utilize free fatty acids for energy production; therefore, supplementation with dietary fat should increases post-ruminal availability of energy (Lourenço et al., 2010). Most fat consumed is in the form of triacylglycerols, which consist of three fatty acids attached by ester bonds to a glycerol backbone. Ruminal microbes can hydrolyze these ester bonds using microbial lipases, forming a glycerol molecule and three free fatty acids. The glycerol molecules can be fermented to propionate, which is absorbed from the rumen and does not contribute H_2 to ruminal metabolic processes (Jenkins, 1993). Gonthier et al. (2004) supplemented omega-3 lipids as raw, micronized, or extruded flaxseed, and observed a decreased

A/P ratios for cows supplemented with flaxseed compared to cows consuming no flaxseed ($P<0.01$). Gonthier et al. (2004) also observed no differences in ruminal pH in cows supplemented with processed or raw flaxseed compared to non-supplemented cows.

3.5.3 Ammonia Concentrations of Ruminal Fluid

Ruminal microbes cannot utilize lipids through beta-oxidation due to anaerobic conditions within the rumen (Lourenço et al., 2010). Ammonia concentrations in ruminal fluid have been used previously as an indicator of microbial protein degradation (Broderick and Kang, 1980). Gonthier et al. (2004) reported that supplementing flaxseed did not affect ruminal concentrations of ammonia or ruminal digestion of crude protein. This observation was similar to our observations on ruminal ammonia concentration from steers consuming our FAB.

3.5.4 Intake, Apparent Duodenal Flow, and Fecal Output of Fatty Acids, OM and NDF

Apparent duodenal flow (g/d) and fecal excretion (g/d) can be used to calculate the estimated disappearance of each individual fatty acid, OM and NDF in different locations of the digestive tract. Extensive biohydrogenation of polyunsaturated fatty acid from the FAB supplement likely occurred, as evidenced by substantial increases in duodenal flow of C16:0 and C18:0 fatty acids compared to the amount of these fatty acids consumed.

Extrusion is a disruptive process that can make nutrients more available to digestion due to chemical and thermal breakdown of complex structures that inhibit digestion of nutrients (Kamau et al., 2020). Lashkari et al. (2015) compared different processing methods for flaxseed, including extrusion, and used an *in situ* method. *In situ* effective disappearance of dry matter degradability was greater ($P<0.05$) for extruded flaxseed compared to roasted, irradiated, and non-processed flaxseed. Extrusion processing could increase the susceptibility of polyunsaturated fatty acids in our FAB supplement to biohydrogenation by ruminal microbes.

This could explain why steers consuming the FAB supplement had greater proportions of duodenal flow of C16:0 and C18:0 fatty acids.

Using internal markers in cattle introduces complications in interpreting data on duodenal flow and fecal excretion of nutrients. Marker techniques assume a homogenous pool in the rumen and a constant rate of out flow of fluid and particulates from the rumen to the intestines (Owens and Hanson, 1992). These conditions unfortunately are not completely true and could lead to the marker not being uniformly mixed with digesta in the rumen when the marker is dosed. This results in either underestimating or overestimating digesta recoveries, which can affect calculations for disappearances or digestion.

Velásquez et al. (2018) compared the use of different external and internal markers fed to Holstein cattle consuming a corn silage-based diet to estimate feed intake. They observed that TiO_2 overestimated fecal recovery of the marker by approximately 200%. De Souza et al. (2015) compared the usage of chromic oxide (Cr_2O_3) and TiO_2 as internal markers for dairy cows and observed that TiO_2 was more accurate in predicting DM and OM digestabilties than Cr_2O_3. Some variation in the total amount of fatty acids consumed compared to the total duodenal flow of fatty acids could be explained by use of TiO_2 as a marker to estimate our total duodenal flow and fecal excretions. Chromium oxide and TiO_2, however, are still valuable tools for estimating nutrient digestion and fecal recovery in cattle (Owens and Hanson, 1992; Titgemeyer et al., 2001).

3.5.5 Coefficients of Ruminal Appearance and Disappearance of Fatty Acids

The conclusion that polyunsaturated fatty acids in our FAB were biohydrogenated was further supported based on the negative coefficient of ruminal appearance and disappearance of C16:0 and C18:0. This observation is further supported based on the coefficients of different polyunsaturated fatty acids being greater than 0 in steers consuming the FAB. Taken together,

this indicates these fatty acids disappeared and were likely biohydrogenated by ruminal microbes.

Protection of unsaturated fatty acids can be challenging since these fatty acids can be toxic to ruminal microbes. Alvarado-Gilis et al. (2015) used two extrusion methods to potentially protect polyunsaturated fatty acids in ground flaxseed. The first method used an extruded blend of ground flaxseed, calcium oxide and molasses and was theorized to produce calcium salts which have been observed to resist biohydrogenation. The second method used an extruded blend of ground flaxseed, soybean meal, molasses, and baker's yeast. This extrusion method was thought to allow the formation of Maillard products which would have protective properties for the unsaturated fatty acids contained within proteins from the flaxseed and soybean meal. Both extruded products were fed to steers for 12 days and jugular blood samples from these steers were used to assess the amount of protection these two extrusion methods had on the polyunsaturated fatty acids flaxseed products. In either method, the extruded products failed ($P>0.1$) to protect the polyunsaturated fatty acids from the ground flaxseed from microbial biohydrogenation. This was based on there being no differences ($P>0.1$) in the concentrations of polyunsaturated fatty acids from jugular blood samples of steers consuming either extruded products compared to steers consuming a diet devoid of flaxseed (Alvarado-Gilis et al., 2015). This previous finding supports our results where extrusion processing does not appear to afford protection of polyunsaturated fatty acids.

3.6 Conclusion

Omega-3 supplementation in cattle is a topic of interest in ruminant nutrition. Beef is a major component of the human diet, and, if the omega-3 lipid content of beef could be increased, it could have potential health benefits and increased value to consumers and cattle producers. Microbial biohydrogenation of non-protected polyunsaturated fatty acids presents a challenge

since this process alters the majority of the polyunsaturated fats consumed (Jenkins and Bridges, 2007). The extruded FAB supplement fed to steers in this experiment, does not appear to bestow significant protection against biohydrogenation due to the extensive saturation that we extrapolated from duodenal outflow data and the negative coefficient of ruminal appearance and disappearance of C18:0. In spite of this, steers fed the FAB supplement, had roughly a four-fold increase in the amount of ALA (g/d) reaching the duodenum compared to non-supplemented steers. This suggested increased opportunity for absorption and incorporation of more omega-3 fatty acids into tissues and a potential increase in the omega-3 concentration of meat from steers consuming this product.

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Table 3.1 Composition of steers diets (g/kg of DM) containing either the FAB supplement or soybean meal

Item	Control	*greatOplus*
Steam flaked corn	313.00	238.70
Alfalfa hay	150.00	150.00
Corn silage	500.00	500.00
greatOplus supplement[1]	-	100.00
Soybean meal, dehulled	25.70	-
Supplement[2]	11.30	11.30
Nutrient composition,		
Crude protein	105.80	107.60
Net energy for maintenance,	1.72	1.76
Net energy for gain, Mcal/kg	1.01	1.04
Neutral detergent fiber	280.20	304.50
Omega-3 fatty acid	1.80	11.20
Ether extract	26.40	41.90

[1] Extruded flax-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 mananese; 0.50 mg/kg of 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	*greatOplus*
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[‡]	
C6:0	0.00
C8:0	0.00
C10:0	0.00
C11:0	0.00
C12:0	0.03
C14:0	0.15
C14:1 (ω-5)	0.00
C15:0	0.12
C15:1 (ω-5)	0.00
C16:0	14.19
C16:1 (ω-7)	0.19
C17:0	0.18
C17:1 (ω-7)	0.00
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-6t)	0.00
C18:2 (ω-6)	39.90
C20:0	0.39
C18:3 (ω-6)	0.00
C18:3 (ω-3)	94.71
C20:1 (ω-9)	0.49
CLA 9c,11t (ω-7)	0.06

CLA 10t,12c (ω-6)	0.00
CLA 9c,11c: (ω-7)	0.00
C21:0	0.00
CLA 9t,11t (ω-7)	0.05
C20:2 (ω-6)	0.11
C22:0	0.44
C20:3 (ω-6)	0.05
C20:3 (ω-3)	0.13
C22:1 (ω-9)	0.05
C20:4 (ω-6)	0.00
C23:0	0.09
C22:2 (ω-6)	0.00
C20:5 (ω-3)	0.00
C24:0	0.38
C24:1 (ω-9)	0.00
C22:5 (ω-3)	0.00
C22:6 (ω-3)	0.00
Total fatty acid	203.50

[‡] Omega 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. The location of the first carbon with a double bond from the methyl end of the fatty acid is indicated in parentheses, preceded by the omega symbol (ω). Unless otherwise specified with a "t" in the location of the first double bond, the orientation of the double bond is assumed cis. Conjugated linolenic acids (CLA) used a slight modification to the omega nomenclature and this method was used to identify the orientation of each double bond. This modified nomenclature used the acronym CLA to denote conjugated linolenic acid followed by individual numbers that correspond to the location and orientation (c-cis; t-trans) of the double bond from the carboxyl end of the fatty acid. The location of the first double bond from the methyl end was also included.

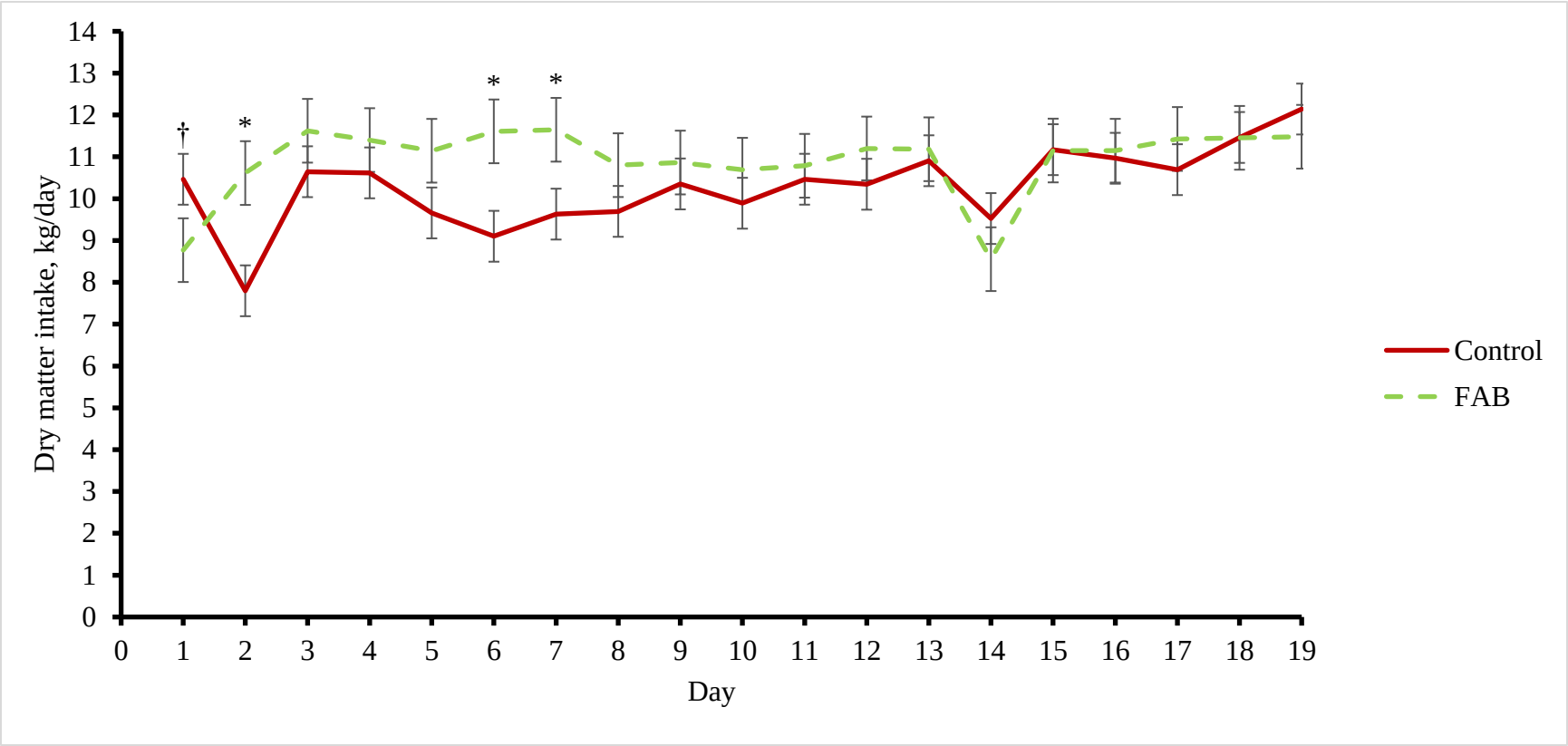

Figure 3.1. Daily dry matter intake (DMI) for steers fed either the control diet or the FAB supplement at a rate of 100 g/kg of the diet.

Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatments on DMI were not different ($P=0.403$). A treatment-by-day interaction ($P<0.001$) was observed; steers fed the FAB supplement, consumed more feed than steers fed the control diet on days 2, 6, and 7 ($P<0.05$). A tendency for greater feed intake was observed on day 1 for steers fed the control diet compared to steers fed the FAB supplement ($0.05<P<0.10$). The symbol * indicates significance ($P<0.05$), and † indicates a tendency ($0.05<P<0.10$).

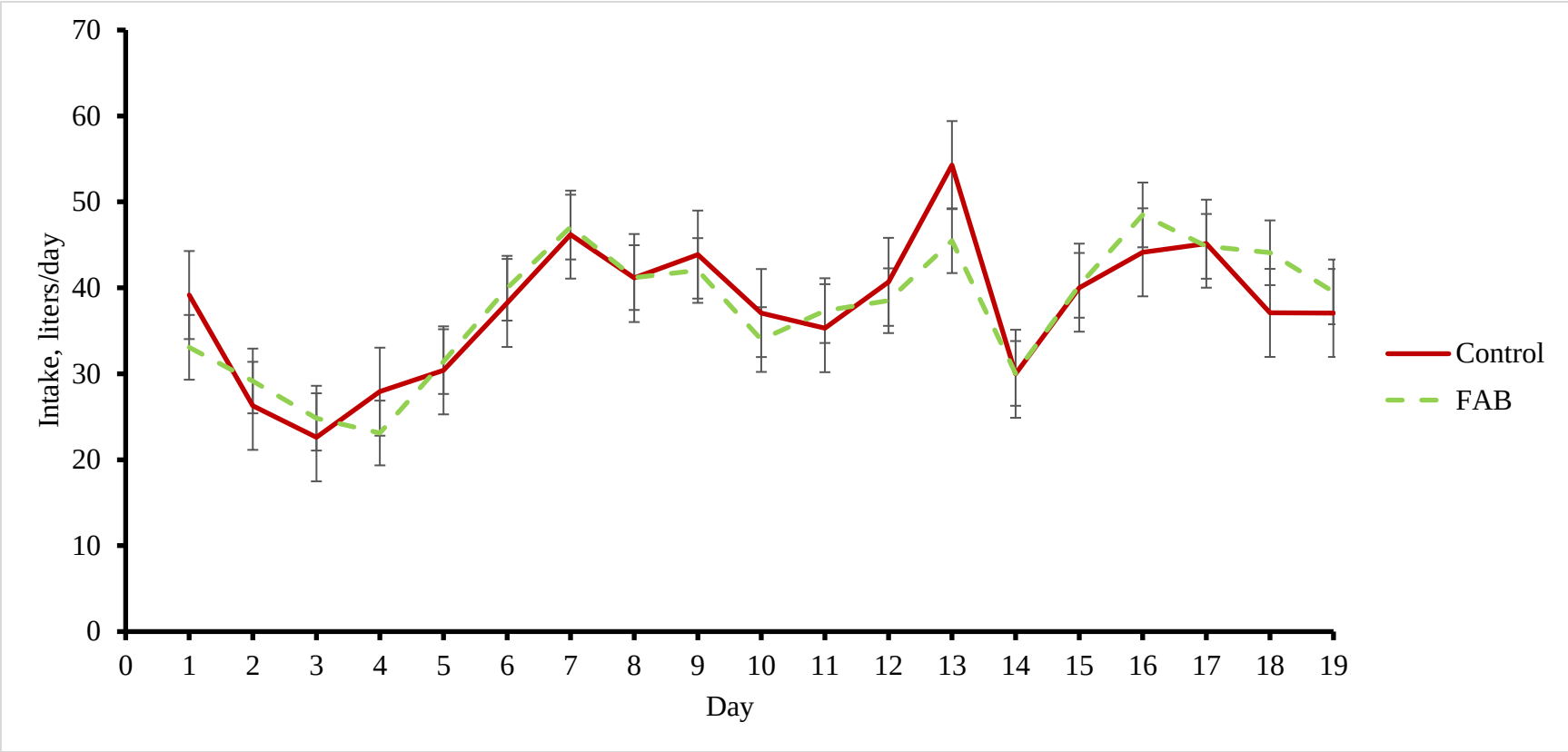

Figure 3.2. Daily water consumption for steers fed either the control diet or the FAB supplement at a rate of 100 g/kg of the diet. Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatments on water consumption were not different (P=0.403). No treatment-by-day interaction was observed (P=0.477).

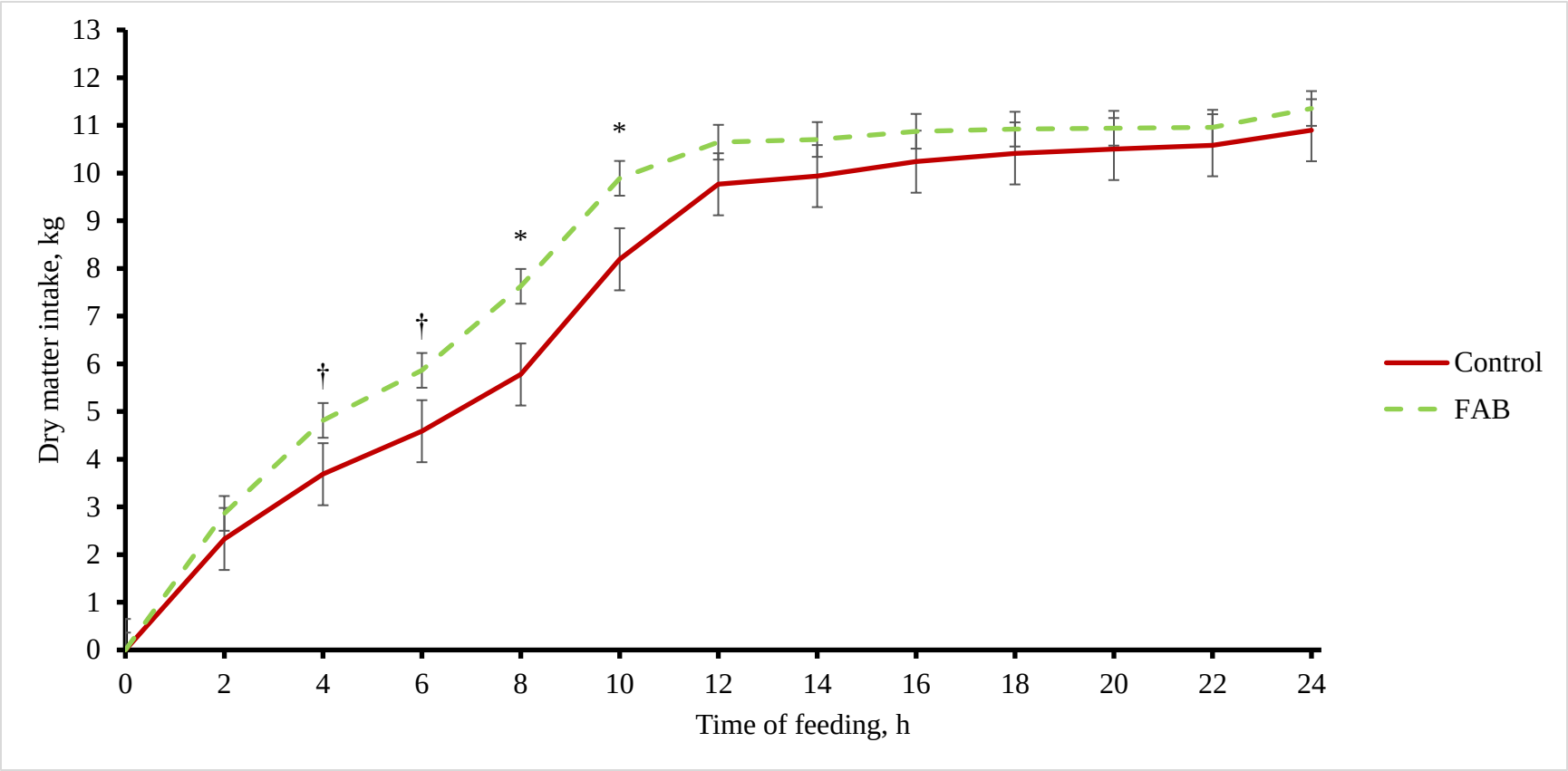

Figure 3.3. Cumulative dry matter intake (DMI) over the collection period for steers fed either the control diet or the FAB supplement at a rate of 100 g/kg of the diet.
Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatments on cumulative DMI were not different (P=0.184). A treatment-by-hour interaction (P=0.001) was observed with steers fed the FAB supplement, consuming greater amount of feed at hour 8 and 10 (P<0.05). Steers fed the FAB supplement, also tended to consume greater amounts of feed at hours 4 and 6 (0.05<P<0.10). The symbol * indicates significance (P<0.05), and † indicates a tendency (0.05<P<0.10).

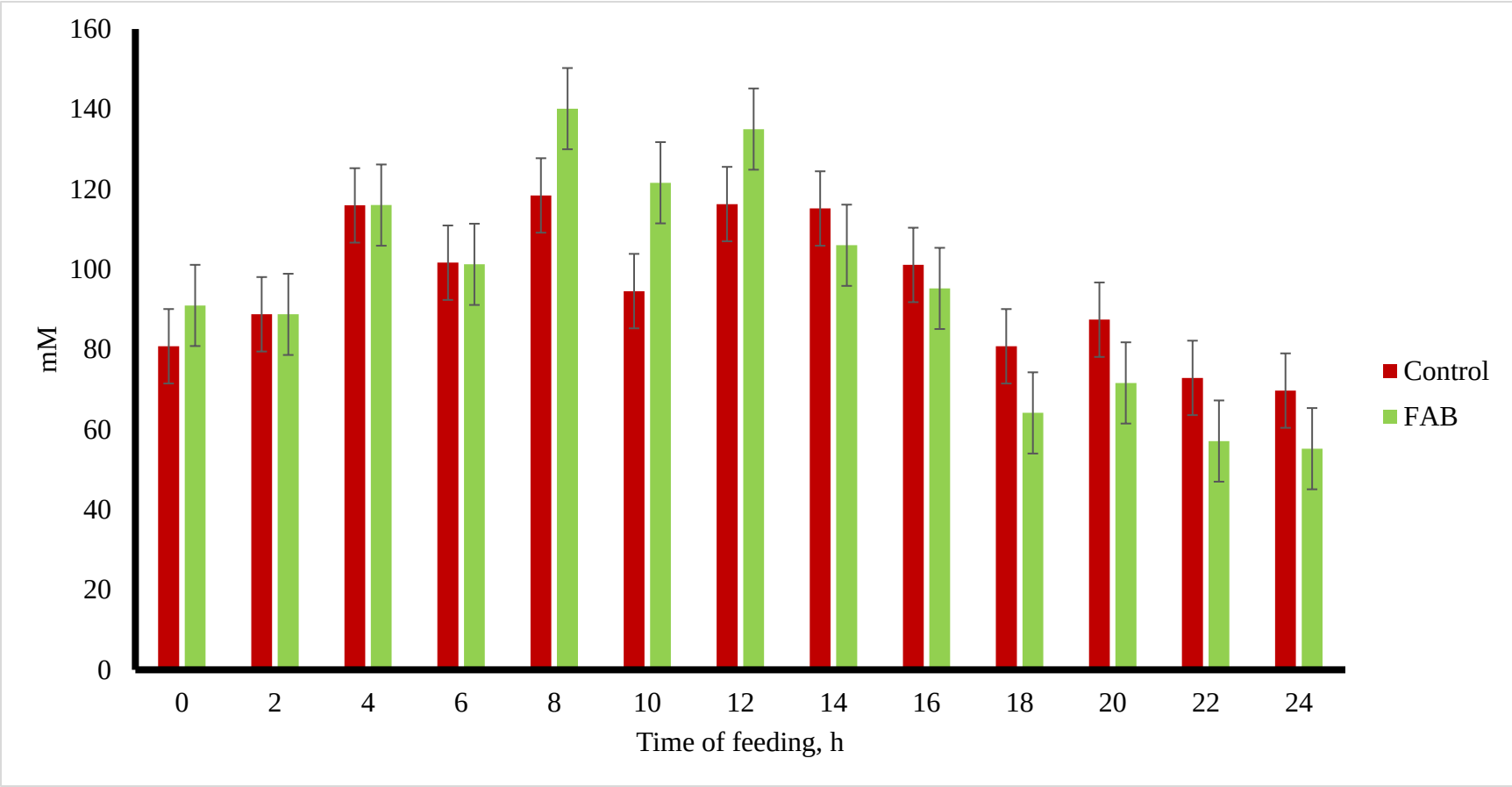

Figure 3.4. Ruminal acetate concentrations during collection interval for steers fed either the control diet or the FAB supplement at a rate of 100 g/kg of the diet.
Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatments on ruminal acetate concentration were not different (P=0.995). No treatment-by-hour interaction was observed (P=0.193).

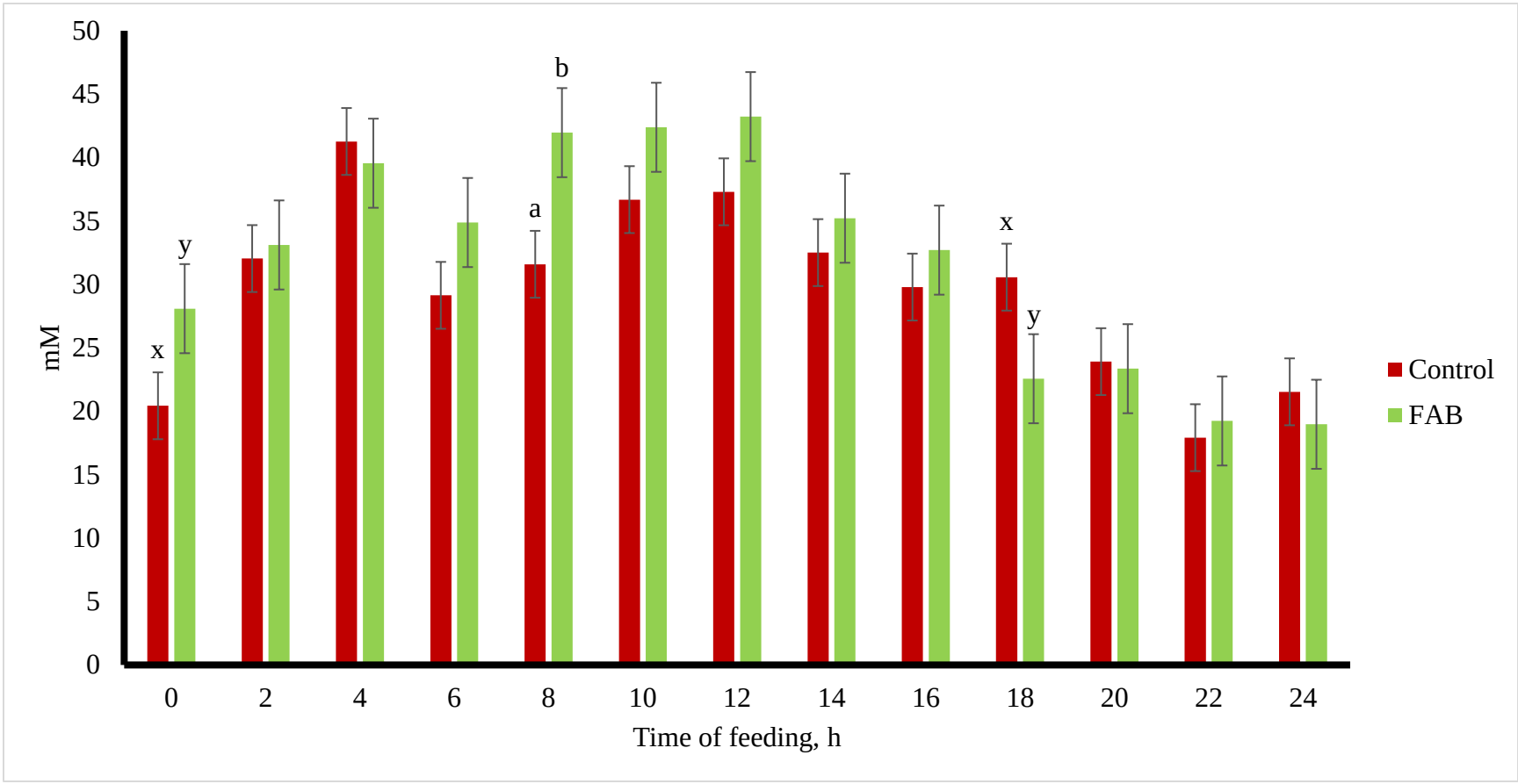

Figure 3.5 Ruminal propionate concentrations during collection for steers fed either the control diet or the FAB supplement at a rate of 100 g/kg of the diet.

Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatments on ruminal propionate concentrations were not different (P=0.333). A treatment-by-hour interaction (P=0.023) was observed. Steers consuming the omega-3 supplement had greater ruminal propionate concentrations at hour 8 (P<0.05) compared to steers fed the control diet and a tendency for greater ruminal propionate concentrations hour 0 compared to steers fed the control diet (0.05<P<0.10). Steers fed the control diet at hour 18, tended to have greater ruminal propionate concentrations compared to steers fed the FAB supplement (0.05<P<0.10). Letters a, b indicates significance (P<0.05), and x, y indicates a tendency (0.05<P<0.10).

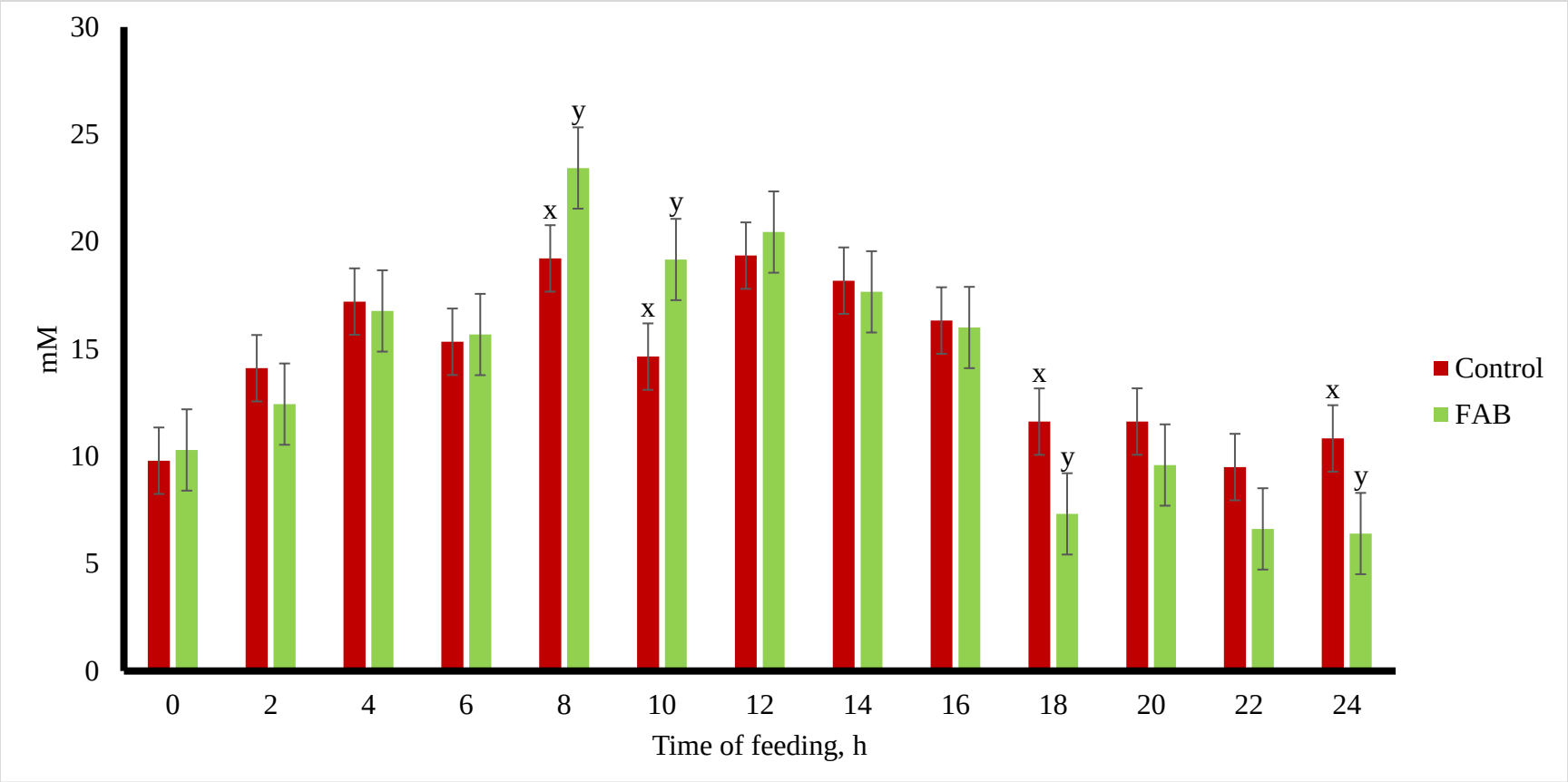

Figure 3.6. Ruminal butyrate concentrations during collection interval for steers fed either the control diet or the FAB supplement at a rate of 100 g/kg of the diet.

Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatments on ruminal butyrate concentrations were not different (P=0.726). A treatment-by-hour interaction (P=0.036) was observed. A tendency for greater ruminal butyrate concentrations was observed for steers fed the FAB supplement at hours 8 and 10 compared to steers fed the control diet (0.05<P<0.10). A tendency for greater ruminal butyrate concentrations was observed for steers consuming the control diet at hours 18 and 24 compared to steers consuming the omega-3 diet (0.05<P<0.10). Letters a, b indicates significance (P<0.05), and x, y indicates a tendency (0.05<P<0.10).

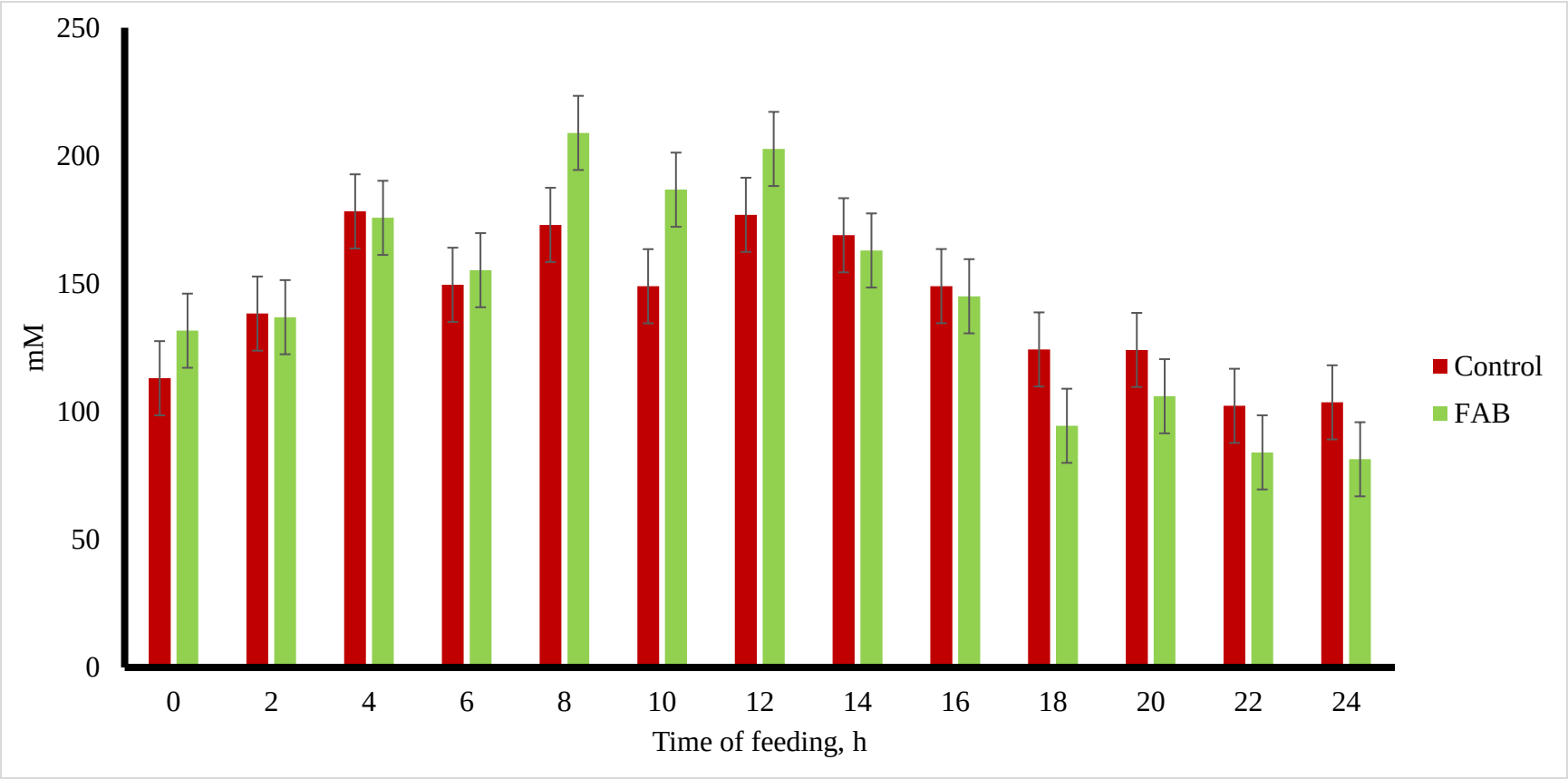

Figure 3.7. Ruminal total volatile fatty acid concentrations during collection interval for steers fed the control diet or the FAB supplement at a rate of 100 g/kg of the diet.
Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatments on ruminal total volatile fatty acid concentrations were not different (P=0.853). No treatment-by-hour interaction was observed (P=0.125).

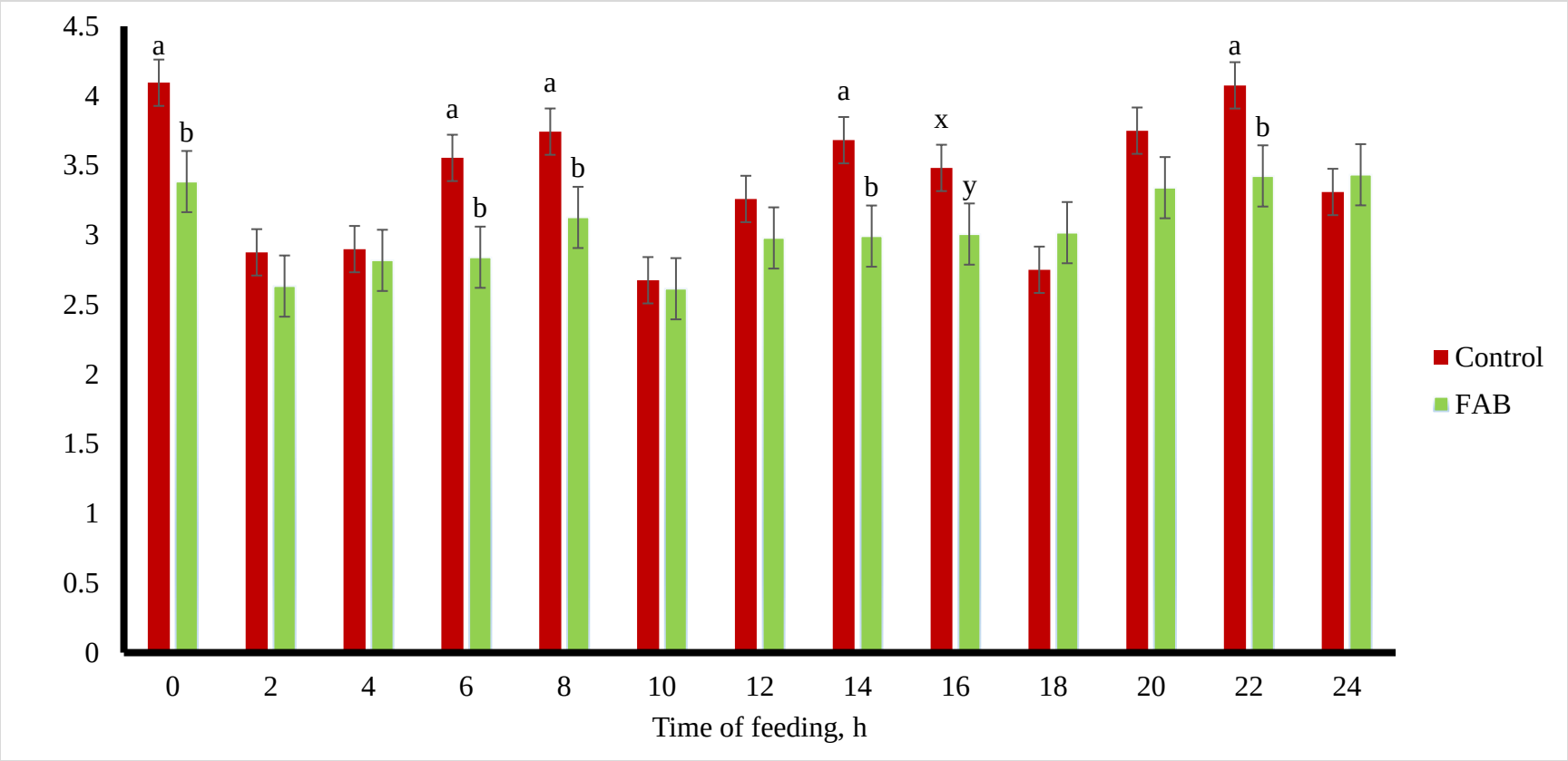

Figure 3.8 Ruminal fluid acetate : propionate (A/P) ratio during collection interval for steers fed either the control diet or the FAB supplement at a rate of 100 g/kg of the diet.

Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatment on ruminal A/P ratio tended to be lower for steers fed the FAB supplement ($P=0.092$). A treatment-by-hour interaction ($P=0.001$) was observed. Steers consuming the FAB supplement, had a decreased ruminal fluid A/P ratio at hours 0, 6, 8, 14, and 22 compared to steers fed the control diet ($P<0.05$). A tendency for decreased A/P ratio was observed during hour 16 for steers fed the control diet ($0.05<P<0.10$). Letters a, and b indicates significance ($P<0.05$), and x, and y indicates a tendency ($0.05<P<0.10$).

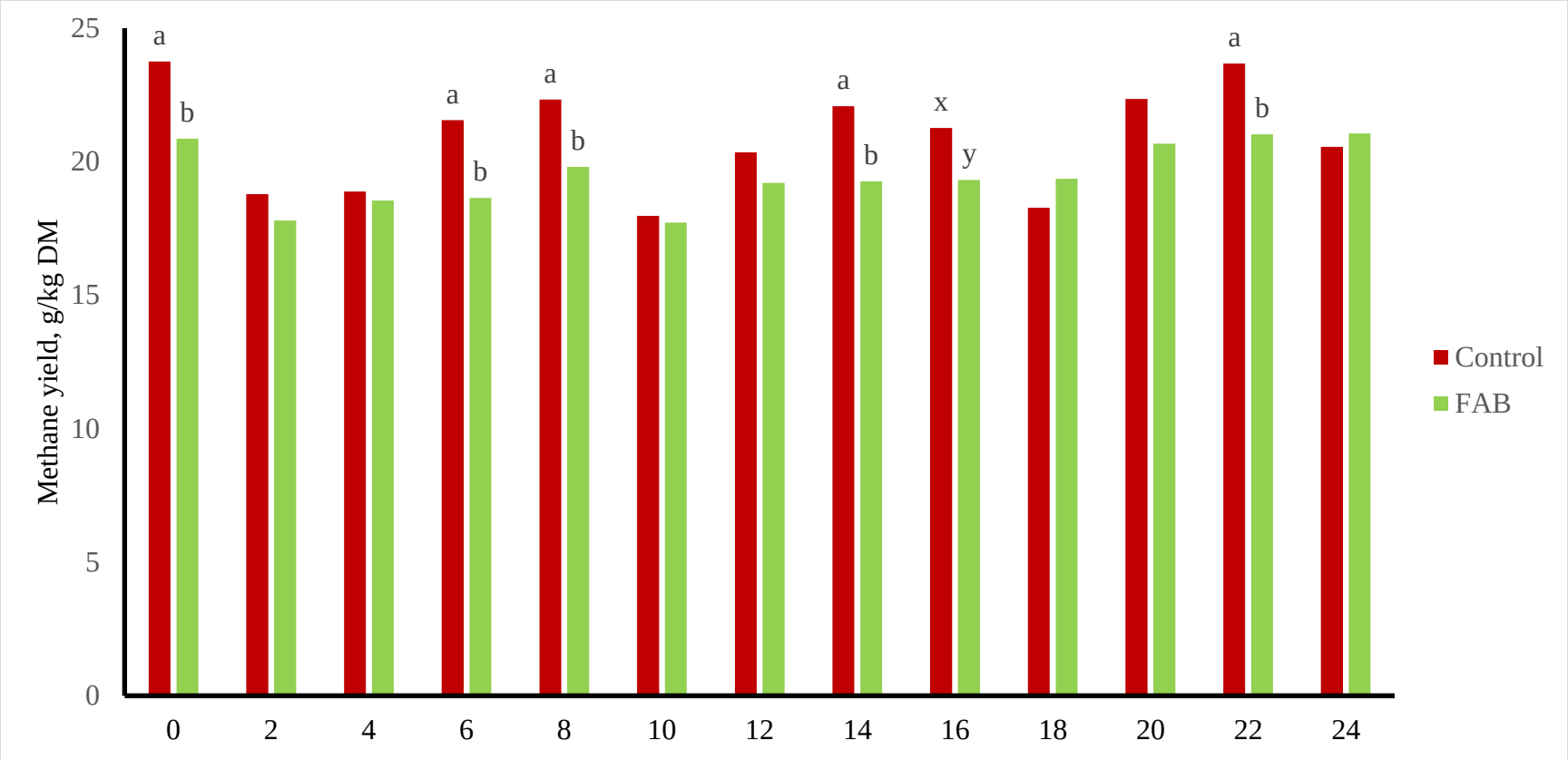

Figure 3.9. Estimated methane yield during collection interval for steers fed either the control diet or the FAB supplement at a rate of 100 g/kg of the diet.

Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatment on methane yield tended to be lower for steers fed the FAB supplement ($P=0.092$). A treatment-by-hour interaction ($P=0.001$) was observed. Steers consuming the FAB supplement had decreased methane yield for hours 0, 6, 8, 14, and 22 compared to steers consuming the control diet ($P<0.05$). A tendency for decreased methane yield was observed during hour 16 for steers fed the FAB supplement compared to steers consuming the control diet ($0.05<P<0.10$). Letters a, and b indicates significance ($P<0.05$), and x, and y indicates a tendency ($0.05<P<0.10$).

Methane Yield was calculated using the following formula "MY = 4.08×(A/P) + 7.05" (William et al., 2019)

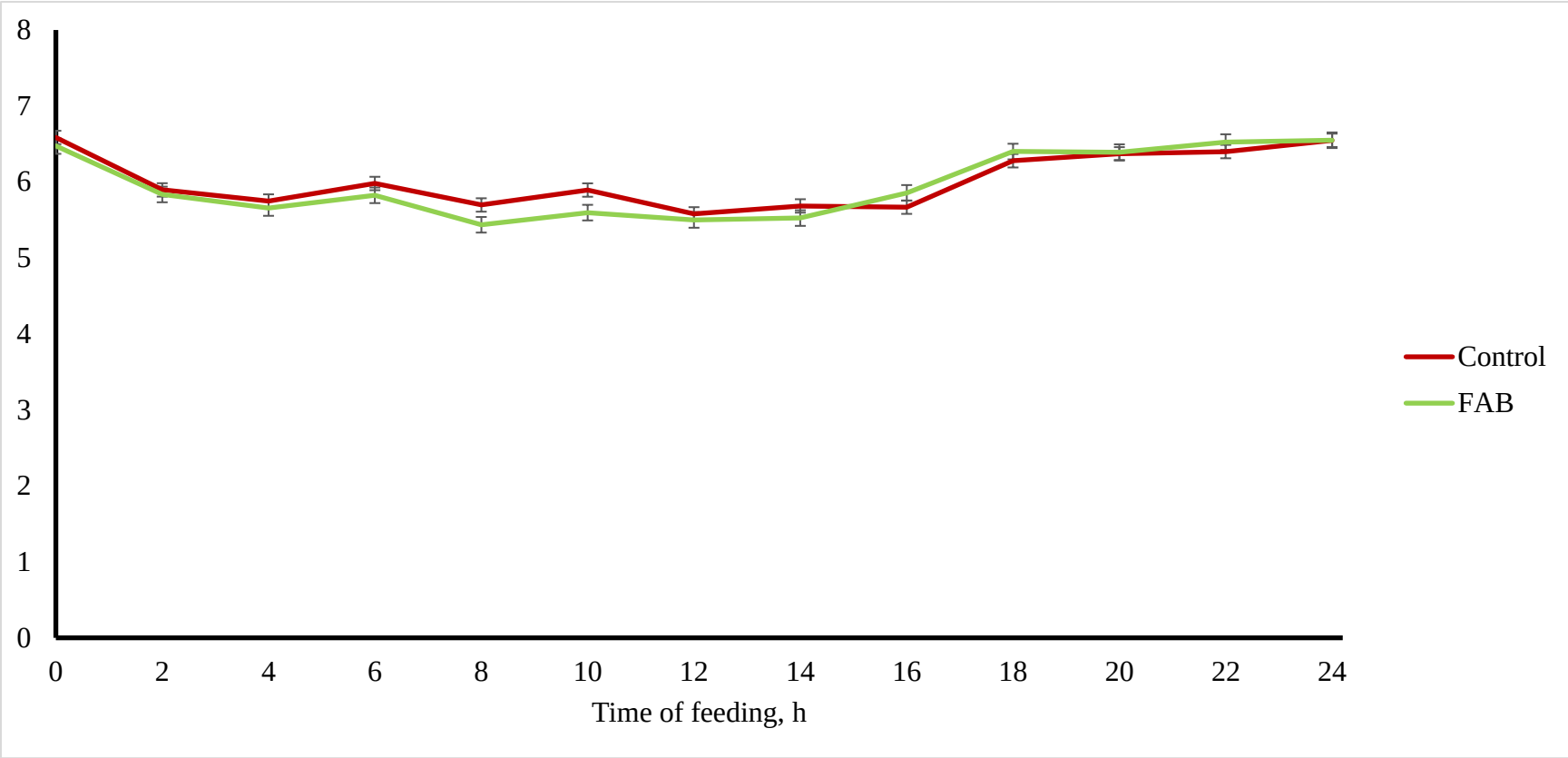

Figure 3.10. Ruminal fluid pH during the collection period for steers fed either control diet or the FAB supplement at a rate of 100 g/kg of the diet.
Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatment on ruminal fluid pH were not different (P=0.418). No treatment-by-hour interaction was observed (P=0.127).

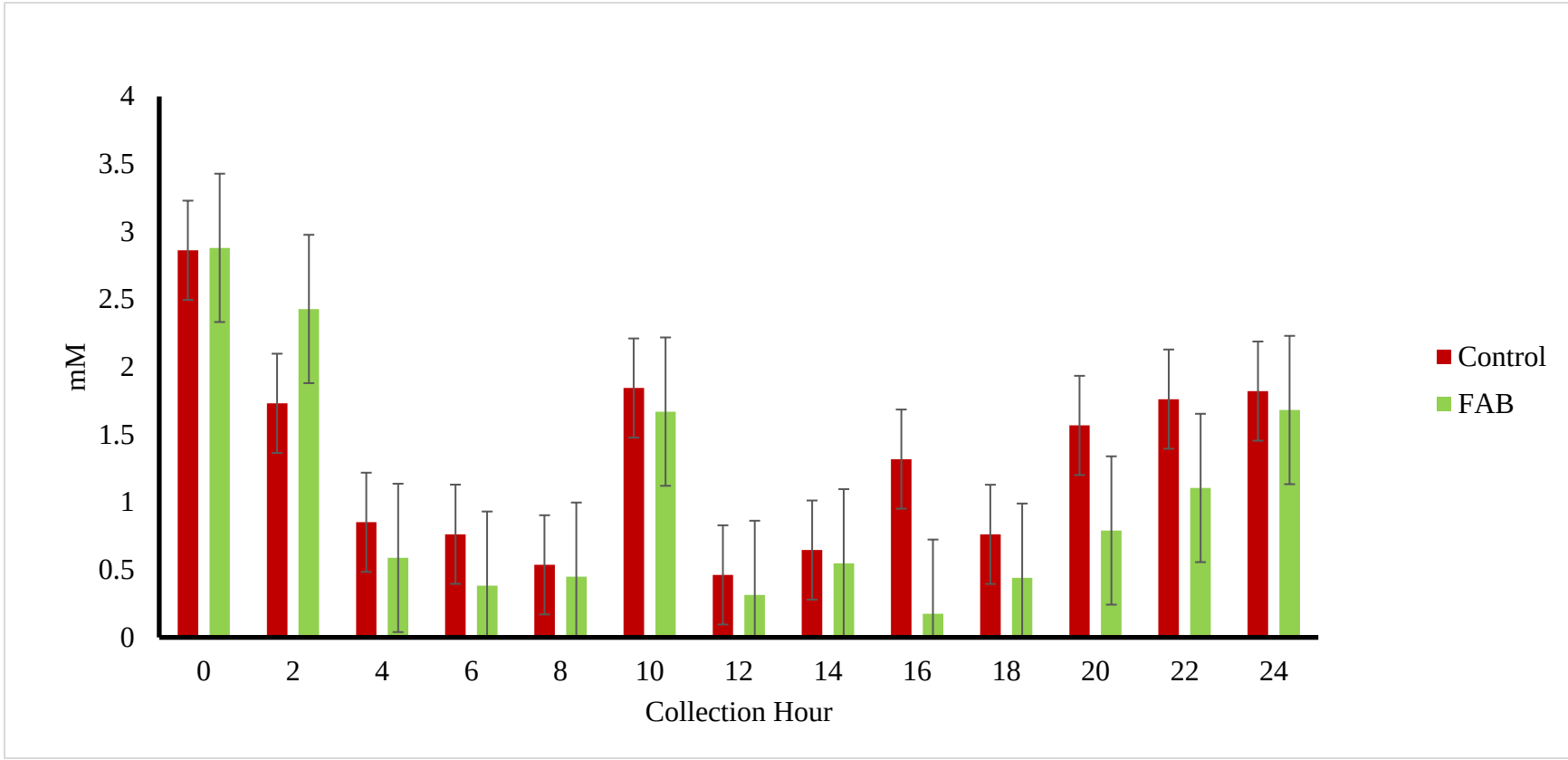

Figure 3.11. Ruminal fluid ammonia concentrations during the collection period for steers fed either the control diet or the FAB supplement at a rate of 100 g/kg of the diet.
Values are least-square means (LSM) ± standard error of the means (SEM). Main effects of treatments on ruminal fluid ammonia concentrations were not different (P=0.546). No treatment-by-hour interaction was observed (P=0.442).

Table 3.3 Apparent total intake of nutrients, ruminal flow of nutrients, and fecal excretion of nutrients

	Intake (g)				Ruminal flow (g)				Fecal excretion (g)			
Item	C[1]	FAB[2]	SEM[3]	P-Value T[e]	C	FAB	SEM	P-Value T	C	FAB	SEM	P-Value T
OM[a]	10,423	11,464	355.8	0.077	4,604	5,039	239.2	0.255	2,274	2,669	125.3	0.041
NDF[b]	3,068	3659	108.6	0.009	NP[†]	NP	-	-	1,487	1,763	75.7	0.024
FA[‡]												
C6:0	0.1	0.1	0.004	0.068	0.4	0.4	0.10	0.824	0.1	0.1	0.02	0.774
C8:0	ND[c]	ND	-	-	ND	ND	-	-	0.02	0.03	0.01	0.571
C10:0	ND	ND	-	-	0.2	0.1	0.05	0.441	0.05	0.04	0.01	0.359
C11:0	0.1	0.1	0.005	0.068	ND	ND	-	-	0.08	0.09	0.01	0.564
C12:0	0.6	0.7	0.02	0.036	1.2	1.3	0.06		0.4	0.4	0.02	0.640
C14:0	0.9	1.1	0.03	0.004	3.1	3.4	0.20	0.225	1.0	1.0	0.07	0.464
C14:1 (ω-5)	ND	ND	-	-	1.2	1.2	0.20	0.837	0.3	0.4	0.03	0.263
C15:0	0.5	0.7	0.02	0.002	2.6	3.2	0.13	0.016	1.1	1.3	0.07	0.205
C15:1 (ω-5)	ND	ND	-	-	0.3	0.1	0.04	0.025	0.08	0.12	0.01	0.009
C16:0	46.6	61.7	1.74	0.001	33.5	47.1	2.20	0.007	8.5	12.9	0.52	0.002
C16:1 (ω-7)	0.6	0.8	0.02	0.001	0.9	1.1	0.06	0.035	0.2	0.2	0.02	0.150
C17:0	0.7	0.9	0.03	0.001	1.5	2.0	0.09	0.008	0.7	1.0	0.05	0.011
C17:1 (ω-7)	ND	ND	-	-	0.1	0.3	0.04	0.015	0.1	0.1	0.01	0.232
C18:0	7.8	16.8	0.40	<0.001	108.2	202.5	11.30	0.002	24.3	63.6	3.38	<0.001
C18:1 (ω-9t)	0.4	0.5	0.02	0.001	1.9	3.5	0.42	0.037	0.2	0.4	0.05	0.024
C18:1 (ω-7t)	ND	ND	-	-	12.3	25.3	2.39	0.010	2.2	4.6	0.32	0.002
C18:1 (ω-9)	61.5	107.6	2.70	<0.001	9.25	17.4	0.98	0.002	2.8	6.2	0.32	<0.001

C18:1 (ω-7)	2.3	4.0	0.10	<0.001	1.7	2.0	0.11	0.075	0.3	0.4	0.02	0.016
C18:2 (ω-6t)	ND	ND	-	-	ND	ND	-	-	ND	ND	-	-
C18:2 (ω-6)	131.9	170.9	4.9	0.002	9.3	11.3	0.93	0.184	3.5	5.0	0.43	0.028
C20:0	1.6	2.1	0.06	0.002	1.7	3.1	0.14	0.001	0.5	0.9	0.04	0.001
C18:3 (ω-6)	ND	ND	-	-	ND	ND	-	-	ND	ND	-	-
C18:3 (ω-3)	20.0	134.7	2.9	<0.001	1.6	6.3	0.48	0.001	0.5	3.5	0.28	0.001
C20:1 (ω-9)	0.8	1.3	0.03	<0.001	0.1	0.3	0.04	0.067	0.04	0.07	0.01	0.005
CLA 9c,11t (ω-7)	0.06	0.14	0.03	<0.001	0.23	0.34	0.04	0.117	0.16	0.23	0.01	0.006
CLA 10t,12c (ω-6)	ND	ND	-	-	0.02	<0.01	0.02	0.405	<0.01	<0.01	0.003	0.405
CLA 9c,11c: (ω-7)	ND	ND	-	-	<0.01	0.09	0.03	0.077	<0.01	0.05	0.008	0.005
C21:0	ND	ND	-	-	ND	ND	-	-	ND	ND	-	-
CLA 9t,11t (ω-7)	0.02	0.08	0.002	<0.001	0.17	0.28	0.04	0.052	0.07	0.1	0.004	0.001
C20:2 (ω-6)	0.2	0.3	0.01	<0.001	ND	ND	-	-	0.04	0.04	0.007	0.344
C22:0	1.16	1.78	0.05	<0.001	1.3	1.8	0.09	0.010	0.6	0.9	0.04	0.003
C20:3 (ω-6)	ND	0.1	0.001	<0.001	0.3	0.2	0.05	0.983	0.02	0.03	0.008	0.340
C20:3 (ω-3)	ND	0.16	0.003	<0.001	ND	ND	-	-	ND	ND	-	-
C22:1 (ω-9)	0.2	0.3	0.01	0.002	0.7	0.8	0.04	0.192	0.38	0.42	0.04	0.440

C20:4 (ω-6)	ND	ND	-	-	0.5	0.5	0.08	0.650	0.1	0.1	0.01	0.581
C23:0	0.5	0.6	0.02	0.003	0.4	0.5	0.03	0.103	0.16	0.22	0.01	0.003
C22:2 (ω-6)	ND	ND	-	-	ND	ND	-	-	ND	ND	-	-
C20:5 (ω-3)	ND	ND	-	-	ND	ND	-	-	ND	ND	-	-
C24:0	1.8	2.4	0.1	0.002	1.6	2.0	0.12	0.071	0.7	1.0	0.04	0.004
C24:1 (ω-9)	ND	ND	-	-	<0.01	0.02	0.01	0.405	0.1	0.1	0.01	0.415
C22:5 (ω-3)	ND	ND	-	-	ND	ND	-	-	ND	ND	-	-
C22:6 (ω-3)	ND	ND	-	-	ND	ND	-	-	ND	ND	-	-
Total fatty acid	280.6	510.2	12.7	<0.001	195.6	338.1	17.1	0.002	49.3	105.5	4.67	<0.001

A- Organic matter
B- Omega-3 diet
C- Not detected
D- Significance was declared at (P<0.05) and a tendency at (0.05<P<0.10)

1. Control diet
2. Extruded flax-algae blend
3. Standard error of means

†-Not performed due to a lack of sufficient sample across treatments.

ǂ-Omega 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. The location of the first carbon with a double bond from the methyl end of the fatty acid is indicated in parentheses, preceded by the omega symbol (ω). Unless otherwise specified with a "t" in the location of the first double bond, the orientation of the double bond is assumed cis. Conjugated linolenic acids (CLA) used a slight modification to the omega nomenclature and this method was used to identify the orientation of each double bond. This modified nomenclature used the acronym CLA to denote conjugated linolenic acid followed by individual numbers that correspond to the location and orientation (c-cis; t-trans) of the double bond from the carboxyl end of the fatty acid. The location of the first double bond from the methyl end was also included.

Table 3.4 Coefficient of apparent ruminal appearance and disappearance of fatty acids

	Coefficient of apparent ruminal appearance and disappearance of fatty acids[1]			
Fatty acid[‡]	**Control**	**FAB[3]**	**SEM[4]**	**P-Value[5]**
C6:0	-4.36[6]	-3.35	0.484	0.280
C8:0	NC[7]	NC	-	-
C10:0	NC	NC	-	-
C11:0	NC	NC	-	-
C12:0	-1.07	-1.00	0.136	0.683
C14:0	-2.63	-2.20	0.250	0.192
C14:1 (ω-5)	NC	NC	-	-
C15:0	-4.31	-3.97	0.276	0.358
C15:1 (ω-5)	NC	NC	-	-
C16:0	0.28	0.23	0.045	0.523
C16:1 (ω-7)	-0.59	-0.42	0.112	0.277
C17:0	-1.18	-1.23	0.142	0.789
C17:1 (ω-7)	NC	NC	-	-
C18:0	-12.92	-11.12	0.960	0.243
C18:1 (ω-9t)	-3.67	-5.49	0.848	0.163
C18:1 (ω-7t)	NC	NC	-	-
C18:1 (ω-9)	0.85	0.84	0.012	0.499
C18:1 (ω-7)	0.28	0.50	0.040	0.008
C18:2 (ω-6t)	NC	NC	-	-
C18:2 (ω-6)	0.93	0.93	0.006	0.535
C20:0	-0.06	-0.46	0.083	0.017
C18:3 (ω-6)	NC	NC	-	-
C18:3 (ω-3)	0.92	0.95	0.006	0.012
C20:1 (ω-9)	0.69	0.77	0.028	0.271
CLA 9c,11t (ω-7)	-3.68	-1.69	0.280	0.035
CLA 10t,12c (ω-6)	NC	NC	-	-
CLA 9c,11c: (ω-7)	NC	NC	-	-

C21:0	NC	NC	-	-
CLA 9t,11t (ω-7)	-13.78	-3.40	1.057	0.011
C20:2 (ω-6)	NC	NC	-	-
C22:0	-0.12	-0.01	0.075	0.304
C20:3 (ω-6)	NC	NC	-	-
C20:3 (ω-3)	NC	NC	-	-
C22:1 (ω-9)	-1.32	-1.07	0.234	0.433
C20:4 (ω-6)	NC	NC	-	-
C23:0	NC	NC	-	-
C22:2 (ω-6)	NC	NC	-	-
C20:5 (ω-3)	NC	NC	-	-
C24:0	NC	NC	-	-
C24:1 (ω-9)	NC	NC	-	-
C22:5 (ω-3)	NC	NC	-	-
C22:6 (ω-3)	NC	NC	-	-
Total fatty acid	0.30	0.33	0.045	0.605

1-Calculated using formula (1 - (duodenal flow of fatty acid/intake of fatty acid))
2-Calculated using the formula (coefficient of apparent ruminal appearance and disappearance of fatty acids×1000)
3-Extruded flax-algae blend
4-Standard error of means
5-Significance was declared at ($P<0.05$) and a tendency at ($0.05<P<0.10$)
6-Negative values represent greater production of particular fatty acid than what was consumed
7-Not calculated due to either not detecting fatty acid or not quantifying enough to perform calculation.

ǂ- Omega 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. The location of the first carbon with a double bond from the methyl end of the fatty acid is indicated in parentheses, preceded by the omega symbol (ω). Unless otherwise specified with a "t" in the location of the first double bond, the orientation of the double bond is assumed cis. Conjugated linolenic acids (CLA) used a slight modification to the omega nomenclature and this method was used to identify the orientation of each double bond. This modified nomenclature used the acronym CLA to denote conjugated linolenic acid followed by individual numbers that correspond to the location and orientation (c-cis; t-trans) of the double bond from the carboxyl end of the fatty acid. The location of the first double bond from the methyl end was also included.

Table 3.5 Apparent intestinal fatty acid disappearance

Fatty acid[ǂ]	Coefficient of apparent intestinal disappearance of fatty acids[1]				Content of intestinal disappearance of fatty acid (mg/g)[2]			
	Control	FAB[3]	SEM[4]	P-Value[5]	Control	FAB	SEM	P-Value
C6:0	0.74	0.73	0.04	0.826	741	729	34.9	0.826
C8:0	NC[6]	NC	-	-	NC	NC	-	-
C10:0	0.87	0.70	0.07	0.306	875	697	68.9	0.306
C11:0	NC	NC	-	-	NC	NC	-	-
C12:0	0.68	0.71	0.02	0.361	679	712	23.0	0.361
C14:0	0.67	0.67	0.02	0.937	670	672	18.0	0.937
C14:1 (ω-5)	0.64	0.57	0.07	0.495	636	569	65.8	0.495
C15:0	0.56	0.59	0.03	0.508	564	592	30.0	0.509
C15:1 (ω-5)	0.73	0.61	0.06	0.224	727	608	58.2	0.224
C16:0	0.74	0.72	0.02	0.411	740	716	19.1	0.411
C16:1 (ω-7)	0.82	0.81	0.02	0.815	818	811	24.6	0.815
C17:0	0.49	0.50	0.03	0.928	499	504	34.4	0.928
C17:1 (ω-7)	0.48	0.49	0.07	0.936	477	485	70.8	0.936
C18:0	0.76	0.67	0.03	0.077	764	673	29.0	0.077
C18:1 (ω-9t)	0.87	0.83	0.03	0.438	867	831	29.9	0.438
C18:1 (ω-7t)	0.81	0.80	0.02	0.737	812	804	15.9	0.737
C18:1 (ω-9)	0.70	0.64	0.02	0.140	696	641	22.2	0.140
C18:1 (ω-7)	0.79	0.77	0.01	0.298	791	770	13.9	0.298
C18:2 (ω-6t)	NC	NC	-	-	NC	NC	-	-
C18:2 (ω-6)	0.62	0.56	0.04	0.302	617	557	36.6	0.302
C20:0	0.67	0.69	0.03	0.742	672	687	30.1	0.742
C18:3 (ω-6)	NC	NC	-	-	NC	NC	-	-
C18:3 (ω-3)	0.64	0.41	0.06	0.039	640	415	57.0	0.039
C20:1 (ω-9)	0.81	0.75	0.03	0.416	811	754	34.0	0.416
CLA 9c,11t (ω-7)	0.44	0.35	0.06	0.287	437	351	56.0	0.287
CLA 10t,12c (ω-6)	NC	NC	-	-	NC	NC	-	-
CLA 9c,11c: (ω-7)	NC	0.71	-	-	NC	712	-	-
C21:0	NC	NC	-	-	NC	NC	NC	NC

CLA 9t,11t (ω-7)	0.71	0.63	0.03	0.175	714	633	30.0	0.175
C20:2 (ω-6)	NC	NC	-	-	NC	NC	-	-
C22:0	0.49	0.48	0.04	0.925	489	483	44.3	0.93
C20:3 (ω-6)	0.92	0.89	0.03	0.421	922	888	29.0	0.421
C20:3 (ω-3)	NC	NC	-	-	NC	NC	-	-
C22:1 (ω-9)	0.45	0.46	0.06	0.957	454	459	64.5	0.957
C20:4 (ω-6)	0.77	0.84	0.04	0.227	771	839	36.0	0.227
C23:0	0.59	0.51	0.04	0.167	588	507	35.4	0.167
C22:2 (ω-6)	NC	NC	-	-	NC	NC	-	-
C20:5 (ω-3)	NC	NC	-	-	NC	NC	-	-
C24:0	0.53	0.47	0.05	0.449	526	471	47.7	0.449
C24:1 (ω-9)	NC	NC	-	-	NC	NC	-	-
C22:5 (ω-3)	NC	NC	-	-	NC	NC	-	-
C22:6 (ω-3)	NC	NC	-	-	NC	NC	-	-
Total fatty acid	0.74	0.68	0.02	0.128	739	677	24.1	0.128

1-Calculated using formula (1 - (fecal excretion of fatty acid/ruminal flow of fatty acid))
2-Calculated using the formula (coefficient of fatty acid disappearance×1000)
3-Extruded flax-algae blend
4-Standard error of means
5-Significance was declared at ($P<0.05$) and a tendency at ($0.05<P<0.10$)
6-Not calculated due to either not detecting fatty acid or not quantifying enough to perform calculation.

ǂ- Omega 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. The location of the first carbon with a double bond from the methyl end of the fatty acid is indicated in parentheses, preceded by the omega symbol (ω). Unless otherwise specified with a "t" in the location of the first double bond, the orientation of the double bond is assumed cis. Conjugated linolenic acids (CLA) used a slight modification to the omega nomenclature and this method was used to identify the orientation of each double bond. This modified nomenclature used the acronym CLA to denote conjugated linolenic acid followed by individual numbers that correspond to the location and orientation (c-cis; t-trans) of the double bond from the carboxyl end of the fatty acid. The location of the first double bond from the methyl end was also included.

Table 3.6 Apparent total tract disappearance of OM and NDF

	Coefficient of apparent total tract disappearance of nutritent[1]				**Content of apparent total tract disappearance of nutritent (g/kg)[2]**			
	Control	**FAB[3]**	**SEM[4]**	**P-Value[5]**	**Control**	**FAB**	**SEM**	**P-Value**
Organic matter	0.78	0.77	0.01	0.272	781	768	8.4	0.272
Neutral detergent fiber	0.53	0.52	0.02	0.805	530	520	17.5	0.805

1-Calculated using formula (1 - (fecal excretion of component/intake of component))
2-Calculated using the formula (coefficient of OM or NDF disappearance×1000)
3-Extruded flax-algae blend
4-Standard error of means
5-Significance was declared at ($P<0.05$) and a tendency at ($0.05<P<0.10$)

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