Corn silage contributions to energy supply and milk fat synthesis of dairy cows

by

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DEDICATION

To my wife Andressa Crescencio Heinzen, your love, support and dedication to me and our family made this dissertation possible. To my sons Antonio Crescencio Heinzen and Augusto Crescencio Heinzen, loving you is what made the effort and work dispense during these years so light and enjoyable.

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ABSTRACT

Adequate energy supply to lactating dairy cows is essential to maximize genetic potential of milk production and efficiency. Corn silage often accounts for more than 50% of diets and therefore, corn starch is one of the main sources of energy fed to lactating dairy cows. However, the energy supply found in form of starch is dependent on its availability to the animal. Several strategies exist to improve starch digestibility in corn silages, including hybrid selection, kernel processing and prolonged storage. However, different ways to assess starch digestibility are used in the dairy industry and research, while ruminal disappearance is the most common procedure, it differs among laboratories, and much is unknown about how the analytical results of starch digestibility assays relates to in vivo digestibility and energy supply. Moreover, high inclusion of corn, in general, in dairy cattle diets are often related to milk fat depression, due to a highly fermentable carbohydrate source and unsaturated fatty acids profile that corn provides. Therefore, a series of experiments were conducted to evaluate a novel technology of corn silage hybrids effects on silage fermentation and starch digestibility, to better understand the sources of variation on starch digestibility assays to improve sample comparison, and to assess the risks of feeding starch and fatty acids from corn silage in milk fat depression.

Two experiments were conducted to evaluate the effects of a genetically-modified corn hybrid with alpha-amylase expressed in the kernel (AMY) on fermentation profile, aerobic stability, nutrient composition, and starch disappearance of whole-plant corn silage (WPCS) and earlage. Both hybrids, AMY and its isogenic counterpart (ISO), were grown in 10 replicated plots (5 for WPCS and 5 for earlage). Samples of each plot were collected at harvest, homogenized, and divided into 5 subsamples which were randomly assigned to 5 storage lengths (0, 30, 60, 90 and 120 d). Minor differences on fermentation profile were observed between AMY and ISO for

WPCS and earlage. Starch concentration was greater for AMY than ISO in WPCS and earlage and greater starch disappearances at 0 h and 6 h were observed for ISO in WPCS and earlage. Minor effects on fermentation profile, microbial counts, aerobic stability, and nutrient composition suggests that AMY can be ensiled for prolonged periods with no concerns for undesirable fermentation or nutrient losses. However, in situ starch disappearance was lower for AMY compared to ISO.

A series of experiments was conducted to investigate variation across time-points, sample grinding size procedures and bag pore sizes used in ruminal starch digestibility assays. Experiment 1: samples of different starch sources submitted three times to multiple laboratories to evaluate variability of starch digestibility assays and the use of different incubation time-points on feedstuff ranking. Greater variation was observed for shorter incubation time-points, while different submission of samples did not influence starch digestibility for corn forage and corn grain samples. Moreover, different incubation time-points can change the ranking of samples. Experiment 2: samples of different starch sources were used to investigate how different grinding sizes and incubation time-point procedures affect starch digestibility assays variation and ranking of samples. Longer incubation time-points and finely ground samples reduced variation of in vitro starch digestibility assays; however, smaller grinding size increased starch digestibility in all starch sources and affected sample ranking. Experiment 3: samples from different starch sources were incubated in situ at 0 h to investigate the loss of particles and their influence on ruminal kinetics. Our results suggest that secondary loss of particles on ruminal incubations at 0 h might be a problem that limits the relationship between bag disappearance and degradation in forage and corn grain samples. Experiment 4: dry ground corn and pure starch samples were washed in water at different temperatures and using different filter types (Dacron polyester in situ bags, Ankom

Technology; DPB, F57 bags, Ankom Technology; F57 or filter papers Whatman G3; WG3) to understand their effects on DM recovery. Samples incubated in room temperature water had greater DM recovery when compared to samples incubated in water at 39º C. Pure starch had greater DM recovery in F57 and WG3 when compared to DPB, however, dry ground corn samples DM recovery was similar across all filter types. Experiment 5: dry ground corn and pure starch samples were incubated in rumen fluid at 0 h, using different filter bags (F57 and DPB) to investigate the effect of sample type and filter bags on DM and starch recovery. F57 bags were able to retain 100% of the starch incubated in pure starch, however, no difference between bags were found for dry ground corn samples for DM or starch recovery. These experiments highlighted the importance of the consideration of specific procedures in starch digestibility assays when ranking samples or generating information for ration formulation.

The objective of the last study was to investigate the effects of dietary fatty acids and starch on milk fat secretion, the fatty acid profile changes in whole-plant corn silage (WPCS) and high-moisture corn (HMC) with different silage management practices as well as the contributions of these nutrients derived from WPCS and HMC to milk fat secretion. Twenty-three published studies that evaluated lipids supplementation to dairy cows were included in the dataset and meta-regressions analysis were conducted to model the effects of: 1) dietary fatty acids, starch and forage concentrations (expressed as % of DM) on milk fatty acids concentration (expressed as g/100g); 2) dietary fatty acids, starch and forage concentrations (expressed as % of DM) on milk fatty acids yield (expressed as g/d); 3) intake of fatty acids (expressed as g/d), starch, and forage (expressed as kg/d) on milk fatty acids concentration (expressed as g/100g); and 4) intake of fatty acids (expressed as g/d), starch and forage (expressed as kg/d) on milk fatty acids yield (expressed as g/d). While predictions of milk fatty acids concentrations are useful, models that predict milk

fatty acids yield had greater performance. In general, starch concentration and starch intake had a positive effect on de novo and total milk fatty acids yield, while unsaturated fatty acids had a negative effect. Samples of WPCS and HMC from previous studies from our laboratory were analyzed for fatty acids profile to evaluate the effects of cutting height and storage length on fatty acids profile of WPCS and the effects of DM concentration and storage length on fatty acids profile of HMC. Increasing cutting height increased unsaturated fatty acids in WPCS, however, storage length and DM concentration effects did not follow a clear pattern of changes in fatty acids profile of WPCS and HMC. Simulations using the generated models were conducted to visualize the contributions of different inclusions of WPCS and HMC in the diet as well as different WPCS and HMC management practices to milk fat secretion. Our results suggest that energy contributions from starch and saturated fatty acids are important in milk fat synthesis predictions, while supporting the general role of unsaturated fatty acids on milk fat depression described in the literature. Management practices like cutting height, dry matter concentration and storage length affect fatty acids profile of WPCS and HMC and it may affect rumen bacteria metabolism and milk fat secretion.

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CHAPTER ONE: LITERATURE REVIEW – CORN CONTRIBUTIONS TO ENERGY SUPPLY AND MILK FAT SYNTHESIS OF DAIRY COWS

INTRODUCTION

Milk production per cow has increased 11% over the past ten years. An average cow in the US would produce approximately 9,800 kg of milk during the whole lactation in 2011 whereas in 2021 the average of milk production was 11,000 kg (USDA, 2023). Increased production is due to the improved genetic potential of dairy cattle; therefore, adequate energy supply is essential for animals to express their genetic potential.

From 2018 to 2022, more than 33 million of hectares of corn was harvested for grain and more than 2.7 million hectares for silage annually in the United States (USDA, 2018 and 2022). Modern lactating dairy cow diets are composed of various sources of corn, and corn silage often accounts for more than 50% of the diet. Corn starch, thus, is the one of the main nutrients fed to dairy cattle that supplies energy. However, efficient utilization of the energy provided by starch is dependent on its digestibility. Researchers and industry fellows have different ways to assess starch digestibility to account for energy supply in ration formulation models or evaluate management practices that increase starch availability. Ruminal disappearance is the most common procedure, and it is widely used to assess starch availability, however, these procedures differ among laboratories, and much is unknown about how the outcome of these procedures relates to in vivo digestibility and energy supply. Because of the lack of standardization, the NASEM (2021) for dairy cattle decided to use book values for total tract starch digestibility of various starch sources.

High inclusion of corn in diets is often related to milk fat depression, due to a combination of highly fermentable carbohydrates and unsaturated fatty acids supply (Baldin et al., 2018). Moreover, ensiling corn changes the endosperm structure and increases starch availability to

digestion (Hoffman et al., 2011). However, little is known about fatty acids changes during silage fermentation (Alves et al., 2011).

Corn kernel

Corn kernels are botanically classified as Caryopsis, single-seeded fruits that develop inside the mature ovary wall produced on the ear, the female inflorescence of the plant. Kernels are composed of four main parts: pericarp, pedicel, endosperm, and germ. The ovary wall develops into the pericarp, which protects the kernels, and it is attached to the cob by the pedicel. The pedicel is an organ that conducts photosynthetic products into the kernel during development to build the structural and nutritional apparatus necessary for embryo growth. The germ contains the plant embryo while the endosperm stores nutrients, mainly starch, that nourish the embryo at germination (Watson, 2003).

After pollination, kernel development occurs in three main phases. The first phase is considered the main cellular division phase, establishing the number of cells and thus, the potential for starch accumulation within the kernel (Jones et al., 1996). This phase is characterized by the rapid increase in the kernel size and weight (Borras and Westgate, 2006). Around 15 d after pollination, a second phase of development starts, which is characterized by the conversion of accumulated sugars into starch and commonly known as the “grain filling” phase. Lastly, the third phase starts around 50 d after pollination, when the separation between the kernels and the rest of the plant occurs, and the formation of an abscission layer (also known as “black layer”) characterizes the end of the third phase and is when the kernel reaches physiological maturity.

Corn Kernel Starch

Starch is a glucose polymer synthetized by kernels' cells and is composed of two different types of polymers: amylose and amylopectin. Amylose is a mostly linear glucose polymer, containing 99% of the glucose residues attached by an alpha bound between carbon 1 and 4 (α-1,4 bound) and 1% of the residues attached by an alpha bound between carbon 1 and 6 (α-1,6), while amylopectin contains around 95% of the glucose residues bound by an α-1,4 connection and the other 5% connected by an α-1,6 bound (Tester et al., 2004). Jeon and collaborators (2010) describe starch biosynthesis highlighting that multiple enzymes are involved in the process. Briefly, sucrose is produced by photosynthesis, and it can be hydrolyzed into glucose and fructose. Glucose isomerization and phosphorylation forms glucose-1-phosphate, which is the precursor of starch linear chains. ADP-glucose phosphorylase is responsible for chain elongation by adding glucosyl linear bounds using molecules of ADP-glucose. Starch polymers are stacked in concentric circles to form what are called starch granules, where the proportion between amylose and amylopectin can influence its form and compact association (Tester et al., 2004). Corn starch granules are reported to be between 2 to 30 µm in size (Tester et al., 2004). Starch in regular corn hybrids contains approximately 25% amylose and 75% amylopectin. Moreover, data shows that amylose proportion increases in relation to amylopectin as plant matures (Li et al., 2007). However, there are mutations that could affect the amylose: amylopectin proportion in corn starch. While synthesis of amylose requires the starch synthase I enzyme that is encoded by a gene called *Waxy1*, amylopectin is synthetized by several starch synthases enzymes (James et al., 2003). Mutations in the *Waxy1* gene increases the amylopectin proportion in starch, these corn lines are generally called waxy corn.

The endosperm represents approximately 80% of the kernel's dry weight and is composed of cells that accumulate starch granules and protein (Hamaker et al., 1995). Starch granules are

surrounded by prolamin proteins (known as zeins, for corn), consisted of 4 subclasses (α, β, γ, δ) and are synthesized in the rough endoplasmic reticulum of the starch-producing organelles (amyloplast; Buchanan et al., 2000). Prolamins association with starch granules is described as a physical limitation to starch access by digestive enzymes (Owens et al., 1986). Electronic microscope work done in the 1960's (Duvick, 1961; Wolf et al., 1969) showed the differences in the corn endosperm structures and allowed researchers to understand the opaque appearance of floury endosperm and the translucid appearance of vitreous endosperm. Zein proteins cross-linkages in vitreous endosperm are extensive, yielding a thicker protein matrix that forms polyhedric shapes and less loose cells, while in floury endosperm the protein matrix is thinner, and cells are less tight (Watson, 2003). These endosperm characteristics affects digestive or bacterial enzymes to access starch and therefore, corn varieties with greater percentages of vitreous endosperm can impair starch digestibility (Philippeau et al., 2000; Correa et al., 2002; Lopes et al., 2009). Endosperm structure and formation aspects are important to dairy nutrition and can improve efficiency when feeding corn as different corn varieties that vary in endosperm type can impact the energetic value of corn.

With the availability of purified enzymes, a chemical procedure to quantify starch in feed samples was reported in the early 1970's (Thivend et al., 1971), and it has been in constant development to better characterize the carbohydrate. Modern chemical procedures consist in the use of amylase and amyloglucosidases enzymes that hydrolyze the glucose linkages in the starch polymers and the resultant glucose residues are measured by colorimetry and starch is calculated (Hall, 2015). However, the enzymes used in the procedures also hydrolyze the same linkages in glycogen and oligosaccharides from animal or microbial sources, even though this pool describes carbohydrates that are potentially available for mammalian digestion it cannot be called starch,

due to the definition of the term to be established as the plant polysaccharide. Therefore, AOAC defines as "Dietary Starch" (Hall, 2015).

Corn Kernel Lipids

Scutella are germ organs where 80% of the lipids of the kernel are located (White and Weber, 2003). Triglycerides form spheres-like organelles and are stored in scutellum cells of the germ and these organelles are remarkably stable inside the cells until the embryo needs it for nourishment. Corn kernels usually contain 3-7% oil, and triglycerides make up most of the lipid content in kernels. Despite the low lipid concentration, corn is the primary source of energy for dairy cows in many countries, which makes corn fatty acids (FA) contribution to energy supply considerable (Baldin et al., 2018).

Triglycerides are fat molecules with energy store function in mammalian and plant tissues. These molecules are composed of a glycerol backbone, and three molecules of FAs. The glycerol molecule has three hydroxyl groups that are bound to the carboxyl group of each FA forming an ester bond (Nelson and Cox, 2004). There are multiple combinations of FA in triglycerides. Using chromatography techniques, researchers were able to identify different types of FA, first by the number of carbons (Plattner et al., 1977), and later by the number of carbons and hydrogen saturation (El-Hamdy and Perkins, 1981). Advanced detection techniques in chromatography systems allowed the identification of several FA besides saturation and isomers that might occur in plants.

Fatty acids are the simplest type of lipids, consisting of a hydrocarbon chain. The naturally occurring plant FAs are composed of an even number of carbons and, most of the chain lengths are longer than 16 carbons, due to its synthetic pathways. However, after synthesis, the carbon

chain can be desaturated by desaturases enzymes. Unsaturated FA has a lower melting point and can change the stability of triglycerides and cell membranes (White and Weber, 2003). Besides the common name of FA, they can be identified by the length of the carbon chain, followed by the number of unsaturation (e.g., C18:1 indicates 18 carbons in the chain with one unsaturation). Moreover, the different positions of the hydrogen atom in the carbon chain can be also identified by *trans* (hydrogen atoms are in opposite sides of the carbon chain and *cis* hydrogen atoms are in the same side of the carbon chain). For example, C18:2 *cis*-9, *trans*-11 is a FA that has a *cis* unsaturation in the carbon 9 and a *trans* unsaturation in the carbon 11.

Corn oil has low levels of saturated fatty acids. A classic review study indicates that C16:0 (11.5% of total FA) and C18:0 (1.8% of total FA) makes up the greater portion of saturated fatty acids in corn oil (Beadle et al., 1965). Therefore, unsaturated FAs are the majority of the lipids in corn oil. The same study reported that C18:1 concentration averaged 26.6% of total FA, while C18:2 concentration averaged 58.7% of total FA (Beadle et al., 1965). Recently, Baldin et al. (2018), compiled research work that reported C18:1 and C18:2 variation including high oil lines, exotic lines, and regular lines from different states in the US and other countries, and the averages reported were similar to Beadle et al. (1965), which indicates that there was little to no shift in corn breeding that resulted in major differences in corn FA profile. This aspect is particularly important when feeding dairy cows, as the FA profile of the diet may affect lipid synthesis in the mammary gland.

Factors affecting corn silage starch and fatty acids concentration

The endosperm differentiation phase occurs between 4 to 10 days post anthesis, a period in which a flower is fully open and functional. During this phase, the number of kernel cells is

defined (Altenbach et al., 2003). Little to no starch deposition is reported to occur in this phase, but the number of cells defines the extent of potential starch deposition, which occurs mostly during kernel development. The peak of starch accumulation occurs during the “grain filling” phase (12 to 35 days post anthesis) and ceases with maturity (40 to 60 days post anthesis) (Olsen, 2001).

Environmental factors can influence the deposition of starch during “grain filling” phase. Bernardes et al. (2018), discussed the effects of a short season limiting the use of corn silage in cold areas, while less detrimental effects of high temperatures are reported in corn. Temperature range for optimal corn yield is between 24 and 30°C, unless rainfall is optimum (Hoeft et al., 2000). Corn ADP-glucose phosphorylase, enzyme responsible for starch chain elongation during starch synthesis, is unstable only in temperatures above 45°C (Greene and Hannah, 1998), however, in good rainfall or irrigation conditions is unlikely to happen in the field. Moreover, nutrient deficiencies, mainly N, can impact starch biosynthetic enzymes activity (Seebauer et al., 2010). Lastly, drought stress can impact starch accumulation because of starch synthases and ADP-glucose phosphorylase activity reduction (Ahmadi and Baker, 2001). In general, heat, nutrients and moisture stress impair gene transcription and activity of various enzymes involved in starch synthesis.

Management practices can be used to increase starch concentration in corn silages. Since accumulation of starch occurs at maturity, delaying harvest increases the proportion of grains in WPCS starch concentration itself each is also beneficial to high-moisture corn (HMC), thereby increasing starch yield per hectare (Buxton and O’Kiely, 2003). However, accumulation of starch happens in synchrony with starch granules packing and protein matrix cross-linkages, which can impair starch digestion. Moreover, greater dry matter concentration might impair silage

fermentation (Ferraretto et al., 2018b). The use of hybrids of greater grain yield has been widely used by producers to increase starch concentration in WPCS (Ferraretto and Shaver, 2015). Lastly, increasing cutting height can also be used as a strategy to increase starch concentration in WPCS (Ferraretto et al., 2018a). Paula et al. (2019) reported a relationship between centimeters of increased cutting height and starch concentration of 0.08%-units. However, greater planting density has been shown to have little to no influence in starch concentration of WPCS (Ferreira et al., 2014). Silage fermentation effects on corn endosperm will be further discussed in this review.

Corn genotype has a greater influence on fatty acid composition than any environmental factor (White and Weber, 2003). Jellum (1970) reported a range of 16 to 64% of C18:1 and 19 to 71% for C18:2 among 788 different corn strains, and a strong negative relationship between these fatty acids. Further, Baldin et al. (2018) reported a negative relationship between C18:3 and C18:2 in WPCS. These relationships are probably due to desaturases activity (Alrefai et al., 1995; Vrinten et al., 2005). Reports of nutrients fertilizer effects on corn oil and fatty acids composition in classical literature agree that there is a minor effect on composition and overall effect on oil yield due to the greater grain yield (Welch, 1969; Jellum et al. 1973). In general, oil content and composition of corn can vary with location and year or season, but it is similar under various environmental conditions (Jellum and Marion, 1966).

Management practices reflect the changes discussed for oil concentration and composition in corn as well due to the increase in kernels DM proportion compared to the whole plant (Khan et al., 2012). Few studies reported changes in fatty acids profile with ensiling. Alves et al. (2011) reported a decrease in C18:2 and C18:3 concentrations with ensiling. However, Agarussi et al. (2020) and Saylor et al. (2021) reported minor or no changes in WPCS ensiled up to 240 d. However, greater concentrations of C18:2 and lower concentrations of C18:3 in late maturities

were observed (Saylor et al., 2021), probably due to the greater contribution of grains proportionally to the WPCS dry matter. Changes in saturated fatty acids concentration due to environmental conditions or silage management practices are minor (Jellum et al., 1973; Khan et al., 2012).

Corn silage fermentation

Conserving forage as silage started as a strategy in places where forage production was seasonal, and in many parts of the world, silage became an essential component of ruminant diets. As the genetics of dairy cows improved, energy requirements to attend high production also raised, thus, the optimization of area harvested per year is essential and ensiling the surplus of forage allows the dairy producer to plan feed inventory for all seasons.

Corn dry matter yield combined with high energy density is a major advantage over grasses, legumes or other cereals for forage production. Yielding 13 to 20 Mg of dry matter per hectare (Allen et al., 2003) corn silage supply NDF and starch as source of energy. Moreover, because of low N concentration, corn has a low buffering capacity and combined with optimal water-soluble carbohydrates concentration provides propitious lactic acid fermentation when ensiled. McDonald et al. (1991) described these characteristics favors rapid lactic acid fermentation and therefore high recovery of DM and energy (Pahlow et al., 2003). Counts of epiphytic lactic acid bacteria (LAB) in fresh corn plant material varies between the limit of detection (10^1) to 10^7 cfu/g. These bacteria are responsible for the conversion of sugars into lactic acid under anaerobic conditions, decreasing the pH of the forage mass and allowing for undetermined conservation period if the environment remains anaerobic. Hirano and Upper (1991) described that bacteria encountered in corn plants at harvest are obligate aerobic bacteria, located

mainly on the lower leaves and stems, protected from UV radiation and drying (Blakeman, 1981), however, epiphytic LAB numbers increase to a factor of 100 and more during harvesting process, phenomenon called as “chopper inoculation” (Woolford and Pahlow, 1998), allowing spontaneous silage fermentation. Enterobacteria are the second most abundant bacteria active in silage fermentation, important for the initial fermentation phase because of its competition with LAB, producing mainly acetic acid. Bacteria of the genera clostridium consist in low number in standing crops; however, soil and manure contamination can increase the population, and if slow fermentation and slow reduction of pH occurs, this group of bacteria can cause large losses of dry matter (Pahlow et al., 2003). Moreover, some species of yeast and molds are resistant to limited oxygen in the silo and lower pH, and when the silo is open these microorganisms are the primary starters of aerobic deterioration.

Silage fermentation process is usually described in four different phases. The first phase is called the initial aerobic phase, it starts at harvest and chop of the standing crop, and it can last for a few hours or days depending on how quickly the silo is sealed and the amount of oxygen that remained trapped inside the silo (Pahlow et al., 2003). When the oxygen inside the silo is depleted, the second phase begins and it is called the primary fermentation phase, lasting between a couple days to weeks depending on the ensiling conditions (Pahlow et al., 2003). Competition between LAB, enterobacteria, clostridia and yeast occurs until the pH is low enough to LAB to domain the microbial population and produce primarily lactic acid until stabilization of pH. The stable phase is the third phase, little occurs, only acid tolerant enzymes are still active. Finally, the fourth phase is at opening and the silage mass is exposed to oxygen and aerobic deterioration pace is determined by how well fermentation went (Pahlow et al., 2003).

Typical silage fermentation in corn generates lactic acid at 3 to 6% of the silage dry matter concentration and pH around 3.7 to 4.0 (Kung Jr., 2018). Acetic acid is the acid found as the second highest in typical corn silage fermentation, ranging from 1 to 3% of dry matter. Greater concentration of acetic acid, usually observed in silages inoculated with *L. buchneri*, lead to an improvement in aerobic stability because the acid has strong antifungal properties (Kung Jr., 2018). Butyric acid should not be detected in well-fermented corn silages, the presence of this acid indicates clostridia metabolism. Ethanol and other alcohols can be produced during corn silage fermentation, but its concentration is usually low (0.5 – 1.5% of dry matter) and greater concentration of ethanol is usually related to high yeast counts in the silage mass, however, the addition of amylases before ensiling can generate greater concentrations of ethanol as well. Moreover, ethanol fermentation caused by enterobacteria and/or heterofermentative LAB led to greater loss of dry matter and energy in silages (Rooke and Hatfield, 2003).

Influence of management practices on corn endosperm nutrients

Hybrid selection

Hybrid selection is the first step a corn producer will consider to improve corn silage nutrient content and availability. Moreover, hybrid selection will affect cows' nutrition the whole year. This section of the review will focus on hybrids with alterations of kernel characteristics. Corn hybrid types consist in different modifications in endosperm structure, often related to an increase of crude protein or lipids concentration at the expense of starch concentration or different starch and protein matrix structure. Giuberti et al. (2014) stated that starch alterations in hybrids are usually related to starch composition (amylopectin and amylose ratio) and endosperm structure (floury and vitreous endosperm ratios).

As discussed previously, mutations in the *Waxy1* gene increase the amylopectin concentration in corn starch corn, and in vitro human enzymatic digestion is greater for starch with greater amylopectin concentration (Rendleman Jr., 2000). However, Ferraretto and Shaver (2015) conducted a meta-analysis over data collected from studies from 1995 and 2014 and did not find differences in total-tract digestibility of starch, dry matter intake or milk production between waxy and conventional corn fed to dairy cattle. Endosperm structure alterations is very common in corn, Correa et al. (2002) compared kernel vitreousness from Brazilian hybrids with U.S. hybrids dissected by hand. Brazilian hybrids averaged 73.1% vitreousness, while U.S hybrids averaged 48.2% vitreousness. Across all hybrids analyzed by the authors, in situ starch disappearance was negatively related to vitreousness (R^2 = 0.87). Moreover, the authors showed that even though starch concentration did not change with maturity, vitreousness increased in U.S corn hybrids (Correa et al., 2002). Lopes et al. (2009) evaluated dry rolled corn from vitreous (64% vitreousness), floury (0% vitreousness) and opaque (0% vitreousness) corn varieties. Varieties with less vitreousness had greater in situ starch disappearance at 8 h of incubation and greater total-tract apparent digestibility of starch.

Corn breeding programs to enhance nutritional quality of corn hybrids were conducted in the early 2000's. The main varieties outcomes from these breeding programs were the hybrids known as NutriDense, breed to produce kernels with larger germ (embryo) and therefore, greater concentrations of oil and protein. Benefield et al. (2006) showed lower starch concentrations, but greater crude protein and ether extract for NutriDense grains when compared to conventional dent grain. However, there was no effect of these hybrids in milk yield or components when cows were fed NutriDense grains compared to conventional (Benefield et al., 2006; Ferraretto and Shaver, 2015).

Different high-oil corn varieties were developed in the beginning of the 20th century (White et al., 2003), and garnered attention of the dairy industry in the 1990's (LaCount et al., 1995). Fatty acids in these hybrids have greater concentration of C18:3 and C18:1 than conventional, yet concentrations of C18:2 is usually similar. LaCount et al. (1995) studied the effect of feeding high-oil corn varieties as grain and as WPCS for lactating dairy cows for up to 43 weeks of lactation. No effects were reported for milk yield and components from week 4 to 43 of lactation, however, cows at 4 to 17 weeks of lactation, milk yield tended to be higher while milk protein concentration was lower for cows fed high-oil corn. More recently, Ferraretto and Shaver (2015) reported no effects on dry matter intake and milk yield of dairy cows fed WPCS from NutriDense, high-oil, waxy and conventional corn varieties. However, high-oil varieties had lower milk fat concentration and yield and milk protein concentration when compared with other hybrids (Ferraretto and Shaver, 2015).

Genetically modified corn varieties are widely used in the agriculture industry. Significant development of heat tolerant alpha-amylase enzymes was achieved in the early 2000's (Richardson et al. 2002) and an alpha-amylase enhanced corn hybrid was developed. Wolt and Karaman (2007) showed expression of alpha-amylase (AMY797E) in kernels in these hybrids and stated that little variance in expression happens over generations and across environments. Initially designed for the ethanol industry, enhanced alpha-amylase corn hybrids garnered interest in the dairy industry. Enhanced amylase grain evaluation was conducted by Hu et al. (2010), authors reported no effects of rumen volatile fatty acids concentration and in vitro ruminal starch degradability at 6 h was less than 1% unit greater for high-amylase corn grains ($P < 0.01$). However, greater amylase activity and starch degradation was reported for high-amylase grain incubated in water at 40º and 65º C for 24 h. Differences of starch degradation at 40º C were small (1.99 vs. 1.60% of initial starch),

while the difference was greater at 65º C (10.56 vs. 0.85% of initial starch). The authors stated that the temperature-dependent enzyme activation explains the lack of effect observed in rumen fermentation of enhanced amylase corn.

Recently, the effects of feeding lactating dairy cows with an enhanced alpha-amylase hybrid have been studied. Cueva et al. (2021), reported 2.0 kg/d greater milk yield but similar intake for cows fed enhanced amylase corn silage compared to its isogenic counterpart. However, apparent total-tract digestibility of starch was similar between both hybrids. Krogstad and Bradford (2023) reported no differences in total-tract starch digestibility, dry matter intake or milk yield when feeding enhanced amylase corn silage in diets of low and high starch compared with its isogenic. Lastly, cows fed a combination between enhanced amylase corn silage and corn grain had 1.5 kg/d greater dry matter intake and 2.2 kg/d greater milk yield, however, greater total-tract apparent starch digestibility was reported for the isogenic counterpart (Rebelo et al., 2023). The authors stated that the differences in starch digestibility could be due to a greater particle size of the grains in enhanced amylase corn observed in their study. It is possible that slight differences in maturity had caused differences in processing and therefore, masked the effects on starch digestibility. These studies suggest that there might be benefits of feeding enhanced amylase corn to lactating dairy cows, however, literature is still inconclusive and inconsistent to fully understand the benefits of feeding this type of hybrid.

Ensiling

The fermentation phase of ensiling was thought to last 7 to 45 d (Pahlow et al., 2003), and minimal to no changes in the silage mass would occur after. However, research has shown that fermentation and changes of the forage mass can continue for much longer in WPCS and HMC.

Different studies report pH decline and accumulation of lactic and acetic acid up to 180 d in WPCS (Der Bedrosian et al., 2012; Windle et al., 2014) and in HMC (Stock et al. 1991). Moreover, Bothast et al. (1975) reported bacterial activity in HMC ensiled for up to 200 d. Baron et al. (1986) reported that proteolysis degrades protein in HMC during the fermentation process and Philippeau and Michalet-Doreau (1998) observed that ensiled grain had greater ruminal starch degradability. Later, it was elucidated that during the fermentation process, the hydrophobic protein matrix that surround starch granules in the kernel endosperm is degraded and allows greater access to starch in HMC by rumen bacteria and extend periods of storage can substantially modify the protein matrix (Hoffman et al., 2011). Junges et al. (2017) reported that bacterial enzymes contribute to 60% of the proteolytic activity, followed by kernel enzymes (30%) and fungi (5%) and fermentation end products (5%) in rehydrated corn grain silages. Hoffman et al. (2011) observed a decrease in zein concentration while soluble crude protein and ammonia-N concentration increased in HMC ensiled for 240 d. Positive relationships between ammonia-N ($R^2 = 0.61$) or soluble CP ($R^2 = 0.55$) and in vitro starch disappearance at 7 h were observed in a study containing more than six thousand HMC samples (Ferraretto et al., 2014). It is important to highlight that ammonia-N and soluble crude protein can also derive from proteolysis that occurs in the stover fraction of the corn plant and maturity of ensiled WPCS can influence the concentration of ammonia-N (Der Bedrosian et al., 2012).

While modification in kernel endosperm is well studied, literature is limited regarding germen and lipids content modifications during silage fermentation for WPCS and HMC. Studies reported loss of FAs after ensiling (Dewhurst and King, 1998; Elgersma et al., 2003), however, these losses are probably due to plant respiration before depletion of oxygen inside the silo (Rooke and Hatfield, 2003). Conversely, Alves et al. (2011), studied fatty acids concentrations in fresh

and ensiled ryegrass and corn plant samples and reported increased C18:2 and C18:3 in ryegrass silages. Increase of total and specific FAs concentration may be observed after ensiling due to the dilution effect of sugars depletion. Occurrence of trans C18:1 and C18:2 isomers was reported in grass silages (Lough and Anderson, 1973). Moreover, Ogawa et al. (2005) found that LAB, commonly found in forages, can hydrogenate C18:2 and C18:3. Since whole-plant or fractioned corn silages make up most of modern dairy cow diets, the amount of FA supply from corn (mainly C18:2) is considered a risk factor for milk fat depression. Alves et al. (2011) reported that ensiling decreased C16:0, C18:1 *cis*-9, C18:2 n-6 and C18:3 n-3, while there was an increase in C18:1 *cis*-11 concentrations of total FA in WPCS. Total FA in mg/g of dry matter did not change with ensiling, which suggests a shift in these fatty acids rather than degradation. Moreover, ensiling corn can increase starch ruminal fermentability as discussed previously. Therefore, the interaction between these factors raises the concern that ensiled corn would post milk fat depression risk (Baldin et al., 2018). Recently, Agarussi et al. (2020) studied the effects of ensiling (0 vs. 120 d of storage) on FA profile of WPCS and reported no differences for the major FA found in corn silages (C16:0, C18:0, C18:1, C18:2 and C18:3). However, Saylor et al. (2021) reported increased C18:3 in WPCS after ensiling (0 vs. 30 d).

It is important to highlight that maturity at harvest may affect C18:2 concentrations in WPCS (Saylor et al., 2021). However, Baldin et al. (2018) reported that C18:2 and total FA was positively related to starch concentration, suggesting that the FA concentration in WPCS reflects the FA composition of kernels and its proportion in the silage mass rather than maturity.

Starch contributions to energy

Starch digestion and metabolism

Corn is the main source of starch fed to modern dairy cows. Generally, starch ferments and digests more extensively and rapidly when compared to fiber and is usually included in the diets to meet energy requirements of lactating dairy cows (NASEM, 2021). Starch is usually extensively fermented in the rumen and still can be digested in the small intestine by pancreatic amylases if it escapes rumen fermentation (Owens et al., 1986).

Starch is mainly fermented into propionate in the rumen, being substrate to amylolytic bacteria in the rumen such as prevotella species and *succinomonas amylolytica*. Bacterial amylase enzymes are used by these species to hydrolize α-1,4 and α-1,6 bound in starch polymers and the glucose released is converted into pyruvate. Since the rumen is an anaerobic environment, pyruvate is converted into succinate via the TCA cycle, and afterwards into propionate, as a mechanism to renew NADH coenzymes and as a hydrogen ion sink. Dijkstra (1994) reported that feeding starch increases molar proportion of propionate production in the rumen over many in vivo observations. However, feeding highly digestible carbohydrates, like starch, increases the rate of production of volatile fatty acids (VFA) in the rumen, which can decrease pH, if the rumen is not properly buffered, thus selecting amylolytic and lactate-producing bacteria and consequently lactate production can arise (Mackie and Gilchrist, 1979). Russel and Hino (1985) reported that highly fermentable diets increase the growth rates of *Streptococcus bovis* and this species was related to decreased acetic acid production and increased lactic acid proportions. Depression of ruminal pH below 5.5 is described as acute acidosis, causing digestive disorders and other health problems to dairy cows (Russel and Hino, 1985). In a recent review, Plaizier et al. (2022) discussed that feeding highly fermentable sources of starch reduced ruminal pH to <5.8 or 5.6 for considerable periods of the day. This ruminal condition is considered subacute ruminal acidosis (SARA), which can impair rumen VFA production, productivity and increase inflammatory responses that potentially

affect rumen and overall animal health (Humer et al., 2018). Therefore, diets with high starch concentrations require attention with other nutrients and management to ensure rumen buffering and maintenance of rumen health.

Ruminal fermentability of starch, however, varies from 30 to virtually 100% (Firkins et al., 2001). Due to the natural function of starch for the corn plant, kernels have structural resources that aim to protect starch from insects and birds, and consequently from digestive enzymes. The pericarp is a major physical barrier to starch access in the rumen, being highly resistant to microbial degradation (McAllister et al., 1994). Therefore, particle size of corn kernel plays a major role in starch digestion and utilization. Moreover, the protein matrix of prolamins evolving starch granules in the endosperm is also considered a physical barrier to ruminal digestive enzymes to starch. It has been demonstrated that silage fermentation contributes to the solubility of prolamins, increasing starch ruminal fermentability (Der Bedrosian, et al., 2012; Ferraretto et al., 2015; Ferraretto et al., 2018b).

Undissociated form of propionate can be absorbed by the rumen epithelium via proton exchange between the rumen lumen and epithelium membrane (Dijkstra, 1994). Less than 10% of propionate is metabolized or used by epithelium cells, thus, 90 to 95% of the propionate produced in the rumen reaches the liver through the portal vein, and the liver removes around 90% of the propionate during first pass (Bergman, 1990). Since most of the dietary carbohydrates are fermented in the rumen, glucose blood concentration in well-fed ruminants is usually around half of the concentration in well-fed nonruminants, the ratio between insulin and glucagon favors gluconeogenesis pathways in the liver using propionate as the major source of glucose liver output (Aschenbach et al., 2010), besides, it favors glucose sparing mechanisms in tissues that do not rely on glucose only. This is particularly important to milk synthesis. Glucose is converted into lactose

in the mammary gland and due to the osmotic function of lactose in mammary gland cells, milk yield increases with greater production of ruminal propionate.

Starch that escapes rumen fermentation can be digested by mammalian amylases, released by the pancreas and intestinal mucosa, in the small intestine and absorbed as glucose. Thus, starch digestion in the intestine is theoretically metabolic more efficient than ruminal, because it does not need gluconeogenesis to supply glucose. However, benefits of shifting site of starch digestion to the small intestine are debatable. Owens et al. (1986), infused starch in the small intestine and demonstrated that there is no apparent limit of amylase activity of ruminants in the small intestine, however, infused amylase did not increase starch digestion in beef steers (Remillard and Johnson, 1984), which suggests that the nature of the ruminal fermentation resistant starch is likely more resistant to digestion post-rumen as well. Literature has shown that infusion of glucose might increase milk yield in short-term (Schlei et al., 2007) but suggests a metabolic adaptation and no effects in milk yield in longer infusion periods (Amaral et al., 1990). Infusion of glucose also showed reverse effects on milk yield depending on the dietary starch (Hurtaud et al., 1998; Hurtaud et al., 2000). Starch fermentation in the rumen also contributes to energy supply to microbes and therefore, contributes to other carbohydrates fermentation and microbial protein production.

Large intestine starch fermentation might happen if enough starch resists ruminal and intestinal digestion. However, the fermentation products generated in the large intestine are lost in the feces and it can cause acidosis depending on the amount of starch that reaches the hindgut (Orskov et al., 1970).

Starch digestibility assays

To understand starch contributions to energy supply and be able to rank samples based on starch availability, ruminant nutrition researchers have been investigating chemistry, physical characteristics and biochemistry of starch since the 1910's when the proximate analysis system was developed. However, chemical assays available at the time to properly separate starch in feed samples were lacking. A decade later, Shaw and Norton (1920) evaluating silo construction material reported starch concentration in corn silage for the first time in the Journal of Dairy Science. However, only in 1969, purified enzymes were available and allowed for specific measurements of starch (Smith, 1969) allowing in vitro starch digestibility to be measured after incubation (Tonroy and Perry, 1974). Starch concentration assays have been improving since then, using fewer interferences and artifacts (Hall and Mertens, 2017).

Starch is a major source of energy fed for dairy cows, thus, efficient utilization of starch by the animals is essential to improve efficiency of milk production. Moreover, the high cost of cereal grains drives that premise further. Firkins et al. (2001) reported that total-tract starch digestibility (TTSD) can increase milk yield, milk protein yield and feed efficiency. Starch digestibility varies widely, and it is affected by several factors like maturity, endosperm type, moisture, and silage fermentation (Ferraretto et al., 2013). Therefore, increasing starch digestibility of corn and other starch sources became a goal for farmers and researchers. Starch concentration assays in feeds and feces allowed in vivo TTSD assays to be conducted, and with the increased use of digestion markers in vivo assays garnered popularity in dairy nutrition research, allowing researchers to conduct these assays without total collection of feces. The in vivo assay consists of recording individual starch intake and collecting feces for a period of 24 hours, using total feces collection or digestion markers to measure excreted starch and calculate digestibility.

However, while TTSD is commonly used in research, is virtually impossible to have TTSD assays conducted on farms to assess starch utilization by the cows. Currently, a limited number of methods to access TTSD on farms are available. Sieving fecal material and determining the amount of grain in feces is only a qualitative test to estimate the amount of starch that escaped digestion. An equation to estimate TTSD from fecal starch measurements was developed at the University of Wisconsin based on controlled research studies (Fredin et al., 2014), with a coefficient of determination of 0.94. For each percentage unit of fecal starch, a decrease of 1.25 percentage units in TTSD was reported, the authors also reported that fecal starch can be accurately predicted using NIRS, which allows estimation of TTSD in rapid and cost-effective way (Fredin et al., 2014).

However, there are limitations to in vivo techniques, for example, evaluation of starch digestibility of individual feeds cannot be conducted in vivo. Thus, determining if forage management practices improve starch digestibility cannot be done by pen and TMR measurements due to the interactions with the other feeds in the diet. Hence, the utilization of in vitro or in situ assays are widely used in the industry as well as a research tool to assess ruminal starch digestibility, allowing for evaluation of management practices and raking of feedstuffs prior to feeding using of a reduced number of animals, and its relatively easy, fast and cheap to conduct.

Sources of variation in in vitro and in situ assays

In vitro systems for feed ingredients degradation were initially reported by Tilley and Terry (1963) and further developed by Goering and Van Soest (1970), these assays were well established for investigation of fiber digestibility. The theory that ruminal microbes could convert nitrogen into protein with carbohydrates fermentation as a source of carbon and energy was the main reason that drove starch ruminal digestibility assays forward. With the availability of starch concentration

assays, it was then possible to use in vitro procedures to assess starch digestibility (Tonroy and Perry 1974). Since then, research in starch digestibility grew and led to the understanding that fermentation of different carbohydrates yields different molar proportions of VFA (Strobel and Russel, 1986). Fermentation of starch yields primarily propionate (Murphy et al., 1982), an important VFA that contributes to milk yield.

In vitro procedures are conducted to mimic the rumen environment, rumen fluid inoculum is used together with buffers and artificial saliva in contained glass vials where bags containing the samples are incubated or the content is filtered after incubation for analysis. This technique allows for data collection on ruminal gas production, fermentation profile and ammonia concentration, pH and microbial ecology besides the disappearance of the target nutrient. Uden and Van Soest (1984), discussed limitations of the in vitro techniques in imitating nutrients degradation and utilization and concluded that controlled environment and minimal pH and temperature oscillation in in vitro techniques can impact the outcome. The authors also notice a difference in lag time when comparing in vitro and in situ procedures, and interactions between bags pore size and gas accumulation inside the bags was demonstrated to impact access of ruminal microbes to the sample (Uden and Van Soest, 1984). Moreover, in vitro procedures lack flow of VFA and favors buildup of gas pressure, which can impact microbial metabolism (Tagliapietra et al., 2010). Additionally, in vitro techniques require sample processing to ensure uniformity of sample/fluid contact, which can be a problem when evaluating treatments that affect particle size of feeds. Yáñez-Ruiz et al. (2016) extensively reviewed the history of the use of in vitro techniques for assessment of enteric methane emissions and the number of described methods for inoculum collection, incubation volume, buffering preparations and other aspects of the assay is remarkable.

Nocek (1985) stated that in situ technique development was focused on avoiding laboratory procedure differences and standardizing the variations related to procedures. In situ procedures consist of placing samples in bags that are incubated inside the rumen of a cannulated animal. Samples are ground to a greater particle size than what is usually done in in vitro procedures, placed in nylon bags and incubated inside the rumen for the time desired. After incubation, bags with samples are washed to clean ruminal content that might be attached to the bags, dried, and analyzed for the target nutrient. Even though in situ techniques appear to be closely related to what happens with starch in the rumen, the procedure has limitations. Vazant et al. (1998) provided an in-depth review of various limitations of in situ procedures that can add variation to the results observed and highlighted that the potential interactions between these factors make standardization of the procedures a difficult task. Some of these limitations also apply to in vitro procedures and it will be discussed together with other in vitro procedures literature.

First, the ratio between the sample size and bag surface area influences the microbial access to the sample, and there is a negative relationship between sample size and bag surface area (Figroid et al., 1972; Van Hellen and Ellis, 1977), where increasing the ratio of sample size to bag surface decreased NDF or DM digestibility. However, when size of the bag was changed but the sample size:bag surface area ratio remained the same, no differences were reported (Playne et al., 1978), this effect is probably observed because of inadequate mixing of the rumen contents with the sample inside the bag, thus, impairing bacterial attachment for digestion of carbohydrates. Vanzant et al. (1998) showed that the sample size:bag surface area ratio should be calculated using the following equation: sample size (mg)/(bag width [cm] x bag length [cm] x 2 sides), and suggested that the target ratio should be 10 mg/cm^2 for in situ procedures. This interaction relates to in vitro procedures as well; however, it is easy to control this relationship. Moreover, if great

amounts of sample residues are needed for the analysis, increasing the number of bags is recommended rather than changing sample size:bag surface area ratio.

Second, bag pore size is extremely important for starch digestibility assays, bags generally used in in situ starch digestibility assays have a greater pore size (50 µm) than most starch granules in corn (2 – 30 µm; Tester et al., 2004). Therefore, once starch granules are released from the endosperm protein matrix, it could leave the bag, limiting the relationship between disappearance and degradation of starch. This process is called secondary starch particulate loss (Huhtanen and Sveinbjornsson, 2006) and may result in overestimation of the extent or rate of starch degradation. Thus, starch disappearance rather than digestibility is the more appropriate term when referring to these assays. Pore size of bags used in in vitro procedures are usually smaller than 25 µm, thus, disappearance of starch without degradation might be a limitation in in vitro procedures as well due to the size of starch granules in corn (2 – 30 µm; Tester et al., 2004). Seifried et al. (2015), compared in vitro starch disappearance of different cereal grains incubated with bags varying in pore sizes (50, 20 and 6 µm) and reported that the starch that left the bags after 8 h of incubation for all the different pore sizes was below the detection limit, which suggests that there is minimal secondary starch particle loss for corn starch after 8 h of incubation in the rumen.

The third problem related to the sample preparation is processing, as grinding size affects the extent and rate of starch degradation. Attempting to mimic actual nutrient digestion, it is debatable whether samples should be prepared similar to how it is fed or after mastication and rumination (Nocek and Kohn, 1988). Due to surface area to microbial access of the sample, longer and coarser particles are associated with slower rates of degradation, which increases the variability of the assay, while finely ground samples are usually more uniform, thus, less variable. However, it increases the risk of secondary particle losses and creates unrealistic data. There is

still debate on grinding size of samples for in vitro and in situ starch digestibility procedures. Generally, commercial laboratories grind samples at 3- or 4-mm for in vitro and at 6-mm for in situ procedures. Moreover, different sample types might be affected differently by grinding, for example, comparing corn kernel with different vitreousness using the same sieve can yield samples of slightly different particle size and contribute with variation of the starch digestibility assay (Michalet-Doreau and Cerneau, 1991).

A fourth limitation that can add variation to the starch digestibility procedure is in the details when conducting the assay. Sources of variation such as preincubation, timing of incubation and removal of samples and rising procedures are not extensively studied, however it might represent important variables. Van Milgen et al. (1993) reported differences in digestion rates in corn cobs hydrated or not before in situ incubation, however, the same authors did not observe any differences when incubating forages. This procedural detail is widely used to mimic hydration by saliva and not introduce a serious temperature change in the rumen, however, literature is not conclusive on this topic. Nocek (1985), observed less variation when removing all the bags at the same time rather than inserting them together. This effect was attributed to less disturbance of the rumen environment and less variation coming from washing procedures.

Lastly, diet composition and feed management can influence rumen environment and potentially affect digestion of nutrients in vitro and in situ impairing comparisons of the same samples analyzed in different days and laboratories (Lindberg, 1985). While all the other sources of variation of starch digestibility assays are passive of standardization, is virtually impossible to standardize rumen conditions of different animals.

Despite the limitations of in vitro and in situ assays, the dairy industry and researchers utilize these assays to study management practices on starch digestion. Moreover, ruminal starch

digestibility affects the efficiency of energy utilization by the cows, contribute to microbial protein synthesis, and illustrate ruminal acidosis risks, therefore, improvement of these assays provides useful information for diet formulation of dairy cows and ranking feed based on starch digestibility is more accurate. Generally, in situ or in vitro assays are conducted with three different data collection approaches: single time-point incubations, mechanistic models (using multiple time-points incubations) and in vitro gas production assays. Data generated from these different approaches can be used in different ways.

Single Time-point incubations

Starch digestibility in vitro or in situ are often measured at single time points, because of secondary particulate loss the outcome is usually referred to as starch disappearance, since the assay measures the amount of starch that disappeared from the bag. Widely used in the industry to rank feeds, in vitro or in situ single time-point incubations are less useful to predict total-tract or ruminal in vivo digestibility (NASEM, 2021). Nocek and Tamminga (1991), showed a decent relationship between in situ and in vivo methods for determining rumen degradable starch (R^2 = 0.65), however, the data used in this regression was across different incubations time-points and feedstuffs and does not explain total-tract starch digestibility, due to compensatory digestion of starch in the small intestine (Ferraretto et al., 2013). Twenty years later, Sniffen and Ward (2011) proposed a natural logarithm equation using starch disappearance at 7 h in vitro to predict starch rate of digestion. However, digestion and utilization of starch is still dependent on passage rate of the nutrient and small intestine digestion. Moreover, even though it might help to understand ruminal starch digestion, ration formulation models that use this equation focus on the relationship between microbial protein synthesis and starch rate of digestion to predict total protein supply to

the animal. Moreover, starch fermentation in the rumen is dynamic and to better predict digestion rate, more time-points are likely needed.

Mechanistic digestion models

Mechanistic models were developed to account for the interaction between rate of degradation and passage rate of nutrients, these models have been evolved to predict ruminal carbohydrate digestibility and better predict metabolizable energy and protein in ration formulation (NASEM, 2021). Briefly, the rate of digestion (k_d) is calculated based on a natural logarithm equation, similar to what was discussed previously (Sniffen and Ward, 2011). Usually, a washout time point (0 h) determines the rapidly digested (or disappeared) fraction (fraction A). Then, a slower digested (or disappeared) fraction is calculated as B = 100 – fraction A. Applying a passage rate (k_p) to the model is possible to predict the effective degradability of the nutrient and effective ruminal degradability (ERD) is calculated as: ERD = A + B (kd/[kd+kp]). Passage rates used are values found in literature. The use of several time-points allows to demonstrate different fractions of starch: a rapidly digested fraction, a slower digested fraction and, in some cases, an undigested fraction (Fernandes et al., 2018).

While nutrient fractions and rates of degradation can be calculated for individual feeds, it does not represent in vivo conditions, the lack of accurate data for rates of degradation and passage in vivo complicates the model even further in predicting starch digestion (NASEM, 2021). Therefore, the use of in vitro and in situ procedures to predict starch digestion in vivo is unlikely to be precise.

These models are useful to rank feeds and study management practices. However, standardization of the procedures and time points might be needed to allow better sample

comparison. Fernandes et al. (2018), evaluated the effect of washout method (0 h time point), the sample grinding size and the determination of an indigestible starch fraction on in situ assays for dry ground corn. The authors reported no effects of washout method on fraction A of starch, suggesting that all washout methods tested are adequate. Further, there was a good relationship between a 2-pool model (fractions A and B) and a 3-pool model (fractions A, B and C) which suggests that a 2-pool model can be used to determine starch digestion kinetics. Lastly, the authors suggest that dry corn samples should be ground to pass a 6 mm sieve for in situ assays, and the assay can be conducted using 0, 3 and 48 h of incubation without detrimental effects on degradation rates determination. Seifried et al. (2015), compared different pore size bags for in situ assays and observed greater fraction A for bags with 50 μm porosity when compared to bags with 20 μm porosity, which led to different effective starch degradability values for each pore size.

Gas production

In 1974, Menke and Ehrensvard (1974, as cited in Menke et al. 1979) proposed a method that relied on monitoring fermentation gas production of rumen fluid in vitro batch cultures. The procedure was conducted in large syringes containing rumen fluid, medium and the sample. Gas production was measured based on the syringe plunge dislocation, and the procedure relied on the inverse relationship between gas accumulation and degradation of the feed sample. Later, Menke et al. (1979) reported a good relationship between in vitro gas production and in vivo organic matter apparent digestibility ($R^2 = 0.82$). In the early 1990's, Theodorou et al. (1994) proposed a simple yet sensitive laboratory procedure to study fermentation kinetics, as the authors described. The rumen fluid batch culture was now conducted in a glass flask hermetic closed, and the gas production measurement conducted using a pressure transducer connected to a needle that is

inserted through the rubber bottle closure. Gas production methods since then have been updated (Muetzel and Tavendale, 2014) and widely used in research due to its relatively ease to assess total volume of gas produced, lag time (important measurement that relates to velocity of bacteria attachment and start of fermentation), and rate of degradation of feed samples. However, there is no defined way to distinguish gas produced by specific nutrients, thus this technique only allows one to study feeds rather than nutrients.

Lipids contribution to energy and fat synthesis

Lipid digestion and metabolism

Despite low lipids concentrations in corn kernels and silage, corn is fed to dairy cows in high amounts. Thus, the contribution to energy of dairy cows from lipids derived from corn should be taken into consideration. Moreover, corn is rich in unsaturated fatty acids, mainly C18:1 and C18:2 (Beadle et al., 1965; Baldin et al., 2018), important fatty acids that can contribute to milk fat depression (Jenkins and Harvatine, 2014).

Lipids are not utilized by bacteria or absorbed in the rumen, but it goes through extensive transformation by microbial population. Microbial lipases can hydrolyze triglycerides, the most abundant form of lipids in plants, and other forms of lipids and release free fatty acids (FFA). Ester linkages between glycerol and fatty acids are hydrolyzed by extracellular lipases, and released glycerol can be fermented into VFA (Jenkins and Harvatine, 2014). Moate et al. (2008), analyzed triglycerides disappearance and FFA appearance in rumen fluid and reported lipolysis rate to be between 200 and 400 mg of lipids/L per hour in vitro, which indicates a rapid release of FFA in the rumen environment. However, lipolysis is dose (Beam et al., 2000) and source dependent

(Garton et al., 1961; Palmquist and Kinsey, 1994), varying in extent from 40% to 95% of total lipids. Therefore, the rate of appearance and total ruminal FFA will strongly depend on the diet.

Once FFAs are released in the rumen, biohydrogenation by ruminal bacteria takes place and modification of released FFA occurs. This process is used by bacteria as a hydrogen sink, renewing essential coenzymes in bacteria metabolism and reducing unsaturation of fatty acids. Briefly, biohydrogenation pathways occur in mainly two phases: isomerization and reduction. Isomerases catalyze an essential step before unsaturation (reduction) catalyzed by reductases. For example, under regular rumen conditions, biohydrogenation of C18:2 begins with isomerization of the molecule to *cis*-9, *trans*-11 C18:2, shifting the unsaturation positions, then a reductase hydrogenates one of the unsaturation to C18:1 and finally a second reduction step takes place to release C18:0 (Jenkins and Harvatine, 2014). Different isomers of long-chain fatty acids can be synthesized, being the pathway described is the most common in regular ruminal conditions for C18:2. Similar to lipolysis, biohydrogenation rate and extent are dependent on the amount of available FFA (Moate et al., 2008) and different biohydrogenation pathways can occur depending on rumen conditions (Qiu et al., 2004; Fuentes et al., 2009). Moreover, high concentrations of C18:2 seems to inhibit the final step of biohydrogenation from C18:1 to C18:0 (Noble et al., 1974). Literature describes that different groups of bacteria participate in the different phases of biohydrogenation, while few bacteria strains can metabolize complete biohydrogenation by itself (Harfoot and Hazlewood, 1997; Palmquist et al., 2005), which characterizes biohydrogenation as a dynamic process.

A pool of different intact and modified FFA reaches the duodenum where it will be emulsified and absorbed. Due to biohydrogenation, most of the fatty acids that leaves the rumen are C16:0, C18:0 and C18:1, the latter being present in the *cis*-9 form or *trans* isomers (NASEM,

2021), depending on the fermentation conditions, diet composition and ruminal microbial population. However, different unsaturated fatty acids are often detected in milk, which indicates that ruminal conditions and the quantity of fatty acids fed can shift biohydrogenation and consequently FFAs absorbed.

Milk Fat origin and bihydrogenation induced milk fat depression

Many producers in the US are paid based on milk fat, the most variable component of milk solids, which is greatly influenced by the diets fed to dairy cattle. Therefore, it is essential to understand how milk fatty acids are synthesized and uptake by mammary gland cells in order to assess the dietary effects on fat production and be able to manipulate it by various feeding strategies.

Milk fatty acids are described as derived from two sources: de novo synthesis in the mammary gland and preformed, fatty acids that have dietary or body reserves mobilization origin. However, before isotopes work was common in dairy research, the leading theory was that short-chain fatty acids observed in milk was derived from C18:0 degradation (Hilditch, 1947), since the dairy rations did not have many of the unique fatty acids that were found in milk. A classic study conducted with dairy goats in the early 1950's using acetate isotopes showed clear indications that there was de novo synthesis of fatty acids occurring in mammary gland cells (Popjak et al., 1951), these fatty acids contain 16 or less carbons chains. During the same decade, another study was published demonstrating that mammary gland cells also uptake circulating fatty acids originated from feed, these fatty acids have 16 or longer carbon chains (Glascock et al., 1956).

Acetate absorbed in the rumen can be converted to acetyl-CoA by acetyl-CoA synthase, an enzyme that uses energy in form of ATP to reduce the 2-carbon molecule to acetyl-CoA, that, in

turn can be substrate for fatty acids synthesis (Palmquist, 2006) in the mammary gland or other tissues. On the other hand, non-ruminants use glucose as fatty acids substrate through conversion of pyruvate to acetyl-CoA, via pyruvate dehydrogenase reaction, an enzyme reported to have low activity in ruminants (Bauman et al., 1970), probably due acetate metabolism yielding acetyl-CoA in ruminant cells, which by negative feedback diminish pyruvate dehydrogenase activity, another glucose sparing mechanism (Palmquist, 2006). Acetyl-CoA is the primer for fatty acids synthesis, while malonyl-CoA is the chain elongation substrate for the sequential reaction of β-ketoacylsynthase. The chain length is influenced by several factors, but low to none of labeled C18 fatty acids were found when infusing labeled acetate (Bishop et al., 1969; Palmquist et al., 1969), thus, fatty acids with carbon chains longer than 16 found in milk fat is thought to be derived from bloodstream.

Circulating lipoproteins rich in triacylglycerol are the main source of C18 and longer chain fatty acids in milk, being non-esterified fatty acids (NEFAs) an important source when blood concentrations are high, notably in negative energy balance situations when adipose tissue mobilization increase NEFAs blood concentration (Palmquist, 2006). Lipoprotein lipase and fatty acids binding proteins are involved in the uptake and intracellular transportation of long chain fatty acids (Vyas et al, 2013). Fatty acids uptake by mammary gland cells from blood are characterized as preformed fatty acids, and as it increases, *de novo* synthesis generally decreases (Palmquist, 2006). Moreover, C16 fatty acids can be synthesized *de novo* and originated from diet or tissue mobilization, therefore these fatty acids are considered mixed origin.

Dietary and ruminal conditions, such as high carbohydrates fermentability, high intake of unsaturated fatty acids, ruminal pH and microbial population can reduce milk fat synthesis. Milk fat depression (MFD) has been investigated for at least a century. An itineration of different

theories has been proposed related to the cause of MFD. Van Soest (1963) considered that a limitation in fatty acids absorption was the cause of MFD, however, cows fed high fat diets may also present the problem. Another significant proposed theory was that changes in rumen VFA was the major contributor to MFD, since specific diets could decrease acetate to propionate ratio, causing inadequate supply of acetate for fat synthesis. However, infusions of acetate during MFD had minimal impact on milk fat yield (Davis and Brown, 1970). Moreover, it was proposed that the increase of propionate production in the rumen would increase insulin release, a hormone that increase adipose tissue lipogenesis and decrease lipolysis, even though this theory was shown to be reliable for early lactation cows (Corl et al., 2006), it does not fully explain MFD in other stages of lactation. The most accepted theory, links ruminal biohydrogenation and the formation of specific intermediates with MFD, C18:2 (*trans*-10, *cis*-12) and C18:1 (*trans*-10) concentrations in milk were strongly correlated with MFD (Vyas et al., 2013; Matamoros et al., 2020). Studies show that the infusion of these fatty acids decreased mRNA abundance of enzymes related to milk fatty acids synthesis and uptake (Piperova et al., 2000; Peterson et al., 2003). A coordinated downregulation of lipogenic enzymes occurs and, thus, de novo synthesis in the mammary gland is decreased to a larger extent than uptake of longer chain fatty acids (Jenkins and Harvatine, 2014).

Concluding remarks

Corn silage, whole-plant or fractioned, is an important source of energy for dairy cows, supplying starch and fatty acids derived from the corn kernel. Energy density of diets is a function of the nutrient concentration and its digestibility. Therefore, understanding starch digestibility is key to models that predict energy availability. Moreover, starch digestibility is an important

parameter to rank feeds based on nutritive value or when evaluating strategies that improve starch availability. However, there is a lack of standardization of starch digestibility assays and understanding the sources of variation of the assays is the first step to standardize and improve these assays.

Feeding energy dense diets is often achieved by increasing corn contribution in the diet, this combined with the fatty acids profile of corn might convey milk fat depression problems. Thus, understanding the changes in starch and fatty acids during ensiling helps nutritionists to guide decisions when feeding high energy diets to dairy cattle.

REFERENCES

Agarussi, M. C. N., V. P. Silva, E. M. Paula, D. Vyas, A. T. Adesogan, O. G. Pereira, and L. F. Ferraretto. 2020. Effects of ensiling of whole-plant corn on silage processing score and fermentation and long-chain fatty acid profiles. Appl. Anim. Sci. 36:167-171.

Ahmadi, A., and D. A. Baker. 2001. The effect of water stress on the activities of key regulatory enzymes of the sucrose to starch pathway in wheat. Plant Growth Reg. 35:81-91.

Allen, M.S., J. G. Coors, and G. W. Roth. 2003. Corn Silage. In Silage Science and Technology. Ed. ASA, CSA, and SSSA, Madison, WI.

Alrefai, R., T. G. Berke, and T. R. Rocheford. 1995. Quantitative trait locus analysis of fatty acid concentrations in maize. Genome 38:894–901.

Altenbach, S.B., DuPont, F.M., Kothari, K.M., Chan, R., Johnson, E.L., Lieu, D. 2003. Temperature, water and fertilizer influence the timing of key events during grain development in a US spring wheat. J. of Cereal Sci. 37, 9-20.

Alves, S., A. Cabrita, E. Jerónimo, R. Bessa, and A. Fonseca. 2011. Effect of ensiling and silage additives on fatty acid composition of ryegrass and corn experimental silages. J. Anim. Sci. 89:2537–2545.

Amaral, D. M., J. J. Veenhuizen, J. K. Drackley, M. H. Cooley, A. D. McGilliard, and J. W. Young. 1990. Metabolism of propionate, glucose, and carbon dioxide as affected by exogenous glucose in dairy cows at energy equilibrium. J. Dairy Sci. 73:1244–1254.

Aschenbach, J. R., N. B Kristensen, S. S. Donkin, H. M. Hammon, and G. B. Penner. 2010. Gluconeogenesis in dairy cows: the secret of making sweet milk from sour dough. IUBMB Life. 62(12):869-877.

Baldin, M., Y. Ying, Y. Fan, G. Roth, D. P. Casper, K. J. Harvatine. 2018. Characterization of linoleic acid (C18:2) concentration in commercial corn silage and grain hybrids. J. Dairy Sci. 101: 222-232.

Bauman, D. E., R. E. Brown, C. L. Davis. 1970. Pathways of fatty acid synthesis and reducing equivalent generation in mammary gland of rat, sow, and cow. Arch. Biochem. Biophys. 140:237–244.

Beadle, J. B., D. Just, R. Morgan, and R. Reiners. 1965. Composition of corn oil. J. Am. Oil Chem. Soc. 42:90–95.

Beam, T. M., T. C. Jenkins, P. J. Moate, R. A. Kohn, and D. L. Palmquist. 2000. Effects of amount and source of fat on the rates of lipolysis and biohydrogenation of fatty acids in rumen contents. J. Dairy Sci. 83:2564–2573.

Benefield, B. C., M. Lineiro, I. R. Ipharraguerre, and J. H. Clark. 2006. NutriDense corn grain and corn silage for dairy cows. J. Dairy Sci. J. Dairy Sci. 89:1571–1579.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Reviews, 70(2), 567–590.

Bernardes, T. F., J. L. P. Daniel, A. T. Adesogan, T. A. McAllister, P. Drouin, L. G. Nussio, P. Huhtanen, G. F. Tremblay, G. Bélanger, and Y. Cai. 2018. Silage review: Unique challenges of silages made in hot and cold regions. J. Dairy Sci. 101:4001-4019.

Bishop, C., T. Davies, R. F. Glascock, V. A. Welch. 1969. A further study of bovine serum lipoproteins and an estimation of their contribution to milk fat. Biochem. J. 113:629–633.

Blakeman, J.P. 1981. Microbial ecology of the phylloplane. Academic Press, London, UK.

Borrás, L., and M. E. Westgate. 2006. Predicting maize kernel sink capacity early in development. Field Crop Res. 95:223-233.

Bothast, R. J., G. H. Adams, E. E. Hatfield, and E. B. Lancaster. 1975. Preservation of high moisture corn: A microbiological evaluation. J. Dairy Sci. 58:386–391.

Buchanan, B. B., W. Gruissem, and R. L. Jones. 2000. Biochemistry and Molecular Biology of Plants. Am. Soc. of Plant Physiol., Rockville, MD.

Buxton, D. R., and P. O'Kiely. 2003. Preharvest plant factors affecting ensiling. In: Silage Science and Technology. D. R. Buxton, R. E. Muck, and H. J. Harrison, ed. ASA, CSA, and SSSA, Madison, WI.

Corl B. A., S. T. Butler, W. R. Butler, D. E. Bauman. 2006. Short communication: regulation of milk fat yield and fatty acid composition by insulin. J. Dairy Sci. 89(11):4172–4175.

Correa, C. E. S., R. D. Shaver, M. N. Pereira, J. G. Lauer, and K. Kohn. 2002. Relationship between corn vitreousness and ruminal in situ starch degradability. J. Dairy Sci. 85:3008–012.

Cueva S. F., H. Stefenoni, A. Melgar, S. E. Räisänen, C. F. A. Lage, D. E. Wasson, M. E. Fetter, A. M. Pelaez, G. W. Roth, and A. N. Hristov. 2021. Lactational performance, rumen fermentation, and enteric methane emission of dairy cows fed an amylase-enabled corn silage. J. Dairy Sci. 104:9827–9841.

Davis C. L., and R. E. Brown. 1970. Low-fat milk syndrome. In: Physiology of digestion and metabolism in the ruminant. Oriel Press. Newcastle, UK.

Dewhurst, R. J., and P. J. King. 1998. Effects of extended wilting, shading and chemical additives on the fatty acids in laboratory grass silages. Grass Forage Sci. 53:219–224.

Dijkstra, J. 1994. Production and absorption of volatile fatty acids in the rumen. Livestock Production Science, 39(1), 61–69.

Der Bedrosian, M. C., L. Kung, Jr., and K. E. Nestor, Jr. 2012. The effects of hybrid, maturity and length of storage on the composition and nutritive value of corn silage. J. Dairy Sci. 95:5115-5126.

Duvick, D. N. 1961. Protein granules in maize endosperm cells. Cereal Chem. 38:374-385.

El-Hamdy, A. H., and Perkins, E. G. 1981. High performance reversed phase chromatography of natural triglyceride mixtures: Critical pair separation. Journal of American Oil Chem. Soc. 58:867-872.

Elgersma, A., G. Ellen, H. van der Horst, B. G. Muuse, H. Boer, and S. Tamminga. 2003. Comparison of the fatty acid composition of fresh and ensiled perennial ryegrass (Lolium perenne L.), affected by cultivar and regrowth interval. Anim. Feed Sci. Technol. 108:191–205.

Fernandes, T., C. L. S. Ávila, M. N. Pereira, and L. F. Ferraretto. 2018. Short communication: Effect of washing method, grinding size, and the determination of an indigestible fraction on in situ degradation of starch in mature corn grain. J. Dairy Sci. 101: 9052-9057.

Ferraretto, L. F., and R. D. Shaver. 2015. Effects of whole-plant corn silage hybrid type on intake, digestion, ruminal fermentation, and lactation performance by dairy cows through a meta-analysis. J. Dairy Sci. 98:2662-2675.

Ferraretto, L. F., K. Taysom, D. Taysom, R. D. Shaver, and P. Hoffman. 2014. Relationships between dry matter content, ensiling, ammonia-nitrogen, and ruminal in vitro starch digestibility in high-moisture corn samples. J. Dairy Sci. 97:3221–3227.

Ferraretto, L. F., P. M. Crump, and R. D. Shaver. 2013. Effect of cereal grain type and corn grain harvesting and processing methods on intake, digestion, and milk production by dairy cows through a meta-analysis. J. Dairy Sci. 96:533–550.

Ferraretto, L. F., P. M. Crump, and R. D. Shaver. 2015. Effect of ensiling time and exogenous protease addition to whole-plant corn silage of various hybrids, maturities and chop lengths on nitrogen fractions and ruminal in vitro starch digestibility. J. Dairy Sci. 98:8869-8881.

Ferraretto, L. F., R. D. Shaver, and B. D. Luck. 2018a. Silage review: Recent advances and future technologies for whole-plant and fractionated corn silage harvesting. J. Dairy Sci. 101:3937–3951.

Ferraretto, L. F., R. D. Shaver, J. G. Lauer, L. H. Brown, J. P. Kennicker, R. J. Schmidt, and D. M. Taysom. 2018b. Influence of plant population and maturity, microbial inoculation and ensiling time on fermentation profile, nitrogen fractions and starch digestibility in earlage. In: XVIII International Silage Conference, Bonn, Germany. Pages: 522-523.

Ferreira, G., M. Alfonso, S. Depino, and E. Alessandri. 2014. Effect of planting density on nutritional quality of green-chopped corn for silage. J. Dairy Sci. 97:5918-5921.

Firkins, J. L., M. L. Eastridge, N. R. St-Pierre, and S. M. Noftsger. 2001. Effects of grain variability and processing on starch utilization by lactating dairy cattle. J. Anim. Sci. 79(E. Suppl.):218-238.

Figroid, W., W. H. Hale, and B. Theurer. 1972. An evaluation of the nylon bag technique for estimating rumen utilization of grains. J. Anim. Sci. 35:113-120.

Fredin, S. M., L. F. Ferraretto, M. S. Akins, P. C. Hoffman, and R. D. Shaver. 2014. Fecal starch as an indicator of total-tract starch digestibility by lactating dairy cows. J. Dairy Sci. 97:1862-1871.

Fuentes M. C., S. Calsamiglia, P. W. Cardozo, B. Vlaeminck. 2009. Effect of pH and level of concentrate in the diet on the production of biohydrogenation intermediates in a dual-flow continuous culture. J. Dairy Sci. 92:4456–4466.

Garton, G. A., A. K. Lough, and E. Vioque. 1961. Glyceride hydrolysis and glycerol fermentation by sheep rumen contents. J. Gen. Microbiol. 25:215–218.

Giuberti, G., A. Gallo, F. Masoero, L. F. Ferraretto, P. C. Hoffman, and R. D. Shaver. 2014. Factors affecting starch utilization in large animal food production system: A review. Starke 66:72–90.

Glascock, R.F., W. G. Duncombe, L. R. Reinius. 1956. Studies on the origin of milk fat. 2. The secretion of dietary long-chain fatty acids in milk fat by ruminants. Biochem. J. 62:535- 541.

Goering, H. K., and P. J. Van Soest. 1970. Forage fiber analyses (Apparatus, reagents, procedures and some applications. Agriculture Handbook No. 379. Agricultural Research Service, USDA, Washington, DC.

Greene, T.W. and L. C. Hannah. 1998. Enhanced stability of maize endosperm ADP-glucose pyrophosphorylase is gained through mutants that alter subunit interactions. Proceedings of the National Academy of Sciences of the United States of America 95, 13342-13347.

Hall, M. B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. J. AOAC Int. 98:397-409.

Hall, M. B., and D. R. Mertens. 2017. A 100-year review: carbohydrates – characterization, digestion, and utilization. J. Dairy Sci. 100:10078-10093.

Hamaker, B. R., A. A. Mohamed, J. E. Habben, C. P. Huang, and B. A. Larkins. 1995. Efficient procedure for extracting maize and sorghum kernel proteins reveals higher prolamin contents than the conventional method. Cereal Chem. 72:583–588.

Harfoot, C.G., and G. P. Hazlewood. 1997. Lipid metabolism in the rumen. In: The Rumen Microbial Ecosystem, 2nd ed., Chapman and Hall, London, UK.

Hilditch, T.P. 1947. The Chemical Constitution of Natural Fats. 2nd ed., Chapman and Hall, London, UK.

Hirano, S. S., and C. D. Upper. 1991. Bacterial community dynamics. In: Microbial ecology of leaves. Springer Verlag, New York.

Hoeft, R. G., E. D. Nafziger, R. R. Johnson, and S. R. Aldrich. 2000. Modern corn and soybean production. MCSP Publications, Champaign, IL.

Hoffman, P. C., N. M. Esser, R. D. Shaver, W. K. Coblentz, M. P. Scott, and A. L. Bodnar. 2011. Influence of ensiling time and inoculation on alteration of the starch-protein matrix in high-moisture corn. J. Dairy Sci. 94:2465–2474.

Hu, W., M. E. Persia and L. Kung Jr. 2010. Short communication: In vitro ruminal fermentability of a modified corn cultivar expressing a thermotolerant α-amylase. J. Dairy Sci. 93:4846–4849.

Huhtanen, P., and J. Sveinbjörnsson. 2006. Evaluation of methods for estimating starch digestibility and digestion kinetics in ruminants. Anim. Feed Sci.Technol. 130:95–113.

Humer, E., R. M. Petri, J. R. Aschenbach, B. J. Bradford, G. B. Penner, M. Tafaj, K. -H. Sudekum, and Q. Zebeli. 2018. Invited Review: Practical feeding management recommendations to mitigate the risk of subacute ruminal acidosis in dairy cattle. J. Dairy Sci. 101:872-888.

Hurtaud, C., H. Rulquin, and R. Verite. 1998. Effects of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 1. Diets based on corn silage. J. Dairy Sci. 81:3239–3247.

Hurtaud, C., S. Lemosquet, and H. Rulquin. 2000. Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 2. Diets based on grass silage. J. Dairy Sci. 83:2952–2962.

James, M. G., K. Denyer, and A. M. Myers. 2003. Starch synthesis in the cereal endosperm. Curr. Opin. Plant Biol. 6:215-222.

Jellum, M. D. 1970. Plant introductions of maize as a source of oil with unusual fatty acid composition. J. Agric. Food. Chem. 18:365-370.

Jellum, M. D. and J. E. Marion. 1966. Factors affecting oil content and oil composition of corn (*Zea mays* L.) grain. Crop Sci. 6:41-42.

Jellum, M. D., F. C. Boswell, and F. Y. Young. 1973. Nitrogen and boron effects on protein and oil of corn grain. Agron. J. 65:330-331.

Jenkins, T. C., K. J. Harvatine. 2014. Lipid feeding and milk fat depression. Vet. Clin. Food Anim. 30:623-642.

Jeon, J. S., N. Ryoo, T. R. Hahn, H. Walia, and Y. Nakamura. 2010. Starch biosynthesis in cereal endosperm. Plant Physiol. and Biochem. 48:383-392.

Jones, R. J., B. M. N. Schreiber, and J. A. Roessler. 1996. Kernel sink capacity in maize: genotypic and maternal regulation. Crop Sci. 36:301-306.

Junges, D., G. Morais, M. H. F. Spoto, A. T. Adesogan, L. G. Nussio, and J. L. P. Daniel. 2017. Influence of various proteolytic sources during fermentation of reconstituted corn grain silages. J. Dairy Sci. 100:9048–9051.

Khan, N., J. Cone, V. Fievez, and W. Hendriks. 2012. Causes of variation in fatty acid content and composition in grass and maize silages. Anim. Feed Sci. Technol. 174:36–45.

Krogstad, K. C. and B. J. Bradford. 2023. The effects of feeding α-amylase enhanced corn silage with different dietary starch concentrations to lactating dairy cows on milk production, nutrient digestibility, and blood metabolites. J. Dairy Sci. 106: 4666-4681.

Kung Jr., L., R. D. Shaver, R. J. Grant, and R. J. Schmidt. 2018. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. J. Dairy Sci. 101:4020–4033.

LaCount, D. W., J. K. Drackley, T. M. Cicela, and J. H. Clark. 1995. High oil corn as silage or grain for dairy cows during an entire lactation. J. Dairy Sci. 78:1745–1754.

Li, L., M. Blanco, and J. L. Jane. 2007. Physicochemical properties of endosperm and pericarp starches during maize development. Carbohyd. Polym. 67(4):630-639.

Lindberg, J. E. 1985. Estimation of rumen degradability of feed proteins with the in sacco technique and various in vitro methods. A review. Acta Agric. Scand. Sect. A-Anim. Sci. Suppl. 25:64-97.

Lopes, J. C., R. D. Shaver, P. C. Hoffman, M. S. Akins, S. J. Bertics, H. Gencoglu, and J. G. Coors. 2009. Type of corn endosperm influences nutrient digestibility in lactating dairy cows. J. Dairy Sci. 92:4541–4548.

Lough, A. K., and L. J. Anderson. 1973. Effect of ensilage on lipids of pasture grasses. Proc. Nutr. Soc. 32:61A–62A.

Mackie, R. I., and F. M. C. Gilchrist. 1979. Changes in lactate-producing and lactate-utilizing bacteria in relation to pH in the rumen of sheep during stepwise adaptation to a high concentrate diet. Appl. Eviron. Microbiol. 38:422-430.

Matamoros, C., R. N. Klopp, L. E. Moraes, and K. J. Harvatine. 2020. Meta-analysis of the relationship between milk *trans*-10 C18:1, milk fatty acids <16C, and milk fat production. J. Dairy Sci. 103:10195-10206.

McAllister, T. A., H. D. Bae, G. A. Jones, and K. J. Cheng. 1994. Microbial attachment and feed digestion in the rumen. J. Anim. Sci. 72:3004-3018.

McDonald, P., A. R. Henderson, and S. J. E. Heron. 1991. The Biochemistry of Silage. 2nd ed. Chalcombe, Marlow, UK.

Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider, W. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. J. Agric. Sci. 93:217-222.

Michalet-Doreau, B., and P. Cerneau. 1991. Influence of foodstuff particle size on in situ degradation of nitrogen in the rumen. Anim. Feed Sci. Technol. 35:69-81.

Moate, P. J., R. C. Boston, T. C. Jenkins, and I. J. Lean. 2008. Kinectics of ruminal lipolysis of triacylglycerol and biohydrogenation of long-chain fatty acids: new insights from old data. J. Dairy Sci. 91:731-742.

Muetzel, S. C. H., and M. H. Tavendale. 2014. A fully automated incubation system for the measurement of gas production and gas composition. Anim. Feed Sci. Technol. 196:1–11.

Murphy, M. R., R. L. Baldwin, and L. J. Koong. 1982. Estimation of stoichiometric parameters for rumen fermentation of roughage and concentrate diets. J. Anim. Sci. 55:411–421.

National Academies of Sciences, Engineering, and Medicine. 2021. Nutrient Requirements of Dairy Cattle: Eighth Revised Edition. Washington, DC: The National Academies Press.

Nelson, D., and M. Cox, W. H. Freeman & Co. 2004. Lehninger principles of biochemistry. 4th Ed. New York, NY.

Nocek, J. E. 1985. Evaluation of specific variables affecting in situ estimates of ruminal dry matter and protein digestion. J. Anim. Sci. 60:1347-1358.

Nocek, J. E., and R. A. Kohn. 1988. In situ particle size reduction of alfalfa and timothy hay as influenced by form and particle size. J. Dairy Sci. 71:932-945.

Nocek, J. E., and S. Tamminga. 1991. Site of digestion of starch in the gastrointestinal tract of dairy cows and its effect on milk yield and composition. J. Dairy Sci. 74:3598-3629.

Ogawa, J., S. Kishino, A. Ando, S. Sugimoto, K. Mihara, and S. Shimizu. 2005. Production of conjugated fatty acids by lactic acid bacteria. J. Biosci. Bioeng. 100:355–364.

Olsen, O.A., 2001. Endosperm development: cellularization and cell fate specification. Annual Review of Plant Physiology and Plant Molecular Biology 52, 233-267.

Orskov, E. R., C. Fraser, V. C. Mason and S. O. Mann. 1970. Influence of starch digestion in the large intestine of sheep on caecal fermentation, caecal microflora and faecal nitrogen excretion. Brit. J. Nutr. 24:671.

Owens, F. N., R. A. Zinn, Y. K. Kim. 1986. Limits to starch digestion in the ruminant small intestine. J. Anim. Sci. 63(5):1634-1648.

Pahlow, G., R. E Muck, F. Driehuis, S. J. W. H. Oude Elferink, and S. F. Spoelstra. 2003. Microbiology of Ensiling. In Silage Science and Technology. Ed. ASA, CSA, and SSSA, Madison, WI.

Palmquist, D. L. 2006. Milk fat: origin of fatty acids and influence of nutritional factors thereon. In: Advanced Dairy Chemistry. Vol. 2: Lipids, 3rd ed. Springer, New York, NY.

Palmquist, D.L., C. L. Davis, R. E. Brown, D. S. Sachan. 1969. Availability and metabolism of various substrates in ruminants. V. Entry rate into the body and incorporation into milk fat of D(-)b-hydroxybutyrate. J. Dairy Sci. 52:633–638.

Palmquist, D. L., and D. J. Kinsey. 1994. Lipolysis and biohydrogenation of fish oil by ruminal microorganisms. J. Dairy Sci. 77(Suppl.1):350. (Abstr.).

Palmquist, D.L., A. L. Lock, K. J. Shingfield, D. E. Bauman. 2005. Biosynthesis of conjugated linoleic acid in ruminants and humans. In: Advances in Food and Nutrition Research. Vol. 50. Elsevier Inc., San Diego, CA.

Paula, E. M., B. A. Saylor, J. Goeser, and L. F. Ferraretto. 2019. Influence of cutting height on nutrient composition and yield of whole-plant corn silage through a meta-analysis. J. Dairy Sci. 102(Suppl. 1):104. (Abstr.)

Philippeau, C., and B. Michalet-Doreau. 1998. Influence of genotype and ensiling of corn grain on in situ degradation of starch in the rumen. J. Dairy Sci. 81:2178–2184.

Philippeau, C., J. Landry, and B. Michalet-Doreau. 2000. Influence of the protein distribution of maize endosperm on ruminal starch degradability. J. Sci. Food Agric. 80:404–408.

Plattner, R. D., G. F. Spencer, and R. Kleiman. 1977. Triglyceride separation by reverse phase high performance liquid chromatography. Journal of American Oil Chem. Soc. 54:511-515.

Playne, M. J., W. Khumnualthong, and M. G. Echeuarria. 1978. Factors affecting the digestion of oesophageal fistula samples and hay samples in nylon bags in the rumen of cattle. J. Agric. Sci. 90:193-204.

Plaizier, J. C., F. J. Mullingan, E. W. Neville, L. L. Guan, M. A. Steele, G. B. Penner. 2022. Invited Review: Effect of subacute ruminal acidosis on gut health of dairy cows. J. Dairy Sci. 105:7141-7160.

Popjak, G., T. H. French, S. J. Folley. 1951. Utilization of acetate for milk-fat synthesis in the lactating goat. Biochem. J. 48, 411–416.

Qiu X., M. L. Eastridge, K. E. Griswold, J. L. Firkins. 2004. Effects of substrate, passage rate, and pH in continuous culture on flows of conjugated linoleic acid and trans-C18:1. J. Dairy Sci. 87:3473–3479.

Rebelo, L. R., M. L. Eastridge, J. L. Firkins, and C. Lee. 2023. Effects of corn silage and grain expressing α-amylase on ruminal nutrient digestibility, microbial protein synthesis, and enteric methane emissions in lactating cows. J. Dairy Sci. 106:3932-3946.

Remillard, R. L. and D. E. Johnson. 1984. Starch digestion in the bovine small intestine with and without buffer and amylase infusion. J. Anim. Sci. 59(Suppl. 1):444(Abstr.).

Rendleman Jr., J. A. 2000. Hydrolytic action of alpha-amylase on high-amylose starch of low molecular mass. Biotechnol. Appl. Biochem. 31(3):171-178.

Richardson, T. H., X. Tan, G. Frey, W. Callen, M. Cabell, D. Lam, J. Macomber, J. M. Short, D. E. Robertson, and C. Miller. 2002. A novel, high performance enzyme for starch liquefaction: discovery and optimization of a low pH, thermostable α-amylase. J. of Biolog. Chemistry. 277:26501-26507.

Rooke, J. A., and R. D. Hatfield. 2003. Biochemistry of Ensiling. In: Silage Science and Technology. Ed. ASA, CSA, and SSSA, Madison, WI.

Russel, J. B., and T. Hino. 1985. Regulation of lactate production in *Streptococcus bovis*: a spiraling effect that contributes to rumen acidosis. J. Dairy Sci. 68:1712-1721.

Saylor, B. A., C. L. McCary, E. C. Diepersloot, C. Heinzen Jr., M. R. Pupo, J. O. Gusmão, L. G. Ghizzi, H. Sultana, L. F. Ferraretto. 2021. Effect of Forage Processor Roll Gap Width and Storage Length on Fermentation Profile, Nutrient Composition, Kernel Processing Score, and Starch Disappearance of Whole-Plant Maize Silage Harvested at Three Different Maturities. Agriculture, 11, 574.

Schlei, I., A. Danfær, I. A. Boman, and H. Volden. 2007. Post-ruminal or intravenous infusions of carbohydrates or amino acids to dairy cows 1. Early lactation. Animal 1:501–514.

Seebauer, J.R., G. W. Singletary, P. M. Krumpelman, M. L. Ruffo, and F. E. Below. 2010. Relationship of source and sink in determining kernel composition of maize. J. Exp. Bot. 61:511-519.

Seifried, N., H. Steingaβ, M. Rodehutscord. 2015. In vitro and in situ evaluation of secondary starch particle losses from nylon bags during the incubation of different cereal grains. Anim. Feed Sci. Technol. 210:26-36.

Shaw, R. H., and R. P. Norton. 1920. A comparative study of corn silage in concrete and stave silos. J. Dairy Sci. 3:300–307.

Smith, D. 1969. Removing and analyzing total nonstructural carbohydrates from plant tissue. Wisconsin Agric. Exp. Stn. Res. Report 41, Madison, WI.

Sniffen, C. J., and R. Ward. 2011. Using starch digestibility information in ration balancing. WCDS Adv. Dairy Technol. 23:121–133.

Stock, R. A., M. H. Sindt, R. M. Cleale IV, and R. A. Britton. 1991. High-moisture corn utilization in finishing cattle. J. Anim. Sci. 69:1645–1656.

Strobel, H. J., and J. B. Russell. 1986. Effect of pH and energy spilling on bacterial protein synthesis by carbohydrate-limited cultures of mixed rumen bacteria. J. Dairy Sci. 69:2941–2947.

Tagliapietra, F., M. Cattani, L. Bailoni, and S. Schiavon. 2010. In vitro rumen fermentation: effect of headspace pressure on the gas production kinetics of corn meal and meadow hay. Anim. Feed Sci. Technol. 158:197–201.

Tester, R. F., Karkalas, J., X. Qi. 2004. Starch – Composition, fine structure and architecture. Journal of Cereal Sci. 39:151-165.

Theodorou, M. K., B. A. Williams, M. S. Dhanoa, A. B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Tech. 48:185–197.

Thivend, P., C. Mercier, and A. Guilbot. 1971. Determination of starch with glucoamylase. In: Methods in Carbohydrate Chemistry. Vol. 6. Academic Press, New York, NY.

Tilley, J. M. A., and R. A. Terry. 1963. A two stage technique for in vitro digestion of forage crops. J. Br. Grassl. Soc. 18:104.

Tonroy, B. R., and T. W. Perry. 1974. In vitro digestion of raw, roasted, and pressure-flaked corn. J. Dairy Sci. 57:1508–1511.

Uden, P., and P. J. Van Soest. 1984. Investigations of the in situ bag technique and a comparison of the fermentation in heifers, sheep, ponies and rabbits. J. Anim. Sci. 58:213-221.

USDA. 2023. Crop production 2022 summary. USDA National Agricultural Statistics Service, Washington, DC.

Van Hellen, R. W., and W. C. Ellis. 1977. Sample container porosities for rumen in situ studies. J. Anim. Sci. 44:141-146.

Van Milgen, J., L. L. Berger, and M. R. Murphy. 1993. An integrated, dynamic model of feed hydration and digestion, and subsequent bacterial mass accumulation in the rumen. Br. J. Nutr. 70:471-483.

Van Soest P. J. 1963. Ruminant fat metabolism with particular reference to factors affecting low milk fat and feed efficiency: A review. J. Dairy Sci. 46:204–16.

Vanzant, E. S., R. C. Cochran, and E. C. Titgemeyer. 1998. Standardization of in situ techniques for ruminant feedstuff evaluation. J. Anim. Sci. 76:2717-2729.

Vrinten, P.; Z. Hu, M. A. Munchinsky, G. Rowland, X. Qiu. 2005. Two FAD3 desaturase genes control the level of linolenic acid in flax seed. Plant Physiol. 139:79–87.

Vyas, D., U. Moallem, B. B. Teter, A. R. K. Fardin-Kia, and R. A. Erdman. 2013. Milk fat responses to butterfat infusion during conjugated linoleic acid-induced milk fat depression in lactating dairy cows. J. Dairy Sci. 96:2387-2399.

Watson, S. A. 2003. Description, development, structure, and composition of the corn kernel. In: Corn: Chemistry and Technology. American Assoc. of Cereal Chemists, St. Paul, MN.

Welch, L. F. 1969. Effects of N, P, and K on the percent and yield of oil in corn. Agron. J. 61:890-891.

White, P. J., and Weber, E. J. 2003. Lipids of the kernel. In: Corn: Chemistry and Technology. American Assoc. of Cereal Chemists, St. Paul, MN.

Windle, M. C., N. Walker, and L. Kung Jr. 2014. Effects of an exogenous protease on the fermentation and nutritive value of corn silage harvested at different dry matter contents and ensiled for various lengths of time. J. Dairy Sci. 97:3053–3060.

Wolf, M. J., Khoo, U., and H. L. Seckinger. 1969. Distribution and subcellular structure of endosperm protein in varieties of ordinary and high-lysine maize. Cereal Chem. 46:253-263.

Wolt, J. D., and S. Karaman. 2007. Estimated environmental loads of alpha-amylase from transgenic high-amylase maize. Biomass and Bioenergy. 31:831-835.

Woolford. M. K., and G. Pahlow. 1998. The silage fermentation. In: Microbiology of fermented foods. 2nd ed. Vol. I. Blackie Academic & Professional, London, UK.

Yáñez-Ruiz, D. R., A. Bannink, J. Dijkstra, E. Kebreab, D. P. Morgavi, P. O'Kiely, C. K. Reynolds, A. Schwarm, K. J. Shingfield, Z. Yu, A. N. Hristov. 2016. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants—a review. Anim. Feed Sci. Technol. 216:1–18.

CHAPTER TWO: EFFECT OF A GENETICALLY-MODIFIED CORN HYBRID WITH ALPHA-AMYLASE AND STORAGE LENGTH ON FERMENTATION PROFILE AND STARCH DISAPPEARANCE OF WHOLE-PLANT CORN SILAGE AND EARLAGE

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INTRODUCTION

Whole-plant corn silage (WPCS) is the predominant forage fed to high-producing dairy cows in the United States. Despite its high-energy density, improvements in the nutritional value of WPCS through hybrid selection are desirable to enhance productivity and profitability of dairy operations. In a meta-analysis, Ferraretto and Shaver (2015) observed greater consumption of dry matter and milk yield when cows were fed hybrids selected for greater fiber digestibility, without improving feed efficiency (kg of milk/kg of DMI). However, effects of feeding hybrids varying in kernel characteristics were not observed in that meta-analysis, and potential benefits of different kernel characteristics warrant further research.

A genetically-modified corn hybrid containing an alpha-amylase trait expressed in the kernel endosperm was developed for ethanol production (Singh et al., 2006) and garnered interest by the dairy industry (Cueva et al., 2021). The enzyme expressed in the kernel could hydrolyze starch, thereby increasing the concentration of reducing sugars available during silage fermentation or increasing ruminal and total tract digestibility of starch. However, while milk production and feed efficiency were greater for cows fed the amylase-expressing WPCS compared to its isogenic counterpart, no effects on total tract starch digestibility were reported (Cueva et al., 2021). Lack of effects on starch digestibility could be related to the enzyme having limited activity under typical silage and rumen temperatures (Hu et al., 2010). However, Hellings et al. (2019) reported greater ruminal starch disappearance at 7 h for amylase-expressing WPCS when compared to its isogenic counterpart, even though this response was more pronounced when silos were ensiled and allowed to ferment under higher temperatures (32-40 ^{o} C). Alternatively, silage was stored for 220 d prior to the feeding trial in the study by Cueva et al. (2021). It is well-established that prolonged storage of WPCS and fractionated corn silage (i.e., earlage, high-

moisture corn) increases ruminal in vitro or in situ starch disappearance (Der Bedrosian, et al., 2012; Ferraretto et al., 2015a; Ferraretto et al., 2018). Thus, storage length might have attenuated any effect of amylase activity on starch disappearance during the storage period. Therefore, evaluating this novel hybrid when fermented for shorter periods of time is warranted.

The use of exogenous amylases at ensiling raised concerns about undesired fermentation patterns. Treating WPCS with an exogenous multi-enzyme preparation containing cellulolytic, hemicellulolytic, and amylolytic enzymes reduced starch concentration, increased yeast counts and ethanol concentration, and decreased aerobic stability compared with untreated silage (Spoelstra et al., 1992). Greater yeast growth was attributed to breakdown of starch into simple sugars by the exogenous amylase, thereby increasing the substrate available for yeast proliferation. However, the effect on starch concentration was dose-dependent and concentrations decreased as the dose of the exogenous multi-enzyme increased. Similarly, Fernandes et al. (2022) reported greater losses of DM and starch in rehydrated corn and sorghum grain silages ensiled with amyloglucosidases, an enzyme able to hydrolyze starch, maltose and maltotriose. Furthermore, silage treated with amyloglucosidases had greater ethanol concentration compared with control (Fernandes et al., 2022), but differences were of a lesser magnitude than reported by Spoelstra et al. (1992).

Benefits of genetically-modified WPCS and earlage containing the alpha-amylase trait on silage fermentation and ruminal starch disappearance are not definitive. Therefore, our objective was to evaluate the effects of ensiling this genetically-modified hybrid with alpha-amylase on the fermentation profile, nutrient composition and ruminal in situ starch disappearance of material ensiled for 0, 30, 60, 90 and 120 d in two separate experiments, one evaluating WPCS and the other evaluating earlage. We hypothesized that the presence of an alpha-amylase in the grain could

decrease starch concentration, providing more substrate for yeast and mold growth, and reduce aerobic stability. However, we also hypothesized that greater ruminal starch disappearance would be achieved in the WPCS and earlage with the alpha-amylase trait when compared with its isogenic counterpart and this response would be more pronounced after a short ensiling period.

MATERIAL AND METHODS

Forage production, experimental design, and treatments

A genetically-modified corn hybrid containing alpha-amylase (E116K4 - Enogen®; Syngenta Seeds®, Downers Grove IL; AMY) and its isogenic counterpart (NK1694 NK Seeds®, Downers Grove IL; ISO) were grown in the University of Florida - Plant Science Research & Education Unit (Citra, FL) in two 40 x 30 m field. Eight 40 m rows (76.2 cm between rows) of each hybrid were planted on each side of the field. Hybrids were separated by 10 rows of buffer corn plants plus a 6 m wide alley, as suggested by the manufacturer to avoid cross pollination of the amylase genes to the isogenic counterpart hybrid. The isogenic hybrid was used as buffer and borders of the field (6 rows). Planting density was 75,624 seeds/ha. Twenty treatment plots (5 for WPCS and 5 for earlage for each hybrid) of four rows each (6 linear m) were marked and harvested when plants reached approximately 35% of DM (2/3 to 3/4 kernel milk line for both hybrids). For WPCS, plants from the two inner rows (approximately three linear m of each row) of each hybrid were harvested manually on July 1st, 2019, at approximately 25 cm of cutting height. Plants of each plot were manually placed through a stationary silage chopper (model no. 707 SN: 245797l; CNH Industrial America LLC), chopped forage was homogenized manually, and allocated into 5 subsamples. Each of the 5 subsamples for each plot were randomly assigned to 1 of 5 storage lengths (0, 30, 60, 90 or 120 d of fermentation). Samples (n = 50; 2 corn hybrids × 5 storage lengths

× 5 replicated plots) were ensiled in 20-L buckets (580 kg of fresh material/m^3, on average). Similarly, for earlage, ears were harvested by hand on July 9th, 2019, and processed, as described previously, when the ears reached approximately 65% of DM. Subsamples within plots were homogenized manually and assigned to 1 of the 5 storage lengths. Samples (n = 50; 2 hybrids x 5 storage lengths x 5 replicated plots) were ensiled in 4-L buckets (1015 kg of fresh material/m^3, on average). Earlage and WPCS laboratory silos with the designated storage length of 0 d were not sealed and samples were processed for subsequent analysis immediately after all other silos were filled and sealed. Neither WPCS nor earlage were inoculated. All laboratory silos were weighed and stored at room temperature (approximately 23° C) until reaching the targeted storage length. After each specified storage length was reached, laboratory silos were weighed, opened, and the material from the top 4 cm of the silo was discarded. Then, the remaining material was mixed, and representative subsamples were collected for analysis. All the analysis described henceforward were done for both WPCS and earlage.

Fermentation profile and microbial counts

Extracts of each sample at ensiling (0 d) and silage after each storage length (30, 60, 90 and 120 d) were obtained from the homogenization of 20 g of sample in 200 mL of peptone water (0.1%) in a stomacher (Lab-Blender 400, Tekmar Company, Cincinnati, OH) for 1 minute. Samples were then filtered through two layers of cheesecloth and divided into 2 subsamples. Extracts of 0 d samples were used only to perform yeast and mold counts. For ensiled samples, the first extract subsample was used to determine pH and fermentation products. Measurements of pH were performed with a pH probe (Orion 710+; Thermo Fisher Scientific Inc., Waltham, MA). For fermentation products, 40 mL of extract was acidified with 0.4 mL of 50% sulfuric acid and centrifuged (7,000 × *g*) for 15 min at 4°C. The supernatant was frozen (-20°C) for subsequent

analysis of ammonia-N, organic acids (lactic, acetic, propionic, valeric, butyric and iso-butyric acids) and 1,2-propanediol. Ammonia-N was evaluated by colorimetry using a Technicon Automatic Analyzer (RFA-300, Alpkem Corporation, Clackamas, OR; Noel and Hambleton., 1976). Organic acids and 1,2-propanediol concentrations were determined by high-performance liquid chromatography (HPLC; Merck Hitachi Elite La-Chrome, Hitachi L2400, Tokyo, Japan) as described by Muck and Dickerson (1988). Briefly, a Bio-Rad Aminex HPX-87H ion exclusion column (300 × 7.8 mm id; Bio-Rad Laboratories, Hercules, CA) was used in an isocratic elution system containing 0.015 M sulfuric acid in the mobile phase of HPLC attached to an UV detector (wavelength 210 nm; L-2400, Hitachi) using a flow rate of 0.7 mL/min at 45°C for organic acids analysis. For the 1,2-propanediol analysis, the same system and column was used. However, 0.005 M sulfuric acid was used as the mobile phase and the HPLC was attached to a Refractive Index detector (L-2490, Hitachi) using a flow rate of 0.6 mL/min at 45°C.

The second subsample was used to enumerate yeast and mold populations using Malt Extract Agar (Difco) media acidified right before pouring with lactic acid solution (85 % v/v, 2 ml per 400 ml of liquid agar) in pour plates that were incubated aerobically at 32°C for 48 hours (h) for yeast counts and an additional 72 h for mold counts.

Chemical composition and ruminal in situ starch disappearance

Representative samples collected before ensiling and silages from each silo at opening, were dried at 60°C for 48 h in a forced-air oven, then ground to pass through a 1-mm screen using a Willey mill (Thomas Scientific, Swedesboro, NJ) for determination of nutrient composition. Ether extract (EE) was analyzed according to AOCS (2009; standard procedure AM 5-04) with an ANKOM XT15 Extractor (Ankom Technologies; Macedon, NY, USA). Ash was determined after 8 h of incineration in a furnace held at 600°C (method 942.05, AOAC, 2012). Concentration of

NDF was determined with heat-stable alpha-amylase and inclusive of residual ash (method 2002.04; AOAC, 2012) using Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY). The concentration of WSC was evaluated by the anthrone reaction test (Ministry of Agricultural, Fisheries, and Food, 1986). Frozen samples were sent to Rock River Laboratory, Inc. (Watertown, WI), dried and ground to pass through a 6-mm sieve for determination of ruminal in situ starch disappearance, or a 1-mm screen for determination of crude protein (CP; method 990.03; AOAC, 2012), soluble CP (Krishnamoorthy et al. 1982), and starch following the colorimetric method of Hall (2015) with thermo-stable alpha-amylase (Ankom Technologies, Macedon, NY, USA) and amyloglucosidase (Megazyme E-AMGDF, Bray, Co., Wicklow, Ireland) enzymes. For ruminal in situ starch disappearance, 5 grams of sample were placed into polyester bags (10 × 20 cm, 50 ± 10 μm porosity; Ankom Technology, Macedon, NY). Bags were placed in laundry bags and incubated for 0, 6, 48 and 120 h (Fernandes et al., 2018) in three rumen-cannulated lactating Holstein cows fed a diet consisting of (DM basis) 30% corn silage, 19% alfalfa silage, 5% whole cottonseed and 46% concentrate. After removal, the bags were immersed in icy water and washed in tap water until rinse water was clear. Incubation residues were analyzed for starch concentration as described previously.

Loss of DM and Aerobic stability

Loss of DM was calculated as the difference between the weight of the silo at ensiling and the weight of the silo at opening, discounted the weight of each bucket. The initial and final DM concentration of each forage and silage was used to calculate the amount of DM for each silo.

Aerobic stability was determined at opening by placing wireless temperature sensors (HOBO temperature data logger 64k; Onset Computer Corporation, Cape Cod, MA) at the center of the plastic buckets containing approximately 6 kg of WPCS or 3 kg of earlage. Buckets were

placed in a room with controlled temperature. Sensors were set to record temperatures every 30 minutes for 240 h. Three additional sensors were placed in the same room to record ambient temperature (23.0 ± 0.7° C). The aerobic stability was denoted by the number of h before an increase of 2°C in silage temperature above the silage baseline temperature.

Statistical analysis

Whole-plant corn silage and earlage data were analyzed separately but using the same statistical model for both datasets. Laboratory silo was the experimental unit. Nutrient composition, ammonia-N, starch disappearance, and yeast and mold counts (log transformed) data were analyzed as a completely randomized block design in a 2 x 5 factorial arrangement of treatments, using generalized linear mixed model procedures (PROC GLIMMIX, SAS 9.4; SAS Institute Inc., Cary, NC, USA) with a model including the fixed effects of hybrid and storage length and their interaction. The effect of plots (considered as blocks) was the sole random effect. Data analysis for pH, DM loss, organic acids, and aerobic stability did not include unfermented forage samples (0 d) and thus, were analyzed as a completely randomized block design in a 2 x 4 factorial arrangement of treatments using the same model. Means were determined using the least square means statement and treatment means were compared using the Bonferroni t-test option after a significant overall treatment F-test. Orthogonal contrasts were used to evaluate the effect of ensiling (0 vs. 30 d), for variables containing 0 d, or storage length (30 d vs. 60 d vs. 90 d vs. 120 d). If an interaction was detected ($P \leq 0.05$), effects were partitioned by storage length using the SLICE option. Statistical significance was declared at $P \leq 0.05$. If interactions were not detected ($P > 0.05$), main effect of hybrids or storage length were reported and discussed.

RESULTS AND DISCUSSION

Propionic acid, iso-butyric acid, butyric acid, valeric acid and 1,2-propanediol were not detected in any WPCS or earlage samples. Starch disappearance at 48 and 120 h was 99.9% of starch for all samples. Therefore, data from these variables will not be presented or discussed.

Whole-plant corn silage

Effects of hybrid and storage length on pH, fermentation profile and microbial counts of WPCS are in Table 2.1. There was a hybrid × storage length interaction ($P = 0.001$) on lactic acid concentration, which was similar between hybrids from 30 to 90 d (8.6% of DM, on average), but greater for ISO than AMY at 120 d (8.5 vs. 5.4% of DM). Both hybrids had pH values within the desirable range (3.7 to 4.0; Kung Jr. et al., 2018) and lactic acid slightly greater than the (3 to 6% of DM) reported by Kung Jr. et al. (2018). Previous studies have shown an increase in acetic acid and pH with prolonged storage (Der Bedrosian et al., 2012; Herrmann et al., 2011) due to the conversion of lactic acid into acetic acid (Lindgren et al., 1990). However, despite differences in lactic acid concentration at 120 d, acetic acid and pH remained constant throughout storage for both hybrids. There was a hybrid × storage length interaction for ammonia-N ($P = 0.001$); ammonia-N concentration did not differ between hybrids at 0 d (2.2% of total N, on average) but was 7.8 percentage-units greater, on average, for AMY than ISO at 30, 60, 90, and 120 d. Considering the absence of butyric acid in the silage, ammonia-N was not an indicator of clostridial fermentation (Rooke and Hatfield, 2003). Therefore, greater ammonia-N concentration was likely due to normal deamination of proteins during fermentation (Kung Jr. et al., 2018). However, differences in starch disappearance suggest this difference in the deamination of proteins did not occur in the kernels but primarily in other plant parts. However, this is only speculation and further research is warranted to elucidate the effect of AMY on ammonia-N and prolamin concentrations.

An interaction between hybrid and storage length was also detected for yeast counts (*P* = 0.001); ISO had greater yeast counts than AMY at 0 d (5.9 vs. 4.2 log cfu/g), similar counts at 30 and 60 d (4.2 and 4.5 log cfu/g on average, respectively), but lower counts at 90 (2.9 vs 4.2 log cfu/g) and 120 d (3.0 vs. 4.0 log cfu/g). Previously, adding exogenous amylase to silage increased available sugars, yeast growth, ethanol concentration, and losses of DM and starch (Spoelstra et al., 1992; Fernandes et al., 2022). However, starch and WSC concentrations (Table 2.2) were not affected by kernel expressed-amylase during silage fermentation in the present study, suggesting greater yeast counts and differences in lactic acid concentration may not be related to amylase activity in the silo. Further research is warranted to understand the differences reported for yeast counts. An interaction between hybrid and storage length was observed for DM losses (*P* = 0.001), which were similar for 30, 60 and 90 d, but lower for AMY compared with ISO at 120 d (0.8 vs. 2.3% of DM, respectively). This further supports the premise of kernel-expressed amylase not negatively affecting silage fermentation and nutrient profile. Losses were generally low in this study, less than 1%, except for ISO at 120 d of storage length. Contrary to our hypothesis, AMY silage reached aerobic stability 12.2 h later than ISO (*P* = 0.001). Yeast and molds are primarily responsible for aerobic deterioration (Borreani et al., 2018), but despite the greater yeast counts observed in AMY at 90 and 120 d, aerobic stability was greater for this hybrid. However, the difference in aerobic stability between hybrids is unlikely to be biologically meaningful. As expected, mold counts decreased with ensiling (4.4 at 0 d vs. 2.6 log cfu/g on average; *P* = 0.001; Table 2.A1).

The effects of hybrid and storage length on nutrient composition of WPCS are in Table 2.2. Greater DM (37.4 % vs. 34.2% of as fed; *P* = 0.001) and CP (6.8 vs. 6.3% of DM; *P* = 0.001) concentrations were observed for AMY than ISO. Conversely, ISO had greater NDF (38.4 vs.

34.8% of DM; *P* = 0.001) and ADF (20.1 vs. 18.5% of DM; *P* = 0.001) concentrations. The concentration of NDF increased with ensiling (36.7 vs. 38.1; *P* = 0.01; Table 2.A1). After ensiling, NDF concentration (*P* = 0.001) was greatest for 60 d (40.8% of DM), and lowest for 90 and 120 d (33.8% of DM, on average; Table 2.A1). A similar pattern was observed for storage length effect on ADF concentration, 60 d had 4.1 percentage-units, on average, greater than 120 d (*P* = 0.001; Table 2.A1). An interaction was detected for WSC (*P* = 0.01), where WSC was greater for ISO than AMY (10.8 vs. 9.6% of DM) at 0 d, but not after ensiling (1.0% of DM, on average). Starch concentration was greater for AMY than ISO (41.0 vs. 38.3% of DM on average, respectively; *P* = 0.001). These differences in DM and starch concentrations could be partially related to a slight difference in stage of maturity between hybrids, or alternatively, to hybrids having different proportions of plant parts. Either of these effects would dilute the ratio of leaves and stalk to kernels, thereby increasing the concentration of starch while reducing concentrations of NDF and ADF in WPCS. There was an interaction between hybrid and storage length for EE concentration (*P* = 0.03), where ISO had greater EE at 0 (2.4 vs. 1.5% of DM) and 120 d (3.6 vs. 3.1% of DM) than AMY but not at 30, 60 and 90 d (2.5% of DM on average). An interaction between hybrid and storage length was observed for soluble CP (*P* = 0.01). Soluble CP was greater for ISO than AMY at 30 d (52.2 vs. 47.2% of CP); but lower at 60 d (54.3 vs. 59.5% of CP), whereas no differences were detected at 0, 90 or 120 d. Starch is surrounded by a protein matrix and its degradation is often associated with an increase in soluble CP and starch availability (Hoffman et al., 2011). Yet, starch disappearance data (Table 2.3) in this study suggests that the ammonia-N differences or soluble CP differences occurred at 30 or 60 d did not reflect differences in starch disappearance at the same storage length. Plant and microbial proteases in the silo can degrade plant proteins from either the kernel or the stover fractions into peptides and free amino acids

(Rooke and Hatfield, 2003; Muck et al., 2003), with the subsequent deamination of amino acids by silage microbes into ammonia-N (Grum et al., 1991). Even though positive relationships between starch digestibility and soluble CP (Der Bedrosian et al., 2012; Ferraretto et al., 2015b) or ammonia-N (Ferraretto et al., 2015b) were previously reported in WPCS ensiled across multiple storage lengths, these relationships were moderate.

Effects of hybrid and storage length on starch disappearance of WPCS are in Table 2.3. Contrary to our hypothesis, starch disappearance was 4.3 and 5.2 percentage-units greater at 0 h ($P = 0.03$) and 6 h ($P = 0.001$) for ISO than AMY. Starch disappearance at 0 h was lower ($P = 0.001$) at 0 (37.3% of starch) than 30 d (49.8% of starch; Table 2.A2). Moreover, starch disappearance at 6 h increased with ensiling (66.7 vs. 77.2% starch; $P = 0.001$; Table 2.A2). Starch disappearance at 6 h increased up to 85% of starch with storage length ($P = 0.001$; Table 2.A2). As discussed previously, slight maturity differences (based on DM concentration) between hybrids could partially explain the starch disappearance differences detected in the present study as starch digestibility decreases as maturity progresses (Ferraretto et al., 2018). In contrast to our results, Hellings et al. (2019) observed greater in situ starch disappearance at 0 and 7 h for a corn hybrid with the amylase trait compared to its isogenic counterpart. The selection of amylase enzymes genes for use in corn hybrids began based on Richardson et al. (2002) using starch liquefaction selection criteria that included temperature ranging from 60 to 105° C and pH ranging from 4.25 to 6.25, which may impact the amylase activity under silo or rumen conditions, which could possibly be related to the results observed in the present study. In vivo literature is inconclusive with reports of no effects (Cueva et al., 2021; Krogstad and Bradford, 2023) or a 1%-unit reduction (Rebelo et al., 2023) in total tract starch digestibility when dairy cows were fed diets containing a corn silage hybrid with the amylase trait compared with its isogenic counterpart. Further research

is warranted to elucidate prolamin concentration, starch digestibility effects and the mechanism associated with improved performance when feeding WPCS with the amylase trait to dairy cows.

Earlage

Effects of hybrid and storage length on pH, fermentation products and microbial counts of earlage are in Table 2.4. The pH was lower for ISO than AMY (3.9 vs. 4.1; $P = 0.001$). However, both hybrids had pH within the typical range (Kung Jr. et al., 2018). There was an interaction effect between hybrid and storage length for ammonia-N concentration ($P = 0.001$). Ammonia-N was similar between both hybrids at 0 d, but 7.3 percentage-units greater, on average, for AMY than ISO after ensiling. The greater increase in AMY ammonia-N concentration over time when compared to ISO suggests that protein went through a greater degree of deamination in AMY. This is likely due to normal deamination of protein by desirable fermentation (Kung Jr. et al., 2018) rather than clostridial fermentation, as butyric acid was not detected in any earlage samples. As discussed previously, in the case of earlage samples, ammonia-N production during silage fermentation can derive from kernel or plant material, which could partially explain the absent relationship between N fractions and starch disappearance results (Table 2.6) observed in this study. Lactic and acetic acid concentrations did not differ between ISO and AMY hybrids, suggesting AMY has minimal influence on earlage fermentation profile. Nevertheless, ISO had a 5.9 h greater aerobic stability than AMY ($P = 0.01$). Aerobic stability gradually increased throughout storage length; it went from 37.7 h at 30 d up to 57.0 h at 120 d ($P = 0.001$; Table 2.A2). Dry matter losses were 0.4 percentage-units greater for AMY than ISO ($P = 0.001$) and these losses are likely due to factors other than fermentation patterns.

Effects of hybrid and storage length on nutrient composition of earlage are in Table 2.5. Greater DM (59.4 vs. 57.8% of as fed; *P* = 0.001) and CP (7.8 vs. 7.1% of DM; *P* = 0.001) concentrations were observed for AMY than ISO, respectively. In contrast, AMY had (*P* = 0.01) 2.5 and 1.4 percentage-units lower soluble CP and NDF concentrations than ISO. A hybrid × storage length interaction was observed for EE concentration (*P* = 0.001), which was similar at 0 and 30 d (3.4% of DM, on average) but 1.8 percentage-units greater for AMY than ISO at 60, 90 and 120 d. Greater ash concentration was observed for AMY than ISO (1.7 vs. 1.6% of DM, *P* = 0.001). Ensiling decreased CP (0.4% of DM; *P* = 0.001; Table 2.A2) and NDF (1.6% of DM; *P* = 0.001; Table 2.A2) but increased soluble CP (18.6% of CP; *P* = 0.001; Table 2.A2). Soluble CP concentration gradually increased with storage length (*P* = 0.001; Table 2.A2), which went from 37.9% of CP at 0 d, up to 48.6% of CP at 90 and 120 d.

An interaction between hybrids and storage length was observed for WSC concentration (*P* = 0.01); even though AMY had greater WSC concentration than ISO across all storage lengths, the magnitude of this difference decreased over time. Moreover, starch concentration was greater for AMY than ISO (60.7 vs. 59.6% of DM, respectively; *P* = 0.03). As discussed for WPCS, this result reflects the differences observed in DM concentration and is corroborated by differences observed for CP and NDF concentrations. Moreover, Fernandes et al. (2022) observed a loss of starch and greater WSC when rehydrated corn grain silage was treated with exogenous amyloglucosidases. However, starch concentration did not change as storage length increased in the present study, suggesting the WSC concentration effect is likely due to the greater WSC concentration prior to ensiling rather than amylase activity.

Effects of hybrid and storage length on starch disappearance of earlage are in Table 2.6. Starch disappearance was greater for ISO than AMY at 0 h (70.2 vs. 63.7% of starch, respectively;

P = 0.001) and 6 h (87.5 vs. 84.0% of starch, respectively; *P* = 0.001). Slight differences in DM observed in this study could partially explain the starch disappearance results. Hu et al. (2010) observed greater in vitro starch disappearance at 6 h for amylase-expressed corn (dry ground corn) when compared to its isogenic counterpart; however, the authors stated that the difference was biologically irrelevant (90.6 vs. 89.7% of starch, respectively). The same authors also assessed the amylase activity in the grain by incubating grains from both hybrids in water at 40° and 65°C for 24 h. They reported greater difference between hybrids in starch degradation when grains were incubated at 65°C compared to 40°C (Hu et al., 2010). These results indicate that amylase activity in this hybrid might be low at normal silage and rumen temperatures, which may help to understand the lack of improvements on starch disappearance for AMY in this study. Further research evaluating prolamin concentration in AMY corn is warranted. Starch disappearance at 0 and 6 h increased with ensiling (6.4% for 0 h and 11.9% for 6 h; *P* = 0.001; Table 2.A2) and gradually increased with storage length (*P* = 0.001; Table 2.A2) for both hybrids, it went from 63.4% of starch up to 74.6% of starch for 0 h and from 85.4% of starch up to 91.6% of starch for 6 h incubation.

CONCLUSION

Minor effects of silage fermentation, microbial counts and aerobic stability were observed between a corn hybrid with alpha-amylase and its isogenic counterpart. These results confirm that AMY hybrids can be ensiled for longer periods without concerns of undesirable fermentation or greater losses. Contrary to our hypothesis, however, AMY had lower in situ starch disappearance.

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REFERENCES

AOAC. 2012. Official methods of analysis. 19th ed. AOAC Int., Arlington, VA.

AOCS - AMERICAN OIL CHEMISTS' SOCIETY. 2009. Official methods and recommended practices of the AOCS, 6th ed., Denver: AOCS.

Borreani G., E. Tabacco, R. J. Schmidt, B. J. Holmes, and R. E. Muck. 2018. Silage review: Factors affecting dry matter and quality losses in silages. J. Dairy Sci. 101:3952–3979. https://doi.org/10.3168/jds.2017-13837

Cueva S. F., H. Stefenoni, A. Melgar, S. E. Räisänen, C. F. A. Lage, D. E. Wasson, M. E. Fetter, A. M. Pelaez, G. W. Roth, and A. N. Hristov. 2021. Lactational performance, rumen fermentation, and enteric methane emission of dairy cows fed an amylase-enabled corn silage. J. Dairy Sci. 104:9827–9841. https://doi.org/10.3168/jds.2021-20251.

Der Bedrosian, M. C., L. Kung, Jr., and K. E. Nestor, Jr. 2012. The effects of hybrid, maturity and length of storage on the composition and nutritive value of corn silage. J. Dairy Sci. 95:5115-5126. https://doi.org/10.3168/jds.2011-4833.

Fernandes, T., C. L. S. Ávila, M. N. Pereira, and L. F. Ferraretto. 2018. Short communication: Effect of washing method, grinding size, and the determination of an indigestible fraction on in situ degradation of starch in mature corn grain. J. Dairy Sci. 101: 9052-9057. https://doi.org/10.3168/jds.2018-14870.

Fernandes T., da Silva K. T., Carvalho B. F., Schwan R. F., Pereira R. A. N., Pereira M. N., Ávila C. L. S. 2022. Effect of amylases and storage length on losses, nutritional value, fermentation, and microbiology of silages of corn and sorghum kernels. Anim. Feed Sci. and Technol. 285: 115227. https://doi.org/10.1016/j.anifeedsci.2022.115227.

Ferraretto, L. F., P. M. Crump, and R. D. Shaver. 2015a. Effect of ensiling time and exogenous protease addition to whole-plant corn silage of various hybrids, maturities and chop lengths on nitrogen fractions and ruminal in vitro starch digestibility. J. Dairy Sci. 98:8869-8881. https://doi.org/10.3168/jds.2015-9511.

Ferraretto, L. F., and R. D. Shaver. 2015. Effects of whole-plant corn silage hybrid type on intake, digestion, ruminal fermentation and lactation performance by dairy cows through a meta-analysis. J. Dairy Sci. 98:2662-2675. https://doi.org/10.3168/jds.2014-9045.

Ferraretto, L. F., R. D. Shaver, J. G. Lauer, L. H. Brown, J. P. Kennicker, R. J. Schmidt, and D. M. Taysom. 2018. Influence of plant population and maturity, microbial inoculation and ensiling time on fermentation profile, nitrogen fractions and starch digestibility in earlage. In: XVIII International Silage Conference, Bonn, Germany. Pages: 522-523.

Ferraretto, L. F., R. D. Shaver, S. Massie, R. Singo, D. M. Taysom, and J. P. Brouillette. 2015b. Effect of ensiling time and hybrid type on fermentation profile, nitrogen fractions and ruminal in vitro starch and NDF digestibility in whole-plant corn silage. Prof. Anim. Sci. 31:146-152. https://doi.org/10.15232/pas.2014-01371.

Grum, D. E., W. L. Shockey, and W. P. Weiss. 1991. Electrophoretic examination of alfalfa silage proteins. J. Dairy Sci. 74:146–154. https://doi.org/10.3168/jds.S0022-0302(91)78155-1.

Hall, M. B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. J. AOAC Int. 98:397-409. https://doi.org/10.5740/jaoacint.15-012.

Hellings E., K. Moyer, É. da Silva, C. Novinski, Y. Li, W. Sá, R. Mester, and L. Kung Jr. 2019. Effect of ensiling time and temperature on the fermentation and nutritive value of a corn hybrid containing an α-amylase enzyme trait (Enogen Feed Corn). In: Abstracts of the 2019 American Dairy Science Association annual meeting. Cincinnati, OH. J. Dairy Sci. Vol. 102, Suppl. 1. Pages: 328-329. (Abstr.)

Herrmann, C., M. Heiermann, and C. Idler. 2011. Effects of ensiling, silage additives and storage period on methane formation of biogas crops. Bioresour. Technol. 102: 5153–5161. https://doi.org/10.1016/j.biortech.2011.01.012.

Hoffman, P. C., N. M. Esser, R. D. Shaver, W. K. Coblentz, M. P. Scott and A. L. Bodnar. 2011. Influence of ensiling time and inoculation on alteration of the starch-protein matrix in high-moisture corn. J. Dairy Sci. 94:2465–2474. https://doi.org/10.3168/jds.2010-3562.

Hu, W., M. E. Persia and L. Kung Jr. 2010. Short communication: In vitro ruminal fermentability of a modified corn cultivar expressing a thermotolerant α-amylase. J. Dairy Sci. 93 :4846–4849. https://doi.org/10.3168/jds.2009-2321.

Krishnamoorthy, U., T. V. Muscato, C. J. Sniffen, and P. J. Van Soest. 1982. Nitrogen fractions in selected feedstuffs. J. Dairy Sci. 65:217–225. https://doi.org/10.3168/jds.S0022-0302(82)82180-2.

Krogstad, K. C. and B. J. Bradford. 2023. The effects of feeding α-amylase enhanced corn silage with different dietary starch concentrations to lactating dairy cows on milk production, nutrient digestibility, and blood metabolites. J. Dairy Sci. 106: 4666-4681. https://doi.org/10.3168/jds.2022-23030.

Kung Jr., L., Shaver, R. D., Grant. R. J., Schmidt, J. 2018. Silage review: Interpretation of chemical, microbial and organoleptic components of silages. J. Dairy Sci. 101: 4020-4033. https://doi.org/10.3168/jds.2017-13909.

Lindgren, S.E., Axelsson, L.T., McFeeters, R.F., 1990. Anaerobic L-lactate degradation by Lactobacillus plantarum. FEMS Microbiol. Lett. 66: 209–213. https://doi.org/10.1111/j.1574-6968.1990.tb03998.x.

Muck, R. E., L. E. Moser, and R. E. Pitt. 2003. Postharvest factors affecting enisling. Pages 251–304 in Silage Science and Technology. D. R. Buxton, R. E. Muck, and J. H Harrison, ed. American Society of Agronomy, Madison, WI.

Noel, R. J., and L. G. Hambleton. 1976. Collaborative study of a semi-automated method for the determination of crude protein in animal feeds. J. Assoc. Off. Anal. Chem. 59:134–140. https://doi.org/10.1093/jaoac/59.1.134.

Ministry of Agriculture, Fisheries, and Food. 1986. The analysis of agricultural materials. Reference Book 427. Minist. Agric., Fisheries Food, Her Majesty's Stationery Office, London, UK.

Muck, R. E. and J. T., Dickerson. 1988. Storage temperature effects on proteolysis in alfalfa silage. Trans. ASABE 31, 1005-009. https://doi.org/10.13031/2013.30813.

Rebelo, L. R., M. L. Eastridge, J. L. Firkins, and C. Lee. 2023. Effects of corn silage and grain expressing α-amylase on ruminal nutrient digestibility, microbial protein synthesis, and

enteric methane emissions in lactating cows. J. Dairy Sci. 106:3932-3946. https://doi.org/10.3168/jds.2022-22770.

Rooke, J. A. and R. D., Hatfield. 2003. Biochemistry of Ensiling. Pages 95 – 139 in Buxton, D. R., Muck, R. E., Harrison, H. J. Silage Science and Technology (Agronomy Series No. 42). American Society of Agronomy, Madison, WI.

Singh, V., Batie C.J., Aux G.W., Rausch K.D., Miller C. 2006. Dry-grind processing of corn with endogenous liquefaction enzymes. Cereal Chem. 83(4):317–20. https://doi.org/10.1094/CC-83-0317.

Spoelstra S. F., Piet G van Wikselaar. 1992. The Effects of Ensiling Whole Crop Maize with a Multi-enzyme Preparation on the Chemical Composition of the Resulting Silages. J. Sci. Food Agric. 60: 223-228. https://doi.org/10.1002/jsfa.2740600210.

TABLES

Table 2.1. Effect of hybrid and storage length on pH, fermentation profile, ammonia-N, microbial counts, aerobic stability, and DM losses of whole-plant corn silage.[1]

Item[2]		pH	Ammonia-N, % total N	Lactic acid, % DM	Acetic acid, % DM	Yeast count, log cfu/g	Mold count, log cfu/g	Aerobic stability, h	DM losses, % ensiled
0 d	ISO	-	2.5	-	-	5.9[a]	5.2	-	-
	AMY	-	1.8	-	-	4.2[b]	3.5	-	-
30 d	ISO	3.6	4.4[b]	8.3	2.1	4.0	2.0	54.5	0.8
	AMY	4.2	13.9[a]	9.8	1.4	4.4	2.8	66.4	0.7
60 d	ISO	3.6	4.3[b]	7.9	1.5	4.4	3.3	46.6	0.9
	AMY	3.5	11.7[a]	9.0	1.8	4.5	2.6	69.5	0.7
90 d	ISO	3.6	4.4[b]	7.7	1.1	2.9[b]	2.5	60.8	0.9
	AMY	3.6	10.6[a]	8.6	1.2	4.2[a]	2.9	60.1	0.7
120 d	ISO	3.6	3.6[b]	8.5[a]	0.5	3.0[b]	2.3	63.7	2.3[a]
	AMY	3.6	12.3[a]	5.4[b]	2.1	4.0[a]	2.1	78.3	0.8[b]
Effect of hybrid	ISO	3.6	3.8	8.1	1.3	4.0	3.1	56.4	1.2
	AMY	3.7	10.1	8.2	1.5	4.3	2.8	68.6	0.7
Effect of SL	0 d	-	2.2	-	-	5.0	4.4	-	-
	30 d	3.9	9.1	9.1	1.7	4.2	2.4	60.4	0.7
	60 d	3.6	7.8	8.4	1.6	4.4	2.9	58.1	0.8
	90 d	3.6	4.5	8.2	1.4	3.5	2.7	60.4	0.8
	120 d	3.6	9.8	6.9	0.9	3.5	2.2	71.0	1.5
SEM		0.22	0.56	0.64	0.36	0.35	0.49	5.6	0.12
P-values									
Hybrid		0.40	0.001	0.83	0.34	0.30	0.35	0.01	0.001

SL	0.35	0.001	0.01	0.06	0.001	0.001	0.08	0.001
Hybrid x SL	0.31	0.001	0.001	0.15	0.001	0.11	0.16	0.001

[a-b] Means with different superscripts within the same day differ ($P \leq 0.05$).

[1]Propionic, iso-butyric, butyric and valeric acids and 1,2-propanediol were analyzed but not detected.

[2]ISO – isogenic counterpart; AMY – genetically-modified corn hybrid with expressed alpha-amylase in the kernel. SL: storage length effect: 0, 30, 60, 90 and 120 d.

Table 2.2. Effect of hybrid and storage length on DM concentration and nutrient composition of whole-plant corn silage.

Item[2]		DM, % as fed	WSC, % DM	NDF, % DM	ADF, % DM	Starch, % DM	EE, % DM	Ash, % DM	CP, % DM	Soluble CP, % CP
0 d	ISO	35.5	10.8[a]	38.8	19.2	39.7	2.4[a]	2.5	6.4	28.7
	AMY	38.8	9.6[b]	34.5	17.5	42.6	1.5[b]	3.1	6.7	27.0
30 d	ISO	33.4	0.7	40.6	21.4	38.9	2.4	3.0	6.3	52.2[a]
	AMY	36.8	1.5	35.6	18.8	41.1	2.3	2.8	6.7	47.2[b]
60 d	ISO	33.2	0.7	41.1	21.8	36.5	2.3	3.1	6.3	54.3[b]
	AMY	35.8	1.3	40.6	21.9	38.9	2.6	3.2	7.0	59.5[a]
90 d	ISO	34.0	0.7	36.6	19.3	38.0	3.0	2.9	6.2	57.9
	AMY	37.7	1.1	31.9	17.4	41.3	2.6	2.8	6.8	54.1
120 d	ISO	34.8	0.7	34.8	18.7	38.6	3.6[a]	2.9	6.2	60.2
	AMY	37.7	1.1	31.6	16.8	40.8	3.1[b]	2.8	6.7	60.3
Effect of hybrid	ISO	34.2	2.7	38.4	20.1	38.3	2.7	2.9	6.3	50.6
	AMY	37.4	2.9	34.8	18.5	41.0	2.4	3.0	6.8	49.6
Effect of SL	0 d	37.2	10.2	36.7	18.3	41.2	2.0	2.8	6.6	27.8
	30 d	35.1	1.1	38.1	20.1	40.0	2.3	2.9	6.5	49.7
	60 d	34.5	1.0	40.8	21.8	37.7	2.4	3.2	6.6	56.9
	90 d	35.8	0.9	34.3	18.4	39.6	2.8	2.9	6.5	56.0
	120 d	36.3	0.9	33.2	17.7	39.7	3.3	2.8	6.5	60.2
SEM		0.77	0.29	1.51	0.88	1.45	0.24	0.16	0.17	1.65
P-values										
Hybrid		0.001	0.22	0.001	0.001	0.001	0.01	0.43	0.001	0.25
SL		0.001	0.001	0.001	0.001	0.17	0.001	0.12	0.79	0.001
Hybrid x SL		0.90	0.01	0.31	0.45	0.99	0.03	0.15	0.82	0.01

[a-b] Means with different superscripts within the same day differ ($P \leq 0.05$).

[1]ISO – isogenic counterpart; AMY – genetically-modified corn hybrid with expressed alpha-amylase in the kernel. SL: storage length effect: 0, 30, 60, 90 and 120 d.

Table 2.3. Effect of hybrid and storage length on in situ starch disappearance at 0 and 6 h of whole-plant corn silage.

Item[1]		StarchD 0h, % starch	StarchD 6h, % starch
0 d	ISO	40.8	71.5
	AMY	33.9	61.9
30 d	ISO	51.6	79.9
	AMY	48.0	74.6
60 d	ISO	54.0	82.5
	AMY	47.8	79.3
90 d	ISO	56.9	87.0
	AMY	50.8	82.1
120 d	ISO	53.4	86.9
	AMY	54.3	84.2
Effect of hybrid	ISO	51.3	81.6
	AMY	46.9	76.4
Effect of SL	0 d	37.3	66.7
	30 d	49.8	77.2
	60 d	50.9	80.9
	90 d	53.8	84.5
	120 d	53.8	85.5
SEM		3.23	1.40
P-values			
Hybrid		0.03	0.001
SL		0.001	0.001
Hybrid x SL		0.70	0.14

[a-b] Means with different superscripts within the same day differ ($P \leq 0.05$).

[1]ISO – isogenic counterpart; AMY – genetically-modified corn hybrid with expressed alpha-amylase in the kernel. SL: storage length effect: 0, 30, 60, 90 and 120 d.

Table 2.4. Effect of hybrid and storage length on pH, fermentation profile, ammonia-N, microbial counts, aerobic stability, and DM losses of earlage.[1]

Item[2]		pH	Ammonia-N, % total N	Lactic acid, % DM	Acetic acid, % DM	Yeast count, log cfu/g	Mold count, log cfu/g	Aerobic stability, h	DM losses, % ensiled
0 d	ISO	-	1.4	-	-	3.5	3.2	-	-
	AMY	-	1.3	-	-	3.7	2.4	-	-
30 d	ISO	3.9	2.5[b]	3.1	0.9	4.5	4.2	37.1	1.1
	AMY	4.1	10.3[a]	3.2	1.3	4.3	4.7	38.3	1.3
60 d	ISO	3.9	2.5[b]	3.4	0.8	3.9	4.0	51.2	1.2
	AMY	4.1	12.2[a]	2.7	0.6	4.5	4.5	44.9	1.5
90 d	ISO	3.9	12.0[b]	3.5	0.8	3.7	3.4	56.1	1.2
	AMY	4.0	18.7[a]	3.8	1.0	4.2	3.6	47.7	1.5
120 d	ISO	4.0	12.7[b]	4.3	1.5	3.4	3.6	61.9	1.2
	AMY	4.2	17.7[a]	3.3	0.9	3.5	3.8	52.2	1.5
Effect of hybrid	ISO	3.9	6.3	3.6	1.0	3.8	3.7	51.6	1.1
	AMY	4.1	12.0	3.2	1.0	4.0	3.8	45.7	1.5
Effect of SL	0 d	-	1.3	-	-	3.6	2.8	-	-
	30 d	4.0	6.3	3.1	1.1	4.4	4.5	37.7	1.2
	60 d	4.0	7.4	3.0	0.7	4.2	4.2	48.0	1.3
	90 d	4.0	15.4	3.6	0.9	3.9	3.5	51.9	1.4
	120 d	4.1	15.2	3.8	1.2	3.5	3.7	57.0	1.3
SEM		0.03	0.93	0.32	0.19	0.48	0.41	3.14	1.54
P-values									
Hybrid		0.001	0.001	0.15	0.67	0.37	0.67	0.01	0.001
SL		0.001	0.001	0.06	0.06	0.27	0.001	0.001	0.21
Hybrid x SL		0.93	0.001	0.15	0.10	0.94	0.49	0.31	0.78

[a-b] Means with different superscripts within the same day differ ($P \leq 0.05$).
[1]Propionic, iso-butyric, butyric and valeric acids and 1,2-propanediol were analyzed but not detected.
[2]ISO – isogenic counterpart; AMY – genetically-modified corn hybrid with expressed alpha-amylase in the kernel. SL: storage length effect: 0, 30, 60, 90 and 120 d.

Table 2.5. Effect of hybrid and storage length DM concentration and nutrient composition of earlage.

Item[2]		DM, % as fed	WSC, % DM	NDF, % DM	ADF, % DM	Starch, % DM	EE, % DM	Ash, % DM	CP, % DM	Soluble CP, % CP
0 d	ISO	58.2	2.1[b]	21.1	7.4	60.7	3.3	1.6	7.5	19.1
	AMY	60.6	3.2[a]	20.0	7.1	60.3	3.1	1.7	8.2	19.7
30 d	ISO	58.3	1.1[b]	20.2	8.1	59.5	3.3	1.5	7.1	39.1
	AMY	59.3	1.6[a]	17.7	7.3	61.7	3.8	1.7	7.8	36.7
60 d	ISO	57.8	0.8[b]	17.9	7.1	59.5	2.0[b]	1.6	7.1	43.0
	AMY	58.8	1.3[a]	18.1	7.8	60.2	4.1[a]	1.7	7.8	40.4
90 d	ISO	57.0	0.7[b]	18.2	7.5	59.3	2.4[b]	1.6	7.0	50.6
	AMY	59.4	1.3[a]	14.9	6.4	61.7	4.3[a]	1.6	7.6	44.8
120 d	ISO	57.6	0.7[b]	17.1	7.2	59.2	2.9[b]	1.6	6.7	50.6
	AMY	59.1	1.2[a]	17.0	7.4	59.7	4.3[a]	1.7	7.7	48.5
Effect of hybrid	ISO	57.8	1.1	18.9	7.5	59.6	2.8	1.6	7.1	40.5
	AMY	59.4	1.7	17.5	7.2	60.7	3.9	1.7	7.8	38.0
Effect of SL	0 d	59.4	2.6	20.5	7.3	60.5	3.2	1.7	7.8	19.3
	30 d	58.8	1.3	18.9	7.7	60.6	3.5	1.6	7.4	37.9
	60 d	58.3	1.0	18.0	7.5	59.9	3.1	1.7	7.4	41.7
	90 d	58.2	1.0	16.5	7.0	60.5	3.3	1.6	7.3	47.7
	120 d	58.3	1.0	17.1	7.3	59.4	3.6	1.7	7.2	49.5
SEM		0.39	0.10	0.75	0.33	0.95	0.23	0.03	0.16	1.31
P-values										
Hybrid		0.001	0.001	0.01	0.20	0.03	0.001	0.001	0.001	0.001
SL		0.01	0.001	0.001	0.31	0.47	0.09	0.44	0.001	0.001
Hybrid x SL		0.12	0.01	0.09	0.08	0.27	0.001	0.11	0.57	0.18

[a-b] Means with different superscripts within the same day differ ($P \leq 0.05$).

[1]ISO – isogenic counterpart; AMY – genetically-modified corn hybrid with expressed alpha-amylase in the kernel. SL: storage length effect: 0, 30, 60, 90 and 120 d.

Table 2.6. Effect of hybrid and storage length in situ starch disappearance at 0 and 6 h of earlage.

Item[1]		StarchD 0h, % starch	StarchD 6h, % starch
0 d	ISO	62.3	76.5
	AMY	51.6	70.4
30 d	ISO	66.8	87.6
	AMY	60.0	83.2
60 d	ISO	71.2	89.5
	AMY	66.7	87.3
90 d	ISO	72.9	91.0
	AMY	68.7	89.1
120 d	ISO	77.6	93.0
	AMY	71.6	90.2
Effect of hybrid	ISO	70.2	87.5
	AMY	63.7	84.0
Effect of SL	0 d	57.0	73.5
	30 d	63.4	85.4
	60 d	68.9	88.4
	90 d	70.8	90.0
	120 d	74.6	91.6
SEM		1.95	1.23
P-values			
Hybrid		0.001	0.001
SL		0.001	0.001
Hybrid x SL		0.38	0.28

[a-b] Means with different superscripts within the same day differ ($P \leq 0.05$).
[1]ISO – isogenic counterpart; AMY – genetically-modified corn hybrid with expressed alpha-amylase in the kernel. SL: storage length effect: 0, 30, 60, 90 and 120.

APPENDIX

Table A1. Main effect of storage length on microbial counts, nutrient composition and in situ starch disappearance at 0 and 6 h of whole-plant corn silage, when averaged over hybrids.[1]

	Storage length						*P*-values[2]	
Item	0 d	30 d	60 d	90 d	120 d	SEM	E	SL
DM, % of as fed	37.2	35.1	34.5	35.8	36.3	0.77	0.16	0.05
Mold count, log cfu/g	4.4	2.4	2.9	2.7	2.2	0.37	0.001	0.40
NDF, % DM	36.7	38.1[ab]	40.8[a]	34.3[bc]	33.2[c]	1.40	0.01	0.001
ADF, % DM	18.3	20.1[ab]	21.8[a]	18.4[bc]	17.7[c]	0.75	0.42	0.001
StarchD 0h, % starch	37.3	49.8	50.9	53.8	53.8	2.45	0.001	0.42
StarchD 6h, % starch	66.7	77.2[c]	80.9[b]	84.5[a]	85.5[a]	1.32	0.001	0.001

[a-b] Means with different superscripts denote differences after Bonferroni correction ($P \leq 0.05$).

[1]Main effect are presented only if the interaction with hybrid was not significant (P > 0.05).

[2]E: ensiling effect (0 vs. 30 d) SL: storage length effect (30 vs. 60 vs. 90 vs. 120 d).

Table A2. Main effect of storage length on microbial counts, aerobic stability nutrient composition and in situ starch disappearance at 0 and 6 h of earlage, when averaged over hybrids.[1]

	Storage length						*P*-values[2]	
Item	0 d	30 d	60 d	90 d	120 d	SEM	E	SL
DM, % of as fed	59.4	58.8	58.3	58.2	58.3	0.39	0.06	0.33
pH	-	4.0	4.0	4.0	4.0	0.04	NA	0.06
Mold count, log cfu/g	2.8	4.5	4.2	3.5	3.7	0.29	0.04	0.03
Aerobic stability, h	-	37.7[b]	48.0[a]	51.9[a]	57.0[a]	2.4	NA	0.001
NDF, % DM	20.5	18.9[a]	18.0[ab]	16.5[b]	17.1[ab]	0.60	0.001	0.01
CP, % DM	7.8	7.4	7.4	7.3	7.2	0.17	0.02	0.18
Soluble CP, % CP	19.3	37.9[c]	41.7[b]	47.7[a]	49.5[a]	1.11	0.001	0.001
StarchD 0h, % starch	57.0	63.4[b]	68.9[ab]	70.8[a]	74.6[a]	1.80	0.001	0.001
StarchD 6h, % starch	73.5	85.4[c]	88.4[bc]	90.0[ab]	91.6[a]	1.11	0.001	0.001

[a-b] Means with different superscripts denote differences after Bonferroni correction ($P \leq 0.05$).

[1]Main effect are presented only if the interaction with hybrid was not significant ($P > 0.05$).

[2]E: ensiling effect (0 vs. 30 d) SL: storage length effect (30 vs. 60 vs. 90 vs. 120 d).

CHAPTER THREE: EFFECTS OF INCUBATION TIME-POINT, GRINDING SIZE AND BAG PORE SIZE ON LABORATORIAL STARCH DIGESTIBILITY ASSAYS

INTRODUCTION

Ruminal in situ and in vitro starch digestibility assays are widely used by the industry, even though a standardized model to predict total-tract starch digestibility based on these assays have not been defined. These assays are widely used to rank and evaluate forages and feedstuffs and provide useful information for ration formulation. For example, the Cornell Net Carbohydrate and Protein System (CNCPS) model uses nutrient degradation rates, which may be calculated using in vitro or in situ digestibility assays, to predict ruminal available energy (Fox et al., 2004; Van Amburgh et al., 2015). But the accurate use of laboratorial starch digestibility values depends upon the precision of the assay performed. Commercial laboratories of feed analysis and research laboratories utilize various procedures when conducting starch digestibility assays. Thus, it is important to understand the variation among and within starch assays. Hall and Mertens (2012) conducted a ring test to evaluate the analytical variability of NDF digestibility assays and reported that laboratories that used Goering and Van Soest (1970) procedure were able to reliably rank samples based on 30 h in vitro NDF digestibility. However, the variation of starch digestibility assays performed with different time-points, grinding sizes and bag pore sizes is unknown.

In vitro and in situ digestibility assays are conducted to mimic what happens with feeds in the rumen. Sample processing is required to ensure uniformity of sample/rumen fluid contact, but it is debatable whether samples should be processed to mimic how cows are fed or after mastication and rumination (Nocek and Kohn, 1988). Michalet-Doreau and Cerneau (1991) evaluated sample grinding size influence on in situ nitrogen degradation of feeds in the rumen and reported that nitrogen degradability increases when samples are more finely ground. Grinding samples to a greater extent increase surface area for degradation. Similar

patterns are observed for the degradation of carbohydrates as well (Ferreira and Mertens, 2005). Most common grinding sizes for in vitro and in situ assays range from 1- to 6-mm (Nocek and Kohn, 1988; Vanzant et al., 1998; Fernandes et al., 2018) and grinding samples through a 1-mm sieve has been recently reported to reduce the variation of the assay (Cueva et al., 2023). Moreover, both in vitro and in situ assays rely on the use of filter bags containing feed samples to calculate starch fraction disappearance from the bag after a period of incubation (Hall and Mertens, 2017). Corn starch granules are reported to be between 2 to 30 μm in size (Tester et al., 2004), and thus, there is potential for secondary particulate loss through the pores of the incubation bags, which ranges from 25 to 50 μm. Consequently, starch degradation rates might be overestimated (Philippeau and Michalet-Doreau, 1997). Seifried et al. (2015) reported that secondary losses during in vitro assays were below the starch detection limit for corn grain when evaluating different bag pore sizes at 8 h of incubation. However, shorter incubation time-points (0, 3, and 4 h, for example) have been recently used to detect differences in starch digestibility among corn hybrids that could be masked at 7 h incubation assays (Cueva et al., 2023). Yet, evaluation of filter bag pore sizes and fraction loss degradability of shorter incubation time-points are not available in the literature.

Thus, there is a need to evaluate variation across time-points, sample grinding size procedures and bag pore size used in ruminal starch digestibility assays to better understand and further refine these assays. Therefore, our experimental objectives were to (1) evaluate the variability in starch and water soluble carbohydrates (WSC) concentrations and starch digestibility submitted three times to multiple laboratories and the use of different incubation time-points on feedstuff ranking; (2) evaluate the effects of the combination of incubation

time-point and grinding size on in vitro starch digestibility of various starch sources; (3) evaluate ruminal degradation kinetics of various starch sources after an in situ incubation at 0 h; (4) evaluate the effect of filter bags commonly used in starch digestibility assays and washout water temperature on DM recovery and ruminal degradation kinetics of starch sources at 0 h; (5) evaluate the effect of filter bags on DM and starch recovery of starch sources incubated in rumen fluid at 0 h.

MATERIAL AND METHODS

Experimental procedures and animal use protocol in experiment 3 were approved by the Animal Care Research Committee of the Institute of Food and Agricultural Sciences of the University of Florida as well as by the animal care research committee of the Facolta' Di Scienze Agrarie, Alimentari e Ambientali of the Universita Cattolica del Sacro Cuore (Piacenza, Italy). Experimental procedures and animal use protocols in experiment 4 and 5 were approved by the Research Animal Resource Committee of the College of Agricultural and Life Sciences of the University of Wisconsin-Madison.

Experiment 1 - variability of starch and WSC concentrations and starch digestibility

An experiment was conducted to understand the variability of starch and WSC concentration assays as well as starch digestibility assays performed at multiple time-points. Samples of five starch sources were utilized for this study (Table 1). First, an unground mature dent corn sample (MDC; used in experiments 1, 2 and 3). Other subsamples of this material had been previously used for in vitro starch degradability assays conducted by our laboratory. In addition, whole-plant corn forage (unfermented samples; H1 and H2 CF) or whole-plant corn silage of two corn hybrids (fermented samples; H1 and H2 CS). Briefly, three mini-silos (20 L buckets) of each hybrid were prepared during the last week of June of

2019. Samples of unfermented material were collected, homogenized, divided using a quartering technique into 200 g samples, and frozen at -20°C. Laboratory silos were allowed to ferment for 60 d. One silo of each hybrid was chosen based on fermentation profile (lowest pH and highest lactic acid concentration). Similar to the procedures used for unfermented samples, silage material of each hybrid was homogenized, divided into 200 g samples, and frozen.

Samples were submitted to four commercial laboratories of feed analyses as if these samples were from a random study from our laboratory. Each of the five starch sources were submitted overnight to each laboratory three times within a 6-month period in triplicate for each submission. Samples were resubmitted only after results from the previous run were received. This design ensured three independent in vitro or in situ runs were conducted for each laboratory and that each run contained three replicates. Mature dent corn was submitted undried (but not frozen) and unground.

Samples were analyzed for starch and WSC concentrations using wet chemistry assays and near infrared spectroscopy (NIRS). Each laboratory performed their routine assays. In addition, samples were analyzed for in vitro starch ruminal degradability or in situ starch disappearance. Briefly, for in vitro procedures, dried and ground samples were weighed and placed in F57 bags (F57; 25 µm porosity; Ankom Technology) and incubated in rumen fluid plus media according to the laboratory routine procedure for 0, 3, 7 and 16 h. One laboratory used a separate rumen fluid collection for the 16 h assay. For in situ procedures, dried and ground samples were weighed and placed in dracon polyester bags (DPB; R1020, 10 cm x 20 cm, 50 µm porosity; Ankom Technology) and incubated in three rumen cannulated lactating dairy cows for 0, 3, 7 and 16 h. In vitro and in situ residues were

analyzed for starch concentration as previously described for each laboratory. Incubation time-points not routinely performed by labs were requested as a special assay. Samples were scanned in NIRS for prediction of nutrient composition, starch and WSC concentrations as well as starch digestibility according to each laboratory routine procedures. Briefly, one laboratory used NIRS predictions for WSC and starch concentrations and starch digestibility at 0 and 7 h; another laboratory used NIRS predictions for WSC and starch concentrations; and a third laboratory used NIRS predictions for WSC and starch concentrations and starch digestibility at 7 h.

To assess variability of starch and WSC concentrations and starch digestibility at different incubation time-points across laboratories and submissions, inter-procedure standard deviations and coefficient of variation for each sample were calculated for wet chemistry results and NIRS predictions. To assess variability of starch and WSC concentrations and starch digestibility at different incubation time-points within a laboratory, intra-procedure coefficients of variation for each sample was calculated for wet chemistry results and NIRS predictions when applicable. Moreover, two separate sets of statistical analyses were performed on wet chemistry starch digestibility data (data from 3 laboratories). First, to understand how sample submission affects starch digestibility in different incubation time-points, sample means, standard deviations, and coefficients of variation of the replicates were analyzed as a completely randomized design with a 4 x 3 factorial arrangement of the treatments using generalized linear mixed model procedures (PROC GLIMMIX, SAS 9.4; SAS Institute Inc., Cary, NC, USA) with a model including the fixed effects of incubation time-point and submission and their interaction. The effect of laboratory was the sole random effect. Statistical significance was declared at $P \leq 0.05$. Second, to understand how

incubation time-point affected sample ranking based on starch digestibility, sample means were analyzed as a completely randomized design with a 5 x 4 factorial arrangement of the treatments using the generalized linear mixed model procedures in SAS, with a model including the fixed effects of starch source, incubation time-point and their interaction. The effect of submission within laboratory was the sole random effect. These effects were partitioned using the SLICE option to rank starch sources within each incubation time-point. Statistical significance was declared at $P \leq 0.05$.

Experiment 2 - grinding size and incubation time-point effect on starch digestibility

Triplicate samples of four starch sources (whole-plant corn forage and silage, high-moisture corn, and dry ground corn) were used in this experiment. Frozen material was submitted to a commercial laboratory (Dairyland Laboratories Inc., Arcadia, WI, United States) as a special project. Samples were dried at 60°C for 48 h in an air-forced oven and ground to pass either a 1- or a 4-mm sieve. Three ruminal in vitro starch digestibility (ivSD) runs were conducted. Briefly, 0.5 g of samples were weighed and placed in F57 bags (F57; 25 μm porosity; Ankom Technology) and incubated in rumen fluid plus media according to Richards et al. (1995) for 4 or 7 h, simultaneously. Samples and in vitro residues were analyzed for starch concentration according to a modified method based on Hall et al. (2015), which the hydrolyzed starch analysis is conducted using a YSI 2700 Select Biochemistry analyzer (Marshal Scientific, Hampton, NH, USA).

To assess variability of in vitro starch digestibility, standard deviations and coefficients of variation for each in vitro procedure (different grinding sizes and incubation time-points) were calculated. In vitro starch digestibility means and standard deviations from each starch source were analyzed as a completely randomized design in a 2 x 2 factorial

arrangement of treatments using generalized linear mixed model procedures (PROC GLIMMIX, SAS 9.4; SAS Institute Inc., Cary, NC, USA) with a model including fixed effects of grinding size, incubation time-point and their interaction. Run was considered a random effect. Moreover, combined data from all starch sources were analyzed as a completely randomized design in a 4 x 4 factorial arrangement of the treatments using generalized linear mixed model procedures (PROC GLIMMIX, SAS 9.4; SAS Institute Inc., Cary, NC, USA) with a model including fixed effect of starch source, in vitro procedure, and their interaction. These effects were partitioned using the SLICE option to rank starch sources within each in vitro procedure. Run was considered a random effect. Means were determined using the least square means statement. Statistical significance was declared at $P \leq 0.05$.

Experiment 3: ruminal kinetics of 0 h incubation residues

Samples of different starch sources were used in this experiment: whole-plant corn forage (WPCF) and whole-plant corn silage (WPCS) from two different hybrids (H1, H2); corn kernels from three hybrids (H1, H2 and H3) harvested at three different maturities (early, mid and late maturity); dry ground corn (to pass a 1-mm sieve) and control samples (no inoculation) high-moisture corn finely or coarsely ground (ground using a large-scale woodchipper or using a tub grinder, respectively) from Saylor et al. (2020). Samples were homogenized, split into two subsamples, dried at 60°C for 48 h in an air-forced oven and forage and kernel samples were ground to pass through a 6-mm sieve of a Wiley mill. The first subsample was used for in situ incubation whereas the other subsample was not submitted to any procedure. Briefly, for in situ incubations, 5.0 g of sample was placed in Dacron polyester cloth bags (DPB; R1020, 10 cm x 20 cm, 50 µm porosity; Ankom

Technology) in duplicate and incubated for 0 and 7 h in three lactating cannulated Holstein cows fed a diet consisting of (DM basis) corn silage (38.2%), alfalfa hay (4.0%), dry ground shelled corn (27.3%), soybean meal (14.5%), citrus pulp (9.1%), and minerals and supplements (6.8%). After incubation, the bags were immersed in icy water and washed in tap water until rinse water was clear and washed in a washing machine using the rinse and spin cycle set with room temperature water for 30 m (Roper RTW4516F*, Whirlpool Corp., Benton Harbor, MI). Three independent runs were conducted. The starch and WSC concentrations of original samples and incubation residues were analyzed according to the method of Hall (2015) and by the anthrone reaction test (Ministry of Agricultural, Fisheries, and Food, 1986), respectively.

Incubation residues for each starch source were composited and along with original samples, were submitted to the Universita Cattolica del Sacro Cuore (Piacenza, Italy) for a gas production assay performed as described by Menke and Staingass (1988). Briefly, 220 mg of each sample were weighed and placed into graduated 100-mL glass syringes, 30 mL of rumen fluid solution (buffer to rumen fluid ratio of 2:1, vol/vol) was added. Rumen fluid was collected from 2 cannulated lactating dairy cows fed a diet consisting of (DM basis) corn silage (31.2%), dehydrated alfalfa hay (16.7%), grass hay (4.1%) and concentrate (48.0%). Pressure produced in the glass syringes was measured in psi at 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h and converted to volume of gas by gram of organic matter incubated. Data were fitted in a one-pool exponential model to obtain final gas volume, rate of degradation (kd) and lag time. Ruminal kinetics data from WPCF and WPCS were analyzed separately from corn kernels, ground corn and high-moisture corn samples. For both data sets, final gas

volume, rate of degradation (kd) and lag time averages were compared using a simple t-test on SAS (SAS 9.4; SAS Institute Inc., Cary, NC, USA).

Experiment 4 - dry matter recovery of 0 h incubations in water at different temperatures

Triplicate samples of two different starch sources were used in this experiment, pure corn starch (Thermo Fisher Scientific, Waltham, MA) and dry ground corn. Samples were dried at 60°C for 48 h in an air-forced oven. Samples were placed in Dacron polyester cloth bags (DPB; R1020, 10 cm x 20 cm, 50 µm porosity; Ankom Technology), F57 bags (F57; 25 µm porosity; Ankom Technology) or filter papers (WG3; 6 µm porosity; Whatman G3) and incubated in water at either 23°C or 39°C. Approximately 5.0, 0.5 and 1.0 g of DM were used for DPB, F57 and WG3, respectively. The WG3 samples were incubated in Erlenmeyer flasks before filtration. After the washout, all samples were dried in an air-forced oven set at 60°C for 48 h and DM recovery was calculated. Two independent runs were conducted.

Dry-sieving procedures were performed for characterization of particle-size distribution using a Tyler Ro-Tap Shaker (model RX-812; W.S. Tyler, Mentor, OH, USA) with a set of eight sieves (W.S. Tyler) with nominal square apertures of 4750, 2380, 1180, 590, 300, 150, and 63 µm and pan (ASABE, 2007). Geometric mean particle size (µm) and surface area (cm^2/g) were calculated using a log-normal distribution (Baker and Herrman, 2002).

To study ruminal kinetics parameters of samples incubated in water, the same procedures were used with duplicate samples of pure corn starch and dry ground corn. Samples were placed in Dacron polyester cloth bags (DPB; R1020, 10 cm x 20 cm, 50 µm porosity; Ankom Technology) and F57 bags (F57; 25 µm porosity; Ankom Technology) and incubated in water at 39°C for 0 h. Approximately 5 and 1 g of DM was used for DPB and

F57, respectively. Incubation residues were dried in an air-forced oven set at 60°C for 48 h and submitted to a gas production assay as described by Goering and Van Soest (1970) using an ANKOM RF gas production system (Ankom Technologies; Macedon, NY, USA) along with the original samples. Briefly, 730 mg of each sample were weighed and placed into 200-mL glass bottles containing 100 mL of rumen fluid solution (buffer to rumen fluid ratio of 2:1, vol/vol). Rumen fluid was collected from 2 cannulated lactating dairy cows fed a diet consisting of (DM basis) corn silage (27.4%), alfalfa silage (31.6%), whole cottonseed (5.7%) and concentrate (35.3%). Pressure was measured in psi every 15 minutes during a 24 h period and converted to volume of gas by g of organic matter incubated. Two independent runs were conducted. Data were fitted in a one-pool exponential model to obtain ruminal kinetics parameters (final gas volume, kd, and lag time).

Dry matter recovery data were analyzed as a completely randomized design in a 2 x 3 x 2 factorial arrangement of the treatments using generalized linear mixed model procedures (PROC GLIMMIX, SAS 9.4; SAS Institute Inc., Cary, NC, USA) with a model including fixed effects of starch source, filter type, incubation temperature, and their two- and three-way interactions. Run was considered a random effect. Means were determined using the least square means statement. Statistical significance was declared at $P \leq 0.05$.

The ruminal kinetics parameters data were analyzed as a completely randomized design in a 2 x 3 factorial arrangement of the treatments using generalized linear mixed model procedures (PROC GLIMMIX, SAS 9.4; SAS Institute Inc., Cary, NC, USA) with a model including fixed effects of starch source, filter type and their interaction. Run was considered a random effect. Means were determined using the least square means statement. Statistical significance was declared at $P \leq 0.05$.

Experiment 5 - dry matter and starch recovery of 0 h incubations in rumen fluid

Duplicate samples of the same two starch sources from Experiment 4 were used in this experiment, pure corn starch and dry ground corn. Samples were placed in Dacron polyester cloth bags (DPB; R1020, 10 cm x 20 cm, 50 μm porosity; Ankom Technology) and F57 bags (F57; 25 μm porosity; Ankom Technology). Approximately 5 and 1 g of DM was used for DPB and F57, respectively. Rumen fluid from the two cannulated lactating Holstein cows was collected and filtered. Animals were fed a diet consisting of (DM basis) corn silage (27.4%), alfalfa silage (31.6%), whole cottonseed (5.7%) and concentrate (35.3%). Samples were incubated in separated vials containing rumen fluid for 0 h. After washout, sample bags were placed in icy water to stop any residual fermentation and rinsed in distilled water until rinse water was clear. Four independent runs were conducted. Residues samples were dried in an air-forced oven set at 60°C for 48 h and DM recovery was calculated. Original samples and incubation residues were sent to a commercial laboratory (Dairyland Laboratories Inc., Arcadia, WI, United States) for analysis of starch concentration according to a modified method by Hall et al. (2015) to calculate starch recovery.

Data were analyzed as a completely randomized design in a 2 x 2 factorial arrangement of the treatments using generalized linear mixed model procedures (PROC GLIMMIX, SAS 9.4; SAS Institute Inc., Cary, NC, USA) with a model including fixed effects of starch source, filter type and their interaction. Run was considered a random effect. Means were determined using the least square means statement. Statistical significance was declared at $P \leq 0.05$.

RESULTS

Experiment 1: variability of starch and WSC concentrations and starch disappearance

Inter-procedure standard deviations (SD) and coefficients of variation (CV) across laboratories and submissions for WSC concentration, starch concentration and starch digestibility conducted either by wet chemistry or NIRS are presented in Table 2. Standard deviation is a measure of dispersion of the data in relation to mean, thus, low standard deviation indicates that the data is precisely around the mean. The coefficient of variation is the ratio between the SD and the mean, showing the variability of the data relative to the mean. Starch and WSC concentrations assays were relatively precise for all starch sources, however, starch digestibility data were more variable. The 7 and 16 h assays presented lower SDs, being the 7 h assay less variable for MDC (8.1 SD and 26.9% CV), while the 16 h assay was less variable for corn forage (H1, 10.3 SD and 11.9% CV; H2, 8.0 and 9.3% CV) and silage (H1, 4.3 SD and 4.7% CV; H2, 5.4 and 6.1% CV). It is important to highlight that NIRS predictions for starch digestibility at 0 and 7 h were less variable than for wet chemistry procedures, which may be indicative of the high variation related to rumen fluid fluctuations. However, these curves are based on wet chemistry assays and results should be considered with caution.

Intra-procedure standard deviations (SD) and coefficients of variation (CV) within laboratories for WSC concentration, starch concentration and starch digestibility conducted either by wet chemistry or NIRS are presented in Table 3. The intra-procedure CV of the 7 and 16 h starch digestibility assays indicate less variability for these incubation time-points when compared to 0 and 3 h incubation time. Intra-procedure CVs of samples incubated for 16 h had lower minimum CV than the 7 h assay. However, larger maximum CV was observed for 16 h compared to 7 h incubation time-point.

The effect of incubation time-points and sample submission on starch digestibility means and standard deviations are in Table 4. No interaction between factors were observed either for means, SD and CV ($P > 0.05$). As expected, longer incubation time-points yielded greater starch digestibility means for all starch sources (MDC, $P = 0.001$; H1 CF, $P = 0.001$; H1 CS, $P = 0.001$; H2 CF, $P = 0.001$; H2 CS, $P = 0.001$). There was an effect of submission on starch digestibility means of silage; H1 CS had an increase of 7.4% in starch digestibility means ($P = 0.01$, average across all time-points) from submission 1 to submission 3, while H2 CS had 7.1% increase in starch digestibility means ($P = 0.01$, average across all time-points) from submission 1 and 2 to submission 3. No effect of incubation time-point on SD of replicates was observed for MDC and H1 or H2 CF ($P > 0.05$). Conversely, there was an incubation time-point effect on SD of CS on both hybrids (H1, $P = 0.03$; H2, $P = 0.02$), SD decreased with longer incubation time-points, going from 5.1% for 0 h to 2.5% for 3, 7 and 16 h on average for H1 and from 6.0% for 0 h to 3.0% for 3, 7 and 16 h on average for H2.

The effect of incubation time-points on ranking of starch sources based on starch digestibility is in Figure 1. When starch digestibility was evaluated at 0 h, corn silage samples ($P = 0.01$; 17.5% of starch, on average) were greater than MDC (9.8% of starch), but corn forage samples did not differ from other treatments. However, when evaluated at 3 h, starch digestibility of H1 and H2 CS ($P = 0.001$; 39.1% of starch on average) were greatest, H1 and H2 CF (30.1% of starch) intermediate, and MDC (15.7% of starch) lowest. Similarly, starch digestibility at 7 h was greatest for H1 and H2 CS ($P = 0.001$; 66.9% of starch on average), intermediate for H1 and H2 CF (56.6% of starch on average), and lowest for MDC (30.3% of starch). At 16 h, starch digestibility was lower for MDC ($P = 0.001$; 65.2% of starch) than other treatments (88.4% of starch on average).

Experiment 2: grinding size and incubation time-point effect on starch digestibility

Standard deviations (SD) and coefficients of variation (CV) of in vitro starch disappearance procedures (grinding size and incubation time-points) in WPCS, WPCF, DGC and HMC are presented in Table 5. Across all starch sources, samples ground at 1 mm had lower variation in starch digestibility (4.3 SD and 11.7% CV on average) compared with 4 mm (4.6 SD and 25.8% CV on average), regardless of incubation time-point. But overall, starch digestibility at 7 h with samples ground at 1 mm were the least variable (3.1 SD and 4.5% CV on average).

The effect of grinding size and incubation time-point on in vitro starch digestibility means and standard deviations of different starch sources is in Table 6. There was an interaction between grinding size and incubation time-point for WPCF (P = 0.001), DGC (P = 0.001) and HMC (P = 0.001) means. Decreasing particle size and allowing more time for degradation to occur not only decreased variation but also increased the mean of starch digestibility. The interactions observed are due to the differences in the magnitude of the increase in starch digestibility between 4 and 7 h for samples ground at 4- or 1-mm. Samples of WPCF ground at 4-mm had an increase of 27.0%-units of ivSD mean between 4 and 7 h, while for samples ground at 1-mm the increase was of 37.5%-units of ivSD. Samples of DGC ground at 4-mm had an increase of 27.2%-units of ivSD mean between 4 and 7 h, while for samples ground at 1-mm the increase was of 42.9%-units of ivSD. Samples of HMC ground at 4-mm had an increase of 30.8%-units of ivSD mean between 4 and 7 h, while for samples ground at 1-mm the increase was of 45.7%-units of ivSD This magnitude of increase was not as expressive for WPCS and therefore, no interaction was observed (P = 0.09), samples ground at 4-mm had a 21.8%-units of ivSD increase between 4 and 7 h, while for samples

ground at 1-mm the increase was of 26.3%-units. There was an effect of grinding size on ivSD standard deviation of WPCF (3.0 vs. 1.0% of starch; *P* = 0.01), while no effects were detected for all other feed ingredients standard deviations. When ranking the different starch sources samples according to each in vitro procedure (combination between grinding size and incubation time-point; Figure 2), means were all separated from each other only when 4-mm ground samples were incubated for 7 h (*P* = 0.001; 32.2% DGC, 39.2% WPCF, 43.5% HMC and 57.8% WPCS ivSD). Incubating 4-mm ground samples for 4 h ranked DGC (5.0% ivSD) lowest, WPCF and HMC (12.5% ivSD on average) intermediate, and HMC (36.0% ivSD) greatest. The 1-mm grinding size at 4 h of incubation procedure ranked WPCS (42.6% ivSD) greater than other starch sources (26.13% ivSD). Lastly, the 1-mm 7 h procedure had ivSD values of WPCF (61.2% of starch) lowest, WPCS and HMC (68.6% of starch on average) intermediate, and HMC (75.0% of starch) highest.

Experiment 3: ruminal kinetics of 0 h incubation residues

Starch, WSC concentrations and starch disappearance at 7 h of intact samples and their respective 0 h residues (when appropriate) averages and standard deviations (SD) of different starch sources are presented in Table 7. There was degradation or loss of WSC and starch across all feed samples tested. The only exception was for H3 corn kernels, as starch concentration was greater after 0 h incubation. Generally, samples with greater losses of starch at 0 h had greater starch disappearance at 7 h.

The effect of in situ 0 h incubation on in vitro gas production and ruminal kinetics parameters of corn silage and corn forage is presented in Table 8. Final gas volume was not different between the intact and 0 h residue samples (210.6 ml/g of OM on average; *P* = 0.77). However, 0 h residues had lower kd (0.038 vs. 0.049 h^{-1}; *P* = 0.001) and greater lag

(1.90 vs. 0.58 h; $P = 0.001$). The effect of in situ 0 h incubation on in vitro gas production and ruminal kinetics parameters of corn grain is presented in Table 9. Final gas volume was not different between the intact and 0 h residue samples (266.9 ml/g of OM on average; $P = 0.64$). However, 0 h residues had lower kd (0.043 vs. 0.049 h^{-1}; $P = 0.001$) and greater lag (2.60 vs. 1.83 h; $P = 0.001$) compared with intact samples.

Experiment 4: dry matter recovery of 0 h incubations in water at different temperatures

The main effect of washout temperature on DM recovery is shown in figure 3A. There was lower DM recovery for samples washed in water at 39ºC (84.90 vs. 86.63% of as fed; $P = 0.02$), which suggests that losses of particles are greater when incubating samples in rumen fluid-like temperature. The interaction effect between starch source and filter type on DM recovery is shown in Figure 3B. Pure starch had the highest DM recovery for F57 and WG3 (92.14% of as fed on average), followed by dry ground corn at any filter type (85.30% of as fed on average) and lastly pure starch for DPB (74.38% of as fed; $P = 0.001$). Despite the smaller particle on pure starch (Table 10), F57 and WG3 pore size was able to hold particles better than DPB. However, DG particle loss is similar between all filter types.

The effect of in situ 0 h washout on in vitro gas production and ruminal kinetics parameters of pure starch and dry ground corn is presented in Table 11. No effects of incubation in water were detected for final gas volume ($P = 0.57$) or kd ($P = 0.91$). However, pure starch had greater lag than dry ground corn (2.55 vs. 2.03 h^{-1}; $P = 0.01$).

Experiment 5: dry matter and starch recovery of 0 h incubations in rumen fluid

The effect of starch source and filter type and F57, 25 µm on DM and starch recovery is in Figure 4. There was an interaction for DM recovery Figure 4A; $P = 0.001$), where pure

starch incubated in DPB bags had the lowest DM recovery (43.3% of as fed), followed by dry ground corn incubated in either in DPB or F57 (85.2% of as fed on average), while the greatest DM recovery was observed for pure starch incubated in F57 bags (93.3% of as fed). However, dry ground corn and pure starch DM recovery was not different when incubated in F57 (91.3% of as fed on average). A similar interaction was observed for starch recovery (Figure 4B; $P = 0.001$), where pure starch incubated in DPB had the lowest recovery (59.1% of incubated starch), followed by dry ground corn in either DPB or F57 (93.6% of incubated starch), while pure starch incubated in F57 bags had no loss of starch in rumen fluid at 0 h (100.0% of incubated starch). The starch recovery of dry ground corn and pure starch did not differ when incubated in F57 (98.9% of incubated starch on average).

DISCUSSION

Overall, results of the present study suggest that starch digestibility assays have high variation and there is a need for better standardization and refinement of the procedures to properly use the information generated by these assays. For example, incubation time-points, grinding size and bag pore size used in in vitro and in situ incubations. However, if considering the rumen inoculum intrinsic variation, it is unlikely that all aspects of the procedures could be standardized. Commercial and research laboratories have demonstrated good ability to rank samples by 30 h ruminal in vitro NDF digestibility values (Hall and Mertens, 2012). However, starch is a more rapidly degradable carbohydrate, and the shorter incubation time-points used in the starch digestibility assay increase the variation to a greater extent than the NDF digestibility assays. Strategies to reduce rumen inoculum variation, such as feeding rumen fluid donors a standardized diet, collection of inoculums at a fixed time of the day, pooling rumen fluid from multiple animals (Rymer et al., 2005), and a priming

technique (Goeser and Combs, 2009) have been proposed previously. However, the use of these rumen inoculum standardization techniques by commercial and research laboratories depends upon routine and workflow. Moreover, Hall and Mertens (2008) evaluated sample processing procedures on starch concentration analytical results and reported that dry rolled corn and high-moisture corn samples ground using different mill types (abrasion vs. cutting) had different starch concentration. Because starch concentration analysis influences the results of starch digestibility assays, the grinding procedure can be considered as a source of variation across research and commercial laboratories. Due to the conditions of experiment 1, variability was expected for digestibility assays, as samples were purposely sent in a manner that required different digestibility runs from the commercial laboratories. Runs conducted in different months will naturally vary due to the rumen inoculum daily variation (Uden and Van Soest, 1984). However, good repeatability across different sample submissions and procedures were observed for MDC, H1 and H2 CF samples. There was a submission effect on starch digestibility means of H1 CS and H2 CS, which indicates that corn silage samples may vary more across submissions than corn grain or unfermented corn forage samples. Samples were frozen prior to submission to laboratories; thus, samples should have had similar means across submissions. Possibly, fermented samples are more prone to the effects of intrinsic rumen inoculum variation. However, research is warranted to better understand this effect. Fermented corn samples have greater starch digestibility than unfermented corn due to the solubilization of the endosperm protein matrix (Hoffman et al., 2011). Therefore, longer incubation time-points allowed for a more extensive starch degradation in all samples, decreasing variability, which is a plausible explanation of the incubation time-point effect on SD of H1 CS and H2 CS samples, but no effects on SD of

CF and MDC samples. According to Michalet-Doreau and Ould-Bah (1992), the appropriate time-points to properly describe disappearance rate depends on the shape of the degradation curve of each nutrient, and thus, single time-point incubations are mostly used to rank feeds or evaluate management practices based on starch digestibility. Sniffen and Ward (2011) stated that a 7 h in vitro starch digestibility assay yields similar results than mechanistic models for ruminal starch effective degradability and developed a natural logarithm equation using starch disappearance at 7 h that predicts starch rate of digestion (Sniffen and Ward, 2011). To our knowledge, this is the most used time-point by research and commercial laboratories. Recently, shorter incubation time-points have been suggested to improve starch digestibility assays or sample ranking (Fernandes et al., 2018; Cueva et al., 2023). However, our study suggests that there is no improvement of shorter time-points in sample ranking compared to the 7 h time-point. Possibly, different samples require specific comparisons. For example, Cueva et al. (2023) ranked corn silage hybrids whereas our study ranked starch sources. These factors combined suggest that unfermented samples for starch digestibility analysis are comparable if they are submitted to the same laboratory over time and analyzed using the same grinding size and incubation time-point.

Experiment 2 showed that decreasing particle size or incubating samples for 7 h not only decreased the coefficients of variation but also increased the mean values of starch digestibility. Yet, grinding size only affected standard deviations of the replicates on WPCF. However, means of all starch sources were fully distinguished only when using the 4 mm grinding size and 7 h incubation procedure. In vitro and in situ assays attempt to mimic actual nutrient digestion. Finer grinding sizes increase surface area of the sample to microbial access, increasing the uniformity of the sample particles, thereby decreasing variability of

the assay. Michalet-Doreau and Cerneau (1991) found that grinding size influences particle size and nitrogen disappearance differently depending on the feed. Moreover, Diepersloot et al. (2023), found a quadratic relationship between corn grain mean particle size and degradation rate or in situ starch disappearance at 7 h, which decreased as particle size increased before reaching a plateau at approximately 750 µm. Uden and Van Soest (1984) recommended that due to the lack of rumination, samples should be processed to adequately represent particle size characteristics observed in vivo. Therefore, it is impractical to state that the in vitro procedure with lower variation would always be the best alternative for the evaluation of all feed samples. Recently, Cueva et al. (2023) observed that grinding samples at 1 mm and incubating in vitro for 7 h was capable of differentiating low starch digestibility hybrids, but not separating medium and high digestibility hybrids. Our results show that finer grinding size increased starch digestibility across all starch sources and affected sample ranking. Samples ground at 1-mm and incubated for 7 h had the inverse ranking of HMC and WPCS compared with the other in vitro procedures. Moreover, finely ground samples can increase the risk of secondary particulate losses (Uden and Van Soest, 1984; Huhtanen and Sveinbjornsson, 2006). Thus, caution should be taken when starch digestibility assays are conducted with the combination of smaller grinding size and longer incubation time-points as the magnitude of an effect may be masked. Moreover, in vitro methods require sample processing which may affect starch digestibility and consequently sample ranking. Future studies should consider the evaluation of different indicators related to starch digestibility on sample ranking.

Experiment 3 demonstrated that the disappearance observed in in situ 0 h incubations changed ruminal kinetics in corn silage and grain samples. In situ 0 h incubation affected the

lag time and degradation rate in vitro. Lag time was greater for corn grain incubation residues than the intact samples. However, no differences in gas production rate or total gas volume were found between the intact sample and the washout residue (Schlau et al., 2020). Bags commonly used in in situ assays have a greater pore size (50 μm) than most starch granules (2 – 30 μm; Tester et al., 2004), and if starch granules are released from the endosperm protein matrix, these fine particles could leave the bag without degradation, increasing the risk of secondary particulate loss of starch (Huhtanen and Sveinbjornsson, 2006) explaining the differences found in the present experiment. Seifried et al. (2015) reported that starch particles that left bags with different pore sizes after 8 h of ruminal incubation were not detected in rumen fluid, suggesting that the particles leaving the bags were degraded throughout the incubation. Corn grain particles leaving the bags after a 0 h incubation had shorter lag time and greater gas production per g of DM than intact samples (Schlau et al., 2020), suggesting washout fractions may not represent the intact sample. Yet, little is known about the actual degradation of particles disappearing at 0 h. Our results suggest that secondary particulate loss at 0 h in situ incubation limits the relationship between disappearance and degradation of particles.

Washout fractions of starch are usually used in mechanistic models as fraction A. However, secondary particulate losses can increase with bag porosity (Uden and Van Soest, 1984; Lindberg and Knutsson, 1981). Thus, we conducted two experiments to understand the effects of bags pore size, washout temperature and starch source on recovery of DM and starch after 0 h incubations. Experiment 4 showed that DM recovery was greater when samples were incubated in water at room than water at rumen-like temperature (39º C). Conversely, no effect of washing method, including inserting samples in the rumen for 0 h,

were reported for the estimation of fraction A of starch in dry ground corn samples (Fernandes et al., 2018). Total gas volume and kd of samples and 0 h residues of incubations conducted using DPB or F57 were not different. But lag time of pure starch was greater than dry ground corn, likely due to the presence of more readily available carbohydrates and nitrogen for bacteria attached in dry ground corn. The 0 h incubation losses did not translate into more intensive or faster fermentation, which indicated little to no secondary particulate losses when compared to results observed in experiment 3. Perhaps the 0 h incubation in situ generates greater secondary particulate losses than washing samples in tap water. As discussed previously, Uden and Van Soest (1984) reported that bags with larger pore sizes can increase cell wall carbohydrates digestibility, the authors highlighted that finely ground samples (< 2-mm) in combination with larger bag pore size can increase secondary particulate losses. Seifried et al. (2015) reported that this is applicable for starch as well. However, in experiment 5, we incubated pure starch in rumen fluid in vitro and despite the very fine particles (Table 10), when the incubation was conducted using F57 bags the recovery of starch was 100%. These data support the premise for pure starch samples, but not for dry ground corn samples, as starch recovery of dry ground corn was not different between F57 (25 µm) and DPB (50 µm) bags. Conversely, Siefried et al. (2015) reported a 7.7%-units increase in fraction A of starch when washing samples of corn grain in tap water using a 50 µm pore size bag compared to a 20 µm pore size bag. Our results reinforce the need for standardization of the combination of bags pore size, grinding size and washout procedure used in in vitro assays whereas grinding size would likely be specific to sample type and assay. Moreover, research is warranted to understand if secondary particulate loss of starch impairs the calculation of fraction A of starch using bags of smaller pore sizes.

CONCLUSION

Large variation was found across laboratorial procedures in starch digestibility assays, while the variation within a procedure was acceptable. The differences in analytical values and variation among laboratories are likely related to the difference in procedures used and therefore, comparisons between samples should be used in appropriate context of each procedure. Different grinding sizes and incubation time-points may change sample ranking, which supports the premise that samples are only comparable if evaluated by the same procedure. The 0 h incubations had the largest variation observed in this study and generated more secondary particulate loss than washing samples using tap water. While many improvements were made on the standardization of this assay over the years, further efforts are warranted to improve reproducibility of starch digestibility assays, improve the precision of the method, and enhance the understanding of starch washout fractions.

REFERENCES

ASABE. 2007. Method of determining and expressing fineness of feed materials by sieving. ANSI/ASAE S319.4:646–649. Am. Soc. Ag. Biol. Eng., St. Joseph, MI.

Baker, S., and T. Herrman. 2002. Evaluating particle size. MF-2051. Kansas State Univ., Manhattan.

Cueva, S. F., M. Harper, G. W. Roth, H. Wells, C. Canale, A. Gallo, F. Masoero, and A. N. Hristov. 2023. Effects of ensiling time on corn silage starch ruminal degradability evaluated in situ or in vitro. J. Dairy Sci. 106: 3961-3974. https://doi.org/10.3168/jds.2022-22817.

Diepersloot, E. C., J. P. Goeser, E. Coons, E. Lynch, J. Karlen, and L. F. Ferraretto. 2023. Relationship between particle size and in situ starch disappearance of corn grain. J. Dairy Sci. 106 (Suppl. 1): (Abstr.). Page 378.

Fernandes, T., C. L. S. Avila, M. N. Pereira, and L. F. Ferraretto. 2018. Short communication: effect of washing method, grinding size and the determination of an indigestible fraction on in situ degradation of starch in mature corn grain. J. Dairy Sci. 101:9052-9057. https://doi.org/10.3168/jds.2018-14870.

Ferreira, G., and D. R. Mertens. 2005. Chemical and physical characteristics of corn silages and their effects on in vitro disappearance. J. Dairy Sci. 88:4414-4425. https://doi.org/10.3168/jds.S0022-0302(05)73128-3.

Fox, D.G., L.O. Tedeschi, T.P. Tylutki, J.B. Russell, M.E. Van Amburgh, L.E. Chase, A.N. Pell, T.R. Overton. 2004. The Cornell Net Carbohydrate and Protein System model for evaluating herd nutrition and nutrient excretion. Anim. Feed Sci. and Technol.112: 29-78. https://doi.org/10.1016/j.anifeedsci.2003.10.006.

Goering, H. K., and P. J. Van Soest. 1970. Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Applications). Agric. Handbook No. 379. ARS-USDA, Washington, DC.

Goeser, J. P., and D. K. Combs. 2009. An alternative method to assess 24-h ruminal in vitro neutral detergent fiber digestibility. J. Dairy Sci. 92:3833-3841. https://doi.org/10.3168/jds.2008-1136.

Hall, M. B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. J. AOAC Int. 98:397-409. https://doi.org/10.5740/jaoacint.15-012.

Hall, M. B. and R. Mertens. 2012. A ring test of in vitro neutral detergent fiber digestibility: Analytical variability and sample ranking. J. Dairy Sci. 95:1992-2003. https://dx.doi.org/10.3168/jds.2011-4802.

Hall, M. B. and R. Mertens. 2017. A 100-year review: carbohydrates – characterization, digestion, and utilization. J. Dairy Sci. 100:10078-10093. https://doi.org/10.3168/jds.2017-13311.

Hoffman, P. C., N. M. Esser, R. D. Shaver, W. K. Coblentz, M. P. Scott, and A. L. Bodnar. 2011. Influence of ensiling time and inoculation on alteration of the starch-protein matrix in high-moisture corn. J. Dairy Sci. 94:2465–2474. https://doi.org/10.3168/jds.2010-3562.

Huhtanen, P., and J. Sveinbjörnsson. 2006. Evaluation of methods for estimating starch digestibility and digestion kinetics in ruminants. Anim. Feed Sci.Technol. 130:95–113. https://doi.org/10.1016/j.anifeedsci.2006.01.021.

Lindberg, J. E. and Knutsson. P. G. 1981. Effect of bag pore size on the loss of particulate matter and on the degradation of cell wall fibre. Agric. Environ. 6: 171-182. https://doi.org/10.1016/0304-1131(81)90009-6.

Menke, K. H. and Steingass, H. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev. 28, 7–55.

Michalet-Doreau, B., and M. Y. Ould-Bah. 1992. In vitro and in sacco methods for the estimation of dietary nitrogen degradability in the rumen: a review. Anim. Feed Sci.Technol. 40:57-86. https://doi.org/10.1016/0377-8401(92)90112-J.

Michalet-Doreau, B., and P. Cerneau. 1991. Influence of foodstuff particle size on in situ degradation of nitrogen in the rumen. Anim. Feed Sci. Technol. 35:69-81. https://doi.org/10.1016/0377-8401(91)90100-7.

Nocek, J. E., and R. A. Kohn. 1988. In situ particle size reduction of alfalfa and timothy hay as influenced by form and particle size. J. Dairy Sci. 71:932-945. https://doi.org/10.3168/jds.S0022-0302(88)79639-3.

Philippeau, C., and B. Michalet-Doreau. 1997. Influence of genotype and stage of maturity of maize on rate of ruminal starch degradation. Anim. Feed Sci. Technol. 68:25–35. https://doi.org/10.1016/S0377-8401(97)00042-4.

Richards, C. J., J. F. Pedersen, R. A. Britton, R. A. Stock, and C. R. Krehbiel. 1995. In vitro starch disappearance procedure modifications. Anim. Feed Sci. Technol. 55:35–45. https://doi.org/10.1016/0377-8401(95)00790-T.

Rymer, C., J. A. Huntington, B. A. Williams, and D. I. Givens. 2005. In vitro cumulative gas production techniques: History, methodological considerations and challenges. Anim. Feed Sci. Technol. 123:9–30. https://doi.org/10.1016/j.anifeedsci.2005.04.055.

Schlau, N., D. R. Mertens, L. F. Ferraretto, and D. Taysom. 2020. Gas production kinetics of in situ washout and remaining fractions. J. Dairy Sci. 103 (Suppl. 1): (Abstr.). Page 149.

Seifried, N., H. Steinga, M. Rodehutscord. 2015. In vitro and in situ evaluation of secondary starch particlelosses from nylon bags during the incubation of different cereal grains. Anim. Feed Sci. Technol. 210:26-36. http://dx.doi.org/10.1016/j.anifeedsci.2015.09.011.

Sniffen, C. J., and R. Ward. 2011. Using starch digestibility information in ration balancing. WCDS Adv. Dairy Technol. 23:121–133.

Tester, R. F., Karkalas, J., X. Qi. 2004. Starch – Composition, fine structure and architecture. Journal of Cereal Sci. 39:151-165. https://doi.org/10.1016/j.jcs.2003.12.001.

Uden, P., and P. J. Van Soest. 1984. Investigations of the in situ bag technique and a comparison of the fermentation in heifers, sheep, ponies and rabbits. J. Anim. Sci. 58:213-221. https://doi.org/10.2527/jas1984.581213x.

Van Amburgh, M. E., E. A. Collao-Saenz, R. J. Higgs, D. A. Ross, E. B. Recktenwald, E. Raffrenato, L. E. Chase, T. R. Overton, J. K. Mills, and A. Foskolos. 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. J. Dairy Sci. 98:6361–6380. https://doi.org/10.3168/jds.2015-9378.

Vanzant, E. S., R. C. Cochran, and E. C. Titgemeyer. 1998. Standardization of in situ techniques for ruminant feedstuff evaluation. J. Anim. Sci. 76:2717-2729. https://doi.org/10.2527/1998.76102717x.

TABLES

Table 3.1. Nutrient composition (average ± SD) of the different starch sources submitted three times to four commercial laboratories (experiment 1)[1].

Item[2]	MDC	H1 CF	H1 CS	H2 CF	H2 CS
DM, % of as-fed	88.3 ± 1.15	33.3 ± 1.13	35.8 ± 1.34	36.4 ± 1.9	34.6 ± 1.36
CP, % of DM	9.7 ± 0.41	6.7 ± 0.60	6.8 ± 0.52	7.2 ± 0.55	7.0 ± 1.20
aNDF, % of DM	9.5 ± 1.71	-	-	-	-
aNDFom[3], % of DM	-	37.3 ± 3.0	35.5 ± 3.14	35.5 ± 3.8	36.1 ± 3.63
EE, % of DM	5.0 ± 0.30	2.4 ± 0.32	3.2 ± 0.42	3.0 ± 0.32	3.3 ± 0.46
Ash, % of DM	1.6 ± 0.15	3.2 ± 0.40	3.2 ± 0.64	3.2 ± 0.44	3.5 ± 0.85

[1]Treatments were mature dent corn (MDC); hybrid 1 corn forage (H1 CF); hybrid 1 corn silage (H1 CS); hybrid 2 corn forage (H2 CF), and hybrid 2 corn silage (H2 CS).

[2]Near-infrared spectroscopy nutrient composition predictions were average across 4 commercial laboratories and 3 submissions for each sample type.

[3]aNDFom was only reported for forage samples.

Table 3.2. Standard deviations (SD) and coefficients of variation (CV) of different starch sources submitted three times to commercial laboratories for analysis of water-soluble carbohydrates (WSC) and starch concentrations and ruminal in vitro or in situ starch digestibility (StarchD) incubated for 0, 3, 7, 16 h (experiment 1)[1]

	MDC		H1 CF		H1 CS		H2 CF		H2 CS	
Item	SD	CV (%)	SD	CV (%)	SD	CV (%)	SD	CV (%)	SD	CV (%)
Wet Chemistry[2]										
WSC, % DM	0.7	20.6	5.1	53.2	0.7	36.2	5.1	51.5	1.7	72.4
Starch % DM	3.3	4.8	5.7	15.7	4.5	10.9	4.4	11.3	5.1	13.0
StarchD, 0 h	10.1	108.1	14.6	98.9	20.8	99.8	17.0	113.6	18.2	107.4
StarchD, 3 h	11.4	74.4	16.3	54.4	20.3	49.9	19.3	66.0	22.3	60.7
StarchD, 7 h	8.1	26.9	8.5	15.0	11.2	16.3	11.3	20.2	10.3	16.2
StarchD, 16 h	21.3	33.0	10.3	11.9	4.3	4.7	8.0	9.3	5.4	6.1
NIRS[3]										
WSC, % DM	0.5	9.0	1.4	18.0	1.3	50.1	1.3	15.8	1.3	51.0
Starch, % DM	2.6	3.8	3.2	8.7	4.5	11.3	4.0	10.7	6.0	15.7
StarchD, 0 h	7.5	31.4	6.9	31.3	6.6	25.4	4.6	15.3	4.5	18.2
StarchD, 7 h	11.7	20.4	6.3	9.8	3.9	5.2	5.5	8.7	5.1	6.9

[1]Treatments were mature dent corn (MDC); hybrid 1 corn forage (H1 CF); hybrid 1 corn silage (H1 CS); hybrid 2 corn forage (H2 CF), and hybrid 2 corn silage (H2 CS).
[2]Four laboratories performed WSC and starch concentrations. Three laboratories performed starch digestibility at 0, 7, 3 and 16 h assays via wet chemistry.
[3]Three laboratories performed starch concentration via near-infrared spectroscopy. Two laboratories performed starch digestibility at 7 h via near-infrared spectroscopy predictions and one laboratory performed WSC concentration and starch digestibility at 0 h via near-infrared spectroscopy predictions.

Table 3.3. Minimums and maximums intra-procedure coefficients of variation (%) of different starch sources submitted three times to commercial laboratories for analysis of water-soluble carbohydrates (WSC) and starch concentrations and ruminal in vitro or in situ starch digestibility (StarchD) incubated for 0, 3, 7, 16 h (experiment 1)[1].

	MDC		H1 CF		H1 CS		H2 CF		H2 CS	
Item	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Wet Chemistry[2]										
WSC, % DM	12.7	27.2	9.0	76.9	22.0	34.1	9.3	64.7	20.8	71.2
Starch % DM	1.2	4.1	6.3	29.5	6.2	12.3	5.2	13.7	8.1	15.3
StarchD, 0 h	15.4	198.3	32.4	140.7	28.5	188.9	25.7	226.3	37.5	164.1
StarchD, 3 h	14.6	150.7	13.2	69.3	7.9	52.2	13.0	134.0	16.4	61.4
StarchD, 7 h	11.9	22.1	7.3	13.8	5.2	6.1	6.9	10.7	5.4	7.8
StarchD, 16 h	1.9	30.2	2.3	16.9	2.1	6.5	3.3	12.9	3.3	6.4
NIRS[3]										
WSC, % DM	-	-	11.5	13.4	7.7	46.4	8.0	15.0	8.1	47.6
Starch, % DM	0.6	3.7	2.4	10.3	1.7	13.0	7.6	14.1	7.7	15.4
StarchD, 7 h	3.4	17.0	2.6	4.2	1.2	3.3	1.7	7.0	1.7	4.8

[1]Treatments were mature dent corn (MDC); hybrid 1 corn forage (H1 CF); hybrid 1 corn silage (H1 CS); hybrid 2 corn forage (H2 CF), and hybrid 2 corn silage (H2 CS).

[2]Four laboratories performed WSC and starch concentrations. Three laboratories performed starch digestibility at 0, 7, 3 and 16 h assays via wet chemistry.

[3]Three laboratories performed starch concentration via near-infrared spectroscopy. Three laboratories performed starch digestibility at 7 h via near-infrared spectroscopy predictions and one laboratory performed WSC concentration (except for MDC) and starch digestibility at 0 h via near-infrared spectroscopy predictions.

Table 3.4. Effect of incubation time-point on in vitro or in situ starch digestibility (StarchD) means and replicates standard deviation (SD) of different starch sources similar samples submitted at three different occasions to four commercial laboratories (experiment 1)[1]

Item[2]	0 h			3 h			7 h			16 h			SEM	*P*-values		
	1st Sub	2nd Sub	3rd Sub	1st Sub	2nd Sub	3rd Sub	1st Sub	2nd Sub	3rd Sub	1st Sub	2nd Sub	3rd Sub		IT	Sub	IT x Sub
StarchD, means																
MDC	11.1	8.0	8.9	14.8	14.7	16.3	31.6	30.5	27.5	64.7	61.0	68.6	7.0	0.001	0.70	0.85
H1 CF	9.5	15.0	19.9	27.9	30.1	32.2	55.4	57.9	57.2	83.1	85.5	89.3	5.8	0.001	0.10	0.95
H1 CS	14.1	19.3	29.1	38.3	37.5	46.2	65.0	70.8	69.8	90.3	91.6	92.3	8.8	0.001	0.01	0.41
H2 CF	13.7	11.7	19.7	29.7	25.8	32.4	52.9	59.0	55.8	84.0	84.4	89.7	7.7	0.001	0.17	0.74
H2 CS	13.2	14.2	25.4	34.2	33.8	41.9	61.0	63.5	67.0	88.9	85.7	91.3	8.3	0.001	0.01	0.88
SD of replicates																
MDC	0.6	2.1	2.6	0.6	1.9	2.7	2.7	2.1	3.2	1.5	2.3	1.9	0.9	0.58	0.18	0.85
H1 CF	4.6	1.8	5.2	5.0	3.5	4.8	6.0	6.4	5.3	2.0	3.6	0.4	2.1	0.18	0.79	0.61
H1 CS	5.0	2.7	7.3	1.6	3.0	5.0	3.4	1.5	2.5	1.2	1.8	1.5	1.4	0.03	0.17	0.44
H2 CF	2.5	2.2	2.8	4.3	6.0	4.5	4.2	3.6	3.8	2.9	4.3	1.4	1.6	0.18	0.65	0.87
H2 CS	4.8	3.4	9.1	3.7	3.8	4.4	2.6	4.0	2.9	1.5	1.7	1.5	1.9	0.02	0.40	0.41

[1]Three laboratories performed starch digestibility at 0, 7, 3 and 16 h assays via wet chemistry. Treatments were mature dent corn (MDC); hybrid 1 corn forage (H1 CF); hybrid 1 corn silage (H1 CS); hybrid 2 corn forage (H2 CF), and hybrid 2 corn silage (H2 CS).

Table 3.5. In vitro starch digestibility (ivSD) standard deviations (SD) and coefficient of variation (CV) in different corn ingredients submitted to a commercial laboratory to be analyzed in different grinding sizes and incubation time-points (experiment 2)[1]

	WPCS		WPCF		DGC		HMC	
ivSD	SD	CV (%)	SD	CV (%)	SD	CV (%)	SD	CV (%)
4 h 1 mm	4.5	10.6	3.7	15.7	7.1	24.2	6.4	25.1
4 h 4 mm	3.4	9.4	6.2	50.4	2.9	22.5	3.8	74.5
7 h 1 mm	2.8	4.1	2.3	3.8	4.0	5.3	3.2	4.7
7 h 4 mm	4.4	7.7	5.3	13.6	5.2	11.9	5.4	16.7

[1]Treatments were whole-plant corn silage (WPCS), whole-plant corn forage (WPCF), dry ground corn (DGC), or high-moisture corn (HMC) ground to pass a 1- or 4-mm sieve size and incubated for 4 or 7 h.

Table 3.6. Effect of grinding size and incubation time-point on in vitro starch digestibility (ivSD) of whole-plant corn forage, whole-plant corn silage, dry ground corn and high-moisture corn (experiment 2)[1]

	4 mm		1 mm			*P*-values		
Item	4 h	7 h	4 h	7 h	SEM	GS	IT	GS x IT
ivSD means								
WPCF	12.2[d]	39.2[b]	23.7[c]	61.2[a]	1.54	0.001	0.001	0.001
WPCS	36.0	57.8	42.6	68.9	1.29	0.001	0.001	0.09
DGC	5.0[d]	32.2[b]	25.4[c]	68.3[a]	1.61	0.001	0.001	0.001
HMC	12.7[d]	43.5[b]	29.3[c]	75.0[a]	1.67	0.001	0.001	0.001
SD of replicates								
WPCF	3.7	2.2	1.0	1.0	0.65	0.01	0.21	0.20
WPCS	2.8	2.9	2.9	2.0	0.73	0.63	0.58	0.48
DGC	2.5	1.7	1.3	1.2	0.45	0.10	0.36	0.39
HMC	1.4	0.8	1.6	1.6	0.28	0.11	0.30	0.28

[a,b]Means in the same row with different superscripts differ ($P \leq 0.05$).
[1]Treatments were whole-plant corn silage (WPCS), whole-plant corn forage (WPCF), dry ground corn (DGC), or high-moisture corn (HMC) ground to pass a 1- or 4-mm sieve and incubated for 4 or 7 h.

Table 3.7. Water-soluble carbohydrates, starch concentration and starch disappearance at 7 h (StarchD) averages and standard deviations (SD) of different feed ingredients and their respective 0 h incubation residues (experiment 3)[1]

	WSC (AVG ± SD)		Starch (AVG ± SD)		StarchD (AVG ± SD)
Item	Sample	0h residue	Sample	0h residue	Sample
Whole-plant corn forage					
H1	6.6 ± 0.4	1.2 ± 0.1	36.6 ± 2.4	26.5 ± 3.1	63.1 ± 3.4
H2	8.2 ± 0.1	1.7 ± 0.4	39.0 ± 1.8	31.6 ± 6.0	70.4 ± 2.0
Whole-plant corn silage					
H1	0.7 ± 0.1	0.5 ± 0.3	34.7 ± 0.4	22.6 ± 4.9	74.3 ± 2.0
H2	0.9 ± 0.0	0.3 ± 0.1	35.1 ± 0.4	26.0 ± 5.0	78.7 ± 2.4
Corn kernels					
H1 early	4.2 ± 0.5	1.6 ± 0.2	74.0 ± 1.0	67.0 ± 3.7	54.9 ± 7.5
H1 mid	3.4 ± 0.0	1.7 ± 0.3	71.1 ± 0.5	68.2 ± 8.1	46.5 ± 9.8
H1 late	2.8 ± 0.0	1.5 ± 0.2	74.5 ± 1.0	66.5 ± 3.8	50.9 ± 4.2
H2 early	4.2 ± 0.2	2.3 ± 0.6	75.1 ± 1.5	73.6 ± 4.8	51.8 ± 7.2
H2 mid	3.4 ± 0.3	1.9 ± 0.4	75.3 ± 0.9	72.0 ± 4.6	50.7 ± 4.2
H2 late	2.7 ± 0.0	1.5 ± 0.2	75.3 ± 2.5	69.8 ± 2.7	43.9 ± 8.3
H3 early	6.4 ± 1.9	2.6 ± 0.4	62.8 ± 2.2	68.9 ± 4.6	46.6 ± 5.8
H3 mid	4.3 ± 0.0	2.3 ± 0.4	63.6 ± 2.9	72.0 ± 4.1	30.7 ± 11.9
H3 late	3.8 ± 0.0	2.2 ± 0.3	64.4 ± 1.7	68.9 ± 3.9	25.0 ± 7.1
Ground corn					
Fine	3.6 ± 0.1	2.6 ± 0.2	67.5 ± 2.5	68.3 ± 3.9	30.4 ± 29.5
High-moisture corn					
Fine	1.3 ± 0.1	0.7 ± 0.3	70.0 ± 2.2	65.1 ± 3.0	74.5 ± 6.2
Coarse	0.9 ± 0.0	0.7 ± 0.1	73.0 ± 2.8	71.1 ± 2.5	31.0 ± 18.5

[1]Samples from whole-plant corn forage (WPCF) and whole-plant corn silage (WPCS) from two different hybrids (H1, H2); corn kernels from three different hybrids harvested at three different maturities (H1, H2 and H3); ground corn; high-moisture corn finely and coarsely ground were used in this experiment.

Table 3.8. Effect of in situ 0 h incubation on final gas volume, degradation rate (kd) and lag time in samples of corn forage and corn silage (experiment 3)[1]

Item	Sample	0 h residue	SEM	*P*-value
Final gas Vol. (ml/g of OM)	211.3	209.9	3.42	0.77
kd (h^{-1})	0.049	0.038	0.0008	0.001
Lag (h^{-1})	0.58	1.90	0.10	0.001

[1]Treatments were either intact samples or 0 h in situ residues from whole-plant corn forage and whole-plant corn silage from two different hybrids.

Table 3.9. Effect of in situ 0 h incubation on final gas volume, degradation rate (kd) and lag time in different corn grain samples (experiment 3)[1]

Item	Sample	0 h residue	SEM	*P*-value
Final gas Vol. (ml/g of OM)	267.6	266.2	2.2	0.64
kd (h^{-1})	0.049	0.043	0.001	0.001
Lag (h^{-1})	1.83	2.60	0.08	0.001

[1]Treatments were either intact samples or 0 h in situ residues from corn kernels from three different hybrids harvested at three different maturities, ground corn, and high-moisture corn finely or coarsely ground.

Table 3.10. Particle size distribution, geometric mean particle size (GMPS) and surface area of pure starch and ground corn samples used in experiments 4 and 5.

Item	Pure starch	Ground corn
Sieve[1], µm		
4750	0.00	0.00
2380	0.00	0.00
1180	0.00	27.33
590	0.00	19.33
300	0.00	21.33
150	21.65	29.33
63	54.24	2.67
Pan	24.11	0.00
GMPS, µm	93.50	748.50
Surface area, cm^2/g	60.50	34.50

[1]Percentage of particles retained on each sieve (DM basis).

Table 3.11. Effect of starch source and filter type on final gas volume, degradation rate, and lag of pure starch and ground corn as original samples or 0 h incubation residues (experiment 4)[1]

	Starch			Ground Corn				*P*-values[2]		
Item	Sample	DPB	F57	Sample	DPB	F57	SEM	S	F	S x F
Gas Vol. (ml/g of OM)	168.4	154.4	166.6	164.7	173.2	192.5	18.08	0.27	0.55	0.57
Kd (h^{-1})	0.017	0.015	0.017	0.017	0.017	0.016	0.003	0.86	0.91	0.91
Lag (h^{-1})	2.56	2.65	2.44	2.21	2.23	1.66	0.25	0.01	0.16	0.55

[1]Treatments were pure starch and ground corn samples intact or incubated in distilled water at 39°C for 0 h using Dracon polyester cloth bags (DPB) or ANKOM F57 bags (F57).
[2]Effects of starch source (S), and filter type (F).

FIGURES

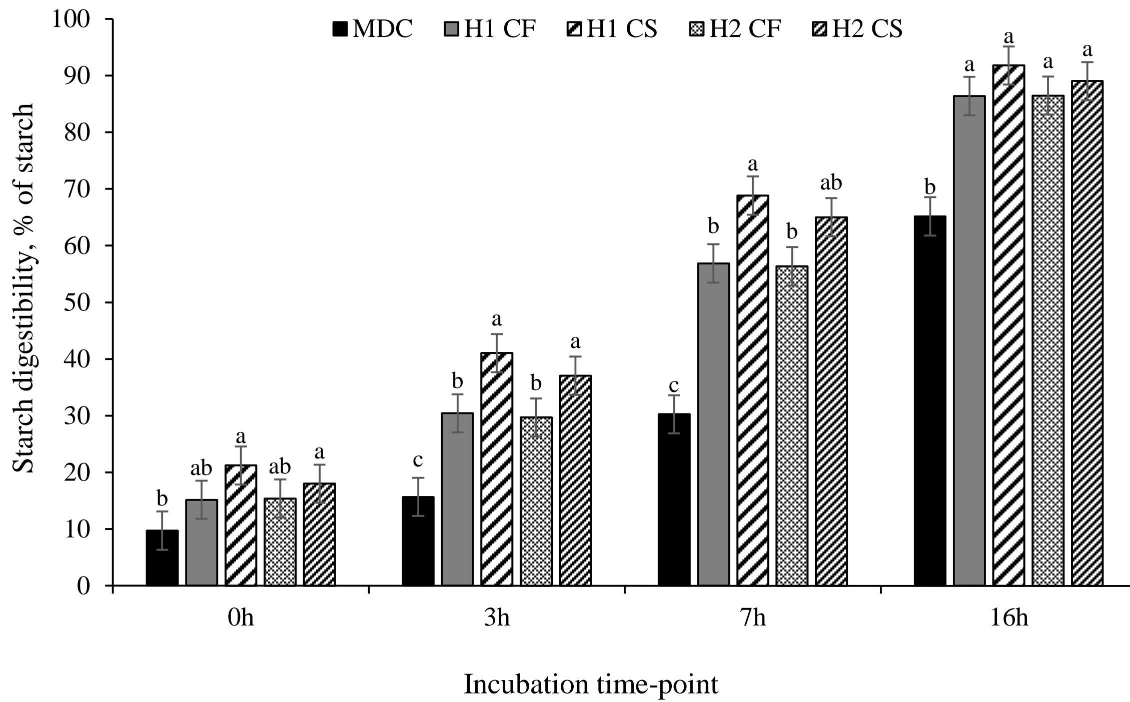


Figure 3.1. Effect of incubation time-point on in vitro or in situ starch digestibility (P = 0.01; SEM: 3.11) of mature dent corn (MDC), hybrid 1 whole-plant corn forage (H1 CF), hybrid 1 whole-plant corn silage (H1 CS), hybrid 2 whole-plant corn forage (H2 CF) and hybrid 2 whole-plant corn silage (H2 CS). Only three laboratories performed starch digestibility 0, 7, 3 and 16 h wet chemistry assays. Different superscripts denote differences between means within an incubation time-point (experiment 1).

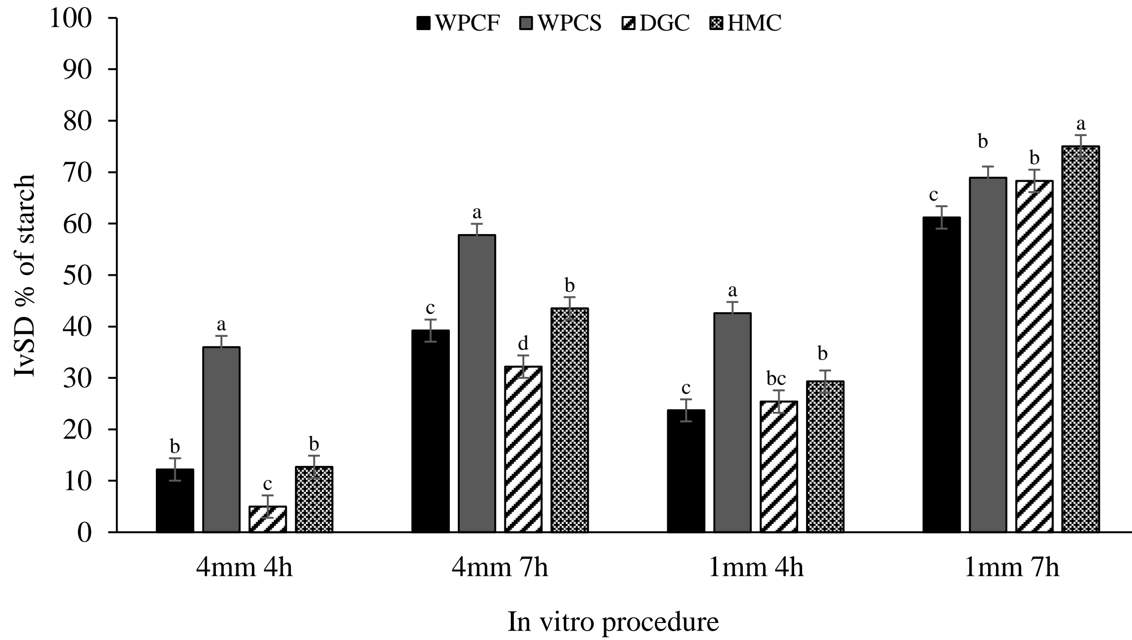


Figure 3.2. Effect of procedure on in vitro starch digestibility (IvSD; $P < 0.01$; SEM: 2.17) of whole-plant corn forage (WPCF), whole-plant corn silage (WPCS), dry ground corn (DGC) and high-moisture corn (HMC) (experiment 2).

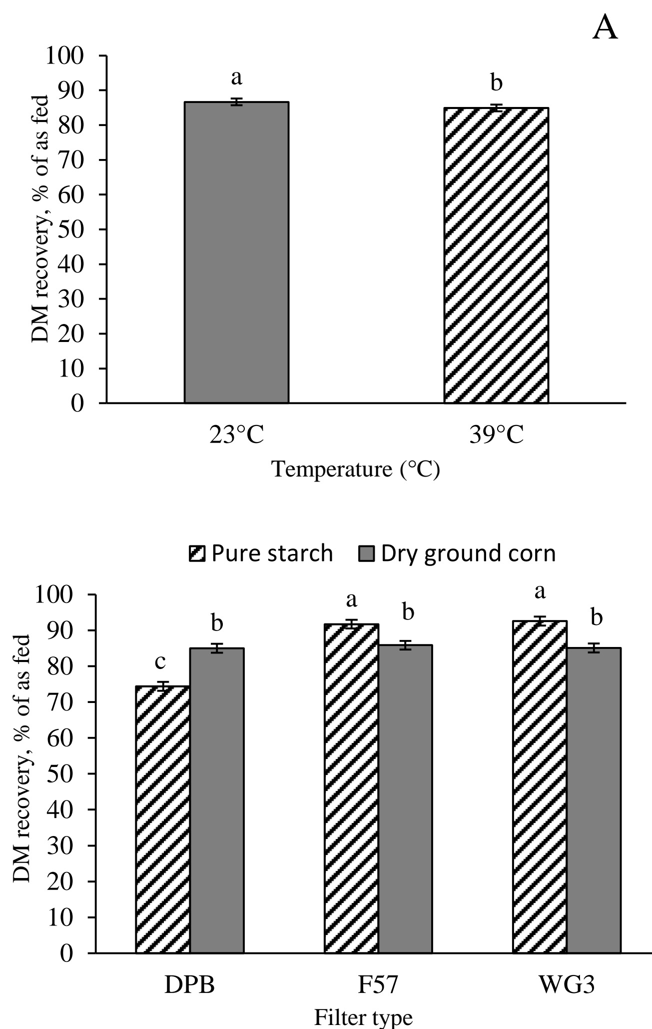


Figure 3.3. Main effect of temperature (Panel A; $P = 0.02$; SEM: 0.98) on DM recovery of samples incubated in water at 0 h, and interaction between of starch source and filter type on DM recovery (Panel B; $P < 0.001$; SEM: 1.21). Treatments were pure starch, and dry ground corn samples incubated in water using different filter types (F57: 25 µm porosity, Ankom Technology; DPB: R1020, 50 µm porosity, Ankom Technology; WG3: 6 µm porosity; Whatman G3) (experiment 4).

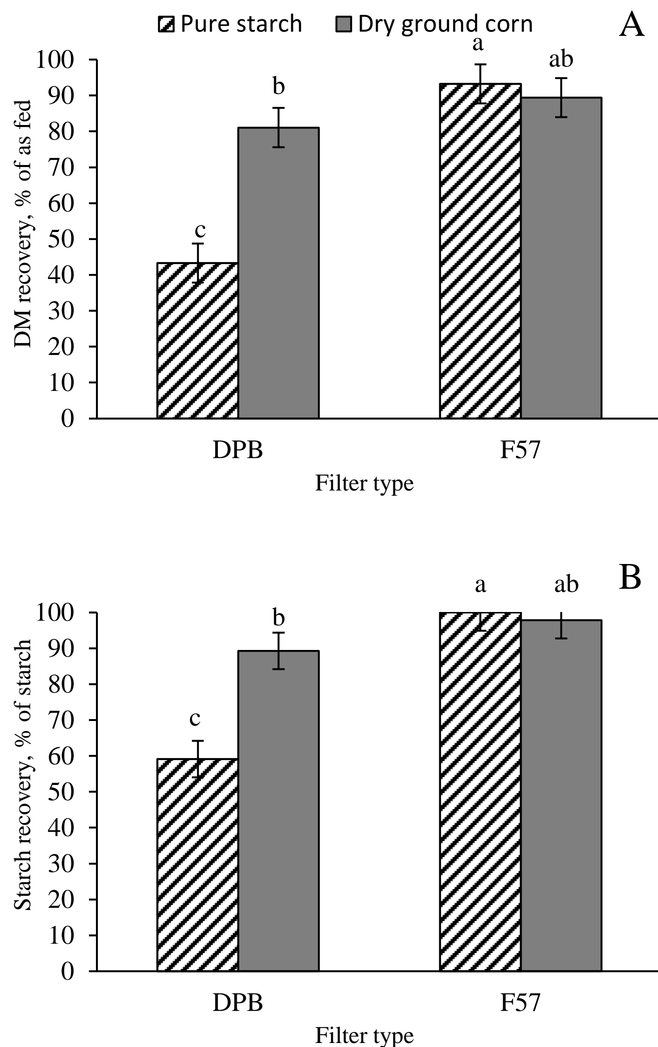


Figure 3.4. Effect of starch source and filter type on DM (panel A; $P = 0.001$; SEM: 5.46) and starch recovery (panel B; $P = 0.001$; SEM: 5.08). Treatments were pure starch, and dry ground corn samples incubated in rumen fluid in vitro using different filter types (F57: 25 µm porosity, Ankom Technology or DPB: R1020, 50 µm porosity, Ankom Technology) (experiment 5).

CHAPTER FOUR: EFFECTS OF DIETARY STARCH AND COMMON FATTY ACIDS ON MILK FATTY ACIDS YIELD AND CONCENTRATION THROUGH A META-ANALYSIS

INTRODUCTION

Diets rich in C18:2 and C18:3 may negatively impact milk fat concentration and yield (He and Armentano, 2011). Incomplete biohydrogenation of these unsaturated fatty acids (UFA) in the rumen generates isomers that escape the rumen, are bioactive in the mammary gland, and inhibit milk fat synthesis (Bauman and Griinari, 2003; Bauman and Lock, 2016). Moreover, the formation of bioactive fatty acids is increased when rapidly degradable carbohydrates and sources of UFA are fed to dairy cows (Griinari et al., 1998). Milk fatty acids are derived from three sources: 1) de novo synthesis in the mammary gland, adipose tissue mobilization or microbial origin (Glasser et al., 2008; Shingfield et al., 2010), these FAs carbon chains are shorter than 16 carbons; 2) preformed fatty acids derived from blood uptake by the mammary gland, carbon chains longer than 16 carbons; and 3) 16-carbons FAs can be generated by de novo synthesis and derived from blood, usually named as mixed. Therefore, UFA isomers-induced milk fat depression decreases de novo synthesis and consequently, total milk fatty acids secretion.

Whole-plant corn silage (WPCS) and high-moisture corn (HMC) are predominant sources of starch fed to high-producing dairy cows in the Upper Midwest of the United States. However, the fat concentration of these feeds also contributes as an energy source. The primary UFAs in corn grains and other parts of the corn plants are C18:1 and C18:2 (Beadle et al., 1965; Baldin et al., 2018). Even though fatty acids concentrations are low, their contribution to the unsaturated fatty acid load requires attention because these feeds make up a great share of the diets. Thus, there are concerns about the contribution of corn silage fatty acids to milk fat depression (Baldin et al., 2018).

Previous studies have shown accumulation of C18:2 as corn maturity increased, either due to n-6 desaturase activity (Alrefai et al., 1995) or reflection of the UFA composition of kernels

proportionally to other plant parts (Baldin et al., 2018; Saylor et al., 2021). Agarussi et al. (2020) reported no changes in fatty acids profile in WPCS ensiled for 120 d. In contrast, Alves et al (2011) reported a decrease in the concentrations of C18:2 and C18:3, even though no changes occurred in total fatty acids concentrations of WPCS ensiled for 63 d. Thus, there are indications that silage management can affect UFA supply, but its relationship with milk fat synthesis requires further exploring.

Therefore, the objectives of this study were (1) to investigate the association of common dietary fatty acids (C16:0, 18:0, C18:1, C18:2, C18:3) and starch on milk fat secretion; (2) to evaluate the effects of common silage management practices in fatty acids profile of WPCS and HMC; (3) and investigate the contributions of these nutrients derived from WPCS and HMC and their different management practices to milk fat secretion. We hypothesized that greater levels of dietary starch combined with UFA will negatively influence milk fat secretion. Moreover, our secondary hypothesis is that silage management practices may alter long-chain fatty acids concentration in WPCS and HMC and influence milk fat secretion.

MATERIAL AND METHODS

Four meta-regressions were conducted to model the effects of: 1) dietary fatty acids, starch, and forage concentrations (expressed as % of DM) on milk fatty acids concentration (expressed as g/100g); 2) dietary fatty acids, starch, and forage concentrations (expressed as % of DM) on milk fatty acids yield (expressed as g/d); 3) intake of fatty acids (expressed as g/d), starch, and forage (expressed as kg/d) on milk fatty acids concentration (expressed as g/100g); and 4) intake of fatty acids (expressed as g/d), starch and forage (expressed as kg/d) on milk fatty acids yield (expressed as g/d). Forage concentrations in the diets were used as a replacement for forage NDF because most of the studies did not report individual forages NDF concentrations. Moreover, the use of

total tract starch digestibility and dietary forage NDF concentration was intended as predictors in the models, but not sufficient data were available in the dataset used for this study to allow for their inclusion.

Dataset

The dataset used in the meta-regression analysis originated from the study by Dorea and Armentano (2017) and updated with studies indexed in the Scielo, Science Direct, Google Scholar and PubMed databases. From Dorea and Armentano (2017), only the studies that fit the criteria of this study, as described later in this section, were included. Milk fatty acids, fat supplementation, oil supplementation, linolenic acid, palmitic acid, linoleic acid, dairy cow were the search terms used in different combinations. Final data search was completed in 2022 and, to ensure that the treatments chosen would be related to ruminal biohydrogenation of fatty acids, the criteria used to select studies were: the study must have fed corn silage in the diet, and the treatments must have pure lipid supplements. Thus, oilseed, seed-processing or rumen-protected fat supplements treatments were not included in the dataset. To be included, the study must also have reported milk fatty acids profile or fatty acids summation by source (de novo, mixed and preformed), milk yield and composition, dietary fatty acid profile and starch concentration, dry matter intake (DMI) and number of animals represented in each treatment. Twenty-three studies were included in the final dataset, describing 76 treatment means and 1,662 dairy cows. Descriptive statistics for the database are described in Table 1.

Dietary intakes of fatty acids (C16:0, C18:0, C18:1, C18:2 and C18:3), starch and forage were calculated based on the concentration of these nutrients in the diet and dry matter intake.

Meta-regression analysis

Data were analyzed using the mixed procedure of SAS (PROC MIXED, SAS 9.4; SAS Institute Inc., Cary, NC, USA). The response variables used in each meta-regression models were total, de novo (<C16), mixed (C16) and preformed (C18) fatty acids expressed as yield in milk (g/d) or milk concentration (g/100g of total fatty acids). Explanatory variables were dietary fatty acids (C16:0, C18:0, C18:1, C18:2 and C18:3), dietary starch and dietary forage expressed as a percentage of dry matter (% DM) or as intake (g/d and kg/d, respectively). Explanatory variables were tested for multicollinearity using variance inflation factor (VIF). The VIF for all variables were below 5, which suggests that multicollinearity was not a problem in our dataset. Explanatory and response variables were included in the model as linear terms.

Models included the fixed effect of dietary fatty acids, starch, and forage. Study effect was considered as a random effect on the intercept of the model, that represents the overall mean (St-Pierre, 2001). Observed data were weighted by the square root of the number of observations in each treatment. Parameters of the models were estimated using the method of maximum likelihood assuming an unstructured covariance. The slope of each explanatory variable in each model was tested to a null hypothesis that stated that the slope is not different from zero. Statistical significance was declared at $P \leq 0.05$, and tendency was declared at $P \leq 0.10$. A cross-validation of the models was conducted using a leave-one-out technique. Concordance correlation coefficient (CCC; Lin, 1989) and root mean square error (RMSE) were calculated for model evaluation. The CCC is reported from 0 (poor model) to 1 (perfect model) and represents the precision and accuracy of the model. Calculation of RMSE reflects the variation unexplained by the explanatory variables on the model, and thus, a lower RMSE indicates a better-fitting model.

Silage management practices

Control samples (without microbial inoculation) of WPCS from 2 cutting height treatments (25 or 65 cm) and from 8 storage length treatments (1, 3, 5, 7, 14, 28, 56 and 90 d) from Diepersloot et al. (2022) and control samples (without microbial inoculation) of HMC from 2 DM concentration treatments (65 and 70% of as fed) and from 4 storage length treatments (7, 14, 28 and 56 d) from Saylor et al. (2022) were dried and ground to pass a 1-mm sieve and sent to Rock River Laboratories for fatty acids profile analysis. Briefly, samples were methylated according to Sukhija and Palmquist (1988) and the gas chromatography analysis was performed using a CP8827 fused-silica column (30 m x 0.32 mm i.d. x 0.25 μm film thickness; Varian Inc.). Fatty acid methyl esters were identified by a flame-ionization detector based on retention time of known standards. Data from WPCS were analyzed as a completely randomized design in a 2 x 8 (2 cutting heights and 8 storage lengths) factorial arrangement of treatments using the GLIMMIX procedure of SAS (SAS 9.4; SAS Institute Inc., Cary, NC, USA), with a model including the fixed effects of cutting height, storage length and their interaction. Orthogonal polynomial contrasts (linear and quadratic) were used to evaluate the main effect of storage length. Statistical significance was declared at $P \leq 0.05$. If an interaction was detected ($P \leq 0.05$), effects were partitioned by storage length using the SLICE option. Data from HMC were analyzed as a completely randomized design in a 2 x 4 (2 DM concentrations and 4 storage lengths) factorial arrangement of treatments using the same SAS procedure, with a model including the fixed effects of DM concentration, storage length and their interaction. Orthogonal polynomial contrasts (linear and quadratic) were used to evaluate the main effect of storage length. Statistical significance was declared at $P \leq 0.05$. If an interaction was detected ($P \leq 0.05$), effects were partitioned by storage length using the SLICE option. The effects of cutting height and storage length on WPCS fatty acids profile and the effects of DM concentration and storage length on HMC fatty acids profile are presented and discussed.

Diet simulations

Simulations using the generated models were conducted to visualize the contributions of different dietary inclusions of WPCS and HMC and the effect of different management practices of WPCS on milk fat secretion. Diets used for the simulation consisted of diet 1: (DM basis) corn silage (30%), alfalfa silage (22.2%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (16.2%) and concentrate (20%); diet 2: corn silage (54%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (14.4%) and concentrate (20%); diet 3: corn silage (30%), alfalfa silage (22.2%), high-moisture corn (14%), whole cottonseed (4.6%), soybean hulls (9.2%) and concentrate (20%); and diet 4: corn silage (54%), high-moisture corn (14%), whole cottonseed (4.6%), soybean hulls (7.4%) and concentrate (20%). Intake of starch and fatty acids was calculated by multiplying dietary concentration by intake of DM, which was set at 32 kg/day (average of the last 3 feeding trials with multiparous Holstein cows conducted by our group). Even though DM intake should change based on the different diets, changing intakes based on the diets would confound the interpretation of the predictions and, thus, the simulations. First, fatty acids and starch averages across storage length of regular cut corn silage (Diepersloot et al., 2022) and HMC (Saylor et al., 2022; 65% DM) were used to conduct simulations with the 4 diets to visualize the inclusion of WPCS (30 vs. 54% DM basis) and HMC (7 vs. 14% DM basis) effect on milk fatty acids. Second, averages across storage length of two cutting heights treatments (25 vs. 65 cm) were used to illustrate the WPCS cutting height management effect on milk fatty acids yield and concentration. Similarly, maturity (1/4 milk line vs. 3/4 milk line) and storage length (30 vs. 240 d) simulations were conducted using WPCS concentrations of starch and fatty acids averaged by processor roll gap width (1 and 3 mm) from Saylor et al. (2021). All three simulations were conducted using diet 1 and 2 as described previously to visualize these effects in two different

inclusions of WPCS (30 vs. 54% DM basis) in the diet. Moreover, fatty acids and starch averages across storage length of two DM concentration treatments (65 vs. 70% of as fed) were used to visualize the effect of HMC DM concentration on milk fatty acids yield and concentration. Similarly, HMC storage length (7 vs. 56 d) simulations were conducted to demonstrate the effect of HMC stored for different periods of time on milk fatty acids yield and concentration. These simulations were conducted using diet 1 and 3 as described previously to visualize these effects in two different inclusions of HMC (7 vs. 14% DM basis) in the diet.

RESULTS AND DISCUSSION

A meta-regression analysis was conducted to understand the effects of the main dietary fatty acids and starch in modern dairy cow diets on total milk fat and fatty acids sources concentration and yield (Tables 2 – 5). Based on CCC and RMSE evaluation, all the models tested for milk fatty acids were reliable. Models to predict milk fatty acids yield had greater CCC than concentration models, suggesting that there was a better-fit of the data when predicting yield. The dilution of milk fatty acids depending on yield was likely a source of variation that affects concentration models, while milk fatty acids yield models did not present this source of variation, which can explain the better fit. However, milk fat concentration is widely used as an indicator of milk fat depression; thus, these models can provide useful information for feeding management evaluation.

Table 2 describes the effects of dietary fatty acids, starch, and forage concentrations (expressed as % of DM) on total milk fat and milk fatty acids classes concentrations (expressed as g/100g). Diet C16:0 had a negative effect on de novo milk fatty acids ($P < 0.001$) and a positive effect on mixed and total milk fatty acids concentration ($P < 0.001$). Palmquist (2006), conferred about greater supply of exogenous long-chain fatty acids reducing de novo synthesis in the mammary gland due to the enzymatic competition, even though it might not reduce total synthesis

of fatty acids. All dietary UFAs had a negative effect on de novo (C18:1, $P < 0.001$; C18:2 $P < 0.001$; C18:3, $P < 0.001$) and mixed milk fatty acids concentration (C18:1, $P < 0.001$; C18:2, $P < 0.001$; C18:3, $P < 0.001$), but positive effects on preformed milk fatty acids concentration (C18:1, $P < 0.001$; C18:2, $P < 0.001$; C18:3, $P < 0.001$). However, only C18:1 ($P = 0.01$) and C18:2 ($P < 0.001$) affected total milk fatty acids concentration negatively, while C18:3 ($P = 0.06$) tended to affect total milk fatty acids concentration negatively. Secretion of C18:1 and C18:2 in the milk were strongly correlated with milk fat depression (Bauman and Lock, 2006; Matamoros et al., 2020). However, the C18:3 relationship with milk fat depression is not conclusive (Mannai et al., 2016; Daley et al., 2022), which agrees with the results found in this study. Dietary starch concentration had a positive effect on mixed milk fatty acids ($P = 0.01$) and tended to increase total milk fatty acids concentration ($P = 0.09$), which can indicate greater energy supply. Starch is the main source of propionic acid producing bacteria in the rumen, a glucogenic precursor that increases milk yield and therefore dilutes milk fat concentration (Molento et al., 2002; Daley et al., 2022). Recently, late lactation cows (194 ± 58 DIM) fed a low or high starch diet (24.3 and 29.6% of DM, respectively), did not have differences in milk yield (Copelin et al., 2021) and rumen fluid concentration of C18:1, C18:2 and C18:3 (Lee et al., 2021), but milk fat concentration was lower for the high starch diet (Copelin et al., 2021). Authors stated that changes in ruminal volatile fatty acids profile and microbial community observed were likely the cause of moderate milk fat depression rather than increased incomplete ruminal biohydrogenation of UFA (Lee et al., 2021). This suggests that well-fed cows should be able to maintain rumen health and have lower ruminal incomplete biohydrogenation which could explain the starch effect observed in this model.

Table 3 describes the effects of dietary fatty acids, starch, and forage concentrations (expressed as % of DM) on total milk fat and milk fatty acids classes yield (expressed as g/d).

Saturated fatty acids did not influence or only tended to influence de novo milk fatty acids yield (C16:0, $P = 0.18$; C18:0, $P = 0.06$). However, there was a positive effect of these fatty acids on preformed milk fatty acids yield (C16:0, $P = 0.02$; C18:0, $P = 0.02$) and C16:0 had a positive effect on mixed milk fatty acids yield ($P < 0.001$). Studies have indicated that C16:0 supply can increase C16:0 yield without changes on de novo synthesis (Stoffel et al., 2016; Dorea and Armentano, 2017), which support our findings. Saturated fatty acids also had a positive effect on total milk fatty acids yield (C16:0, $P < 0.001$; C18:0, $P = 0.02$), reflecting the greater supply of preformed milk fatty acids without decreasing de novo synthesis. Studies from the 1960's reported that feeding pure sources of C16:0 and C18:0 increased milk fat yield similarly (Steele and Moore, 1968; Steele, 1969). Conversely, studies from the last decade have been reporting lower milk fat yield with C18:0 supplementation compared to C16:0 supplementation (Loften et al., 2014; Rico et al., 2014; Piantoni et al., 2015). Indeed, the effect of C16:0 on total milk fatty acids yield was greater than the effect of C18:0, each percentage unit of dietary C16:0 yields 99.48 g/d, while each percentage unit of dietary C18:0 yields 68.15 g/d. Dietary UFA had a negative effect or tended to have a negative effect on de novo (C18:1, $P = 0.06$; C18:2, $P < 0.001$; C18:3, $P < 0.001$) and mixed milk fatty acids yield (C18:1, $P = 0.03$; C18:2 $P < 0.001$; C18:3, $P < 0.001$). However, C18:2 ($P < 0.001$) and C18:3 ($P < 0.001$) had negative effects on total milk fatty acids yield. Dietary starch had a positive effect on de novo ($P = 0.02$), mixed ($P < 0.001$) and total milk fatty acids yield ($P < 0.001$), diets with greater energy increase milk yield and consequently fat yield. During milk fat depression, caused either by unsaturated fatty acids or by highly fermentable diets, both de novo and preformed fatty acids decrease due to a central regulation of fat synthesis mechanisms in the mammary cells (Jenkins and Harvatine, 2014). Thus, it is unlikely that greater concentration of starch in the diets is the sole factor, but rather one of the factors causing milk fat

depression. Weiss and Pinos-Rodriguez (2009) reported that cows fed a 60% forage diet with saturated fat supplementation increased energy intake when compared to cows not supplemented with fat. However, greater energy intake did not result in greater milk fat yield. Conversely, in the same study, cows fed a 40% forage diet with saturated fat supplementation had greater energy intake and increased greater milk fat yield, which suggests that the energy partitioning in those two scenarios was different (Weiss and Pinos-Rodriguez, 2009), a plausible explanation for the findings of this study. Generally, this study shows a negative effect of dietary UFA on de novo and mixed milk fatty acids, while a positive effect is observed for preformed milk fatty acids.

Table 4 describes the effects of fatty acids (expressed as g/d), starch, and forage intake (expressed as kg/d) on total milk fat and milk fatty acids concentrations (expressed as g/100g). Similar to dietary C16:0 concentration, intake of C16:0 had a negative effect on de novo milk fatty acids concentration ($P < 0.001$), while it positively affected mixed and total milk fatty acids concentration ($P < 0.001$ and $P = 0.01$, respectively). As discussed before, this effect was expected. Intake of all UFA had a negative effect on de novo (C18:1, $P = 0.01$; C18:2, $P < 0.001$; C18:3, $P = 0.01$) and mixed milk fatty acids concentration (C18:1, $P = 0.03$; C18:2, $P < 0.001$; C18:3, $P = 0.01$). However, C18:2 negatively affect total milk fatty acids concentration ($P < 0.001$), while C18:1 and C18:3 tended to negatively affect total milk fatty acids concentration ($P = 0.06$ and $P = 0.09$). Conversely, UFA had a positive effect on preformed milk fatty acids concentration (C18:1, $P < 0.001$; C18:2, $P < 0.001$; C18:3, $P < 0.001$), reflecting a greater supply of UFA to the mammary gland. Starch intake had a positive effect on de novo ($P = 0.01$) and mixed fatty acids concentration ($P < 0.001$), but a negative effect on preformed milk fatty acids concentration ($P < 0.001$). Starch intake did not influence total milk fatty acids concentration ($P = 0.31$). The mode of starch intake data used in this study was 6.71 kg/d, cows fed this amount of daily starch were

able to maintain rumen pH above 6.0 (Oba and Allen, 2003; Lee et al., 2021). Perhaps, the dietary starch effects observed in this study can be attributed to increased energy supply. Moreover, milk fatty acids profile of cows fed isoenergetic diets responded differently with ruminal propionate infusion or glucose infusion in the duodenum. Propionate infusion increased de novo milk fatty acids concentration, while glucose infusion increased preformed milk fatty acids concentration (Rigout et al., 2003); this supports that different mechanisms of milk fat depression can be involved when the site of starch digestion is shifted.

Table 5 describes the effects of fatty acids (expressed as g/d), starch, and forage intake (expressed as kg/d) on total milk fat and milk fatty acids yield (expressed as g/d). Intake of C16:0 had a positive effect on mixed and total milk fatty acids yield ($P < 0.001$ and $P < 0.001$, respectively), as expected. Bishop et al. (1969) reported that 5% of infused C16:0 was incorporated into longer-chain preformed milk fatty acids in dairy cows, which may explain the positive effect of C16:0 intake on preformed milk fatty acids yield ($P = 0.02$). Intake of C18:0 tended to increase preformed milk fatty acids ($P = 0.09$) and had a positive effect on total milk fatty acids ($P = 0.03$). Similarly to the observed effects of dietary saturated fatty acids concentration on milk fatty acids yield, C18:0 effect was lower than C16:0 (0.21 g/d per unit of C18:0 intake vs. 0.31 g/d per unit of C16:0 intake). Whereas intake of C18:2 and C18:3 had a negative effect on de novo (C18:2, $P < 0.001$; C18:3, $P = 0.01$), mixed (C18:2, $P = 0.01$; C18:3, $P = 0.01$) and total milk fatty acids yield (C18:2, $P = 0.01$; C18:3, $P = 0.01$). As discussed previously these results were expected. Starch intake had a positive effect on de novo ($P < 0.001$), mixed ($P < 0.001$), preformed ($P = 0.03$) and total milk fatty acids yield ($P < 0.001$). As discussed previously, greater energy supply provided by greater starch intake can increase milk yield and consequently milk fatty acids yield.

The effects of cutting height and storage length on concentrations of C16:0 of WPCS are in Figure 1A ($P = 0.01$). Regular cut WPCS had greater C16:0 concentrations at 3, 5, 28 and 90 d, while high cut WPCS had greater concentrations at 14 d. No difference between cutting heights were observed at 1, 7 and 56 d. The effects of cutting height and storage length on concentrations of C18:0 of WPCS are in Figure 1B ($P = 0.04$). Concentrations of C18:0 of high cut WPCS were lower at 3 and 90 d, while no difference between cutting height was observed for the other storage lengths. The effects of cutting height and storage length on concentrations of C18:2 of WPCS are in Figure 1C ($P = 0.02$). High cut WPCS had grater concentrations of C18:2 than regular cut WPCS at 3, 5, 28 and 90 d, while no difference between cutting height was observed for the other storage lengths. The effects of cutting height and storage length on total fatty acids concentrations of WPCS are in Figure 1D ($P = 0.01$). High cut WPCS height had greater concentration of total fatty acids than regular cut WPCS only at 5, 56 and 90 d of storage length.

Agarussi et al. (2020) did not observe differences in C16:0 and C18:0 concentrations of WPCS ensiled for 120 d compared to unfermented samples. However, Alves et al. (2011) reported a decrease of 0.38 g/100g in C18:0 concentration when ensiling WPCS for 63 d. In general, these results did not show an apparent pattern of WPCS fatty acids concentrations across storage length.

The main effect of storage length on WPCS fatty acids concentration profile is in Table 6. There was an effect of cutting height on C18:1 ($P = 0.001$) and C18:3 ($P = 0.001$). Concentration of C18:1 increased with higher cutting height (22.0 vs. 22.8 g/100g), while concentration of C18:3 decreased (7.7 vs. 6.2 g/100g). This reflects the greater amount of grain in WPCS with higher cutting height, as C18:1 is found primarily in the kernel while C18:3 is more present in the leaves and stalk of corn plants (Khan et al., 2012).

The main effect of storage length on WPCS fatty acids concentration profile is in Table 7. There was a storage length effect on C18:1 (P = 0.02) and C18:2 (P = 0.02). Concentrations of C18:1 decreased linearly with storage length; it went from 22.8 g/100g for 1 d to 21.9 g/100g. While C18:2 concentrations increased linearly from 51.4 g/100g (1 d) up to 53.2 g/100g (90 d). The effects of storage length in silage fatty acids are not well understood. Baldin et al. (2018) reported negative correlation between C18:1 and C18:2 in corn grain, which supports the results observed in this study. Saylor et al. (2021) reported minor changes of storage length on C18:2 and C18:3, while Alves et al. (2011) reported an increase in C18:1 and a decrease in C18:2 and C18:3 concentrations when ensiling WPCS for 63 d. Concentrations of C16:1 decreased linearly with storage length, it went from 0.12 g/100g to 0.10 g/100g. Overall, the differences observed in WPCS in this study fatty acids may affect bacteria metabolism of lipids in the rumen and consequently milk fat synthesis.

The effects of DM concentration and storage length on concentrations of C16:0 of HMC are in Figure 2A (P = 0.001). Low DM HMC had lower C16:0 concentrations at 7 (12.1 vs. 12.7 g/100g) and 56 d (11.6 vs. 11.9 g/100g), while no difference between low and high DM was observed at 14 and 28 d. The effects of DM concentration and storage length on concentrations of C18:0 of HMC are in Figure 2B (P = 0.03). Concentration of C18:0 was greater for high DM only at 7 d (1.5 vs. 1.4 g/100g), while no difference was observed at 14, 28 and 56 d.

The main effect of DM concentration on HMC fatty acids concentration profile is in Table 8. High-moisture corn with greater DM had greater C18:1 concentration (28.8 vs. 28.5 g/100g; P = 0.03), but lower concentration of C18:2 (55.9 vs. 56.3 g/100g; P = 0.001). The main effect of storage length on HMC fatty acids concentration profile is in Table 9. Concentration of C18:1 decreased linearly with storage length (P = 0.001), 7 and 14 d had the highest concentration of

C18:1 (29.0 g/100g on average), while 28 and 56 d had the lowest concentrations (28.4 g/100g on average). Conversely, concentrations of C18:2 ($P = 0.001$) and C18:3 ($P = 0.001$) increased with storage length, HMC ensiled for 7 d had 55.4 g/100g of C18:2 on average, while 56 d had 56.8 g/100g on average. Concentrations of C18:3 went from 1.0 g/100g on HMC stored for 7 d to 1.1 g/100g for 56 d. Gardner (1970) reported that lipoxygenases in corn grain can hydrolyze C18:2 and C18:3. However, our results showed a slight increase in these fatty acids with storage length, which suggests that lipoxygenases are probably not active during silage fermentation. Alves et al. (2011) discussed the presence of biohydrogenation intermediates in ryegrass silage and suggested that perhaps lactic acid bacteria or other microorganisms can biohydrogenate fatty acids during silage fermentation. However, few studies on fatty acids changes in WPCS and HMC are available in the literature and research is warranted to understand metabolism of lipids by lactic acid bacteria in silage.

Simulations with the models using different diets were conducted to understand the impact of dietary levels and intake of WPCS and HMC on milk fatty acids concentration and yield (Table 10 – 15). Predicted milk fatty acids using 4 different diets with 30 or 54% WPCS (DM basis) and 7 or 14% HMC (DM basis) are in Table 10. Intake inputs were calculated based on an animal consuming 32 kg of DM per day. All models predicted similar patterns for the different diets. Greater inclusion of WPCS and HMC in the diets generally increased proportions of de novo and mixed milk fatty acids, while decreased preformed milk fatty acids proportions. However, greater inclusion of WPCS and HMC in the diets generally increased the yield of all classes and total milk fatty acids, which suggests that greater inclusions of WPCS and HMC does not contribute greatly to milk fat depression on well-fed cows.

Predicted milk fatty acids from high (54% DM basis) or regular (30% DM basis) inclusion of WPCS harvest at regular or high cutting height are in Table 11. For diet 1 (30% WPCS DM basis), high cut WPCS seems to have minimum effect on prediction of classes and total milk fatty acids concentration and yield. However, predictions of classes and total milk fatty acids on diet 2 (54% WPCS DM basis) seem more pronounced, generally showing an increase in milk fatty acids yield while minor changes are observed in milk fatty acids concentrations. Predicted milk fatty acids concentration and yield from high (54% DM basis) or regular (30% DM basis) inclusion of WPCS harvested early or late are in Table 12. Like predictions based on WPCS cutting height (Table 11), WPCS maturity seems to have minor changes in milk fatty acids concentrations. However, late maturity WPCS increases predictions of milk fatty acids yield. Late maturity WPCS had greater concentration of C18:1, C18:2 and starch, which suggests that the increased starch concentration in late maturity plays a greater role than the accumulation of UFA in the predictions. Predicted milk fatty acids concentration and yield from high (54% DM basis) or regular (30% DM basis) inclusion of WPCS stored for 30 d or 240 d are in Table 13. Storage length did not notably impact total or classes of milk fatty acids for all the models compared to the predictions using WPCS cutting height and maturity. Minor differences were reported for UFA and starch concentrations of WPCS ensiled for 30 or 240 d (Saylor et al., 2021), which supports the predictions of our models.

Predicted milk fatty acids concentration and yield from regular (7% DM basis) or high (14% DM basis) inclusion of HMC with 65 or 70% DM concentration are in Table 14. Concentration of DM in HMC seems to have minimal effect on predicted milk fatty acids concentration and yield. Predicted milk fatty acids from regular (7% DM basis) or high (14% DM basis) inclusion of HMC store for 7 or 56 d are in Table 15. Similarly, HMC store for 7 or 56 d effect on predicted milk

fatty acids concentration and yield seems to be minimal. The relatively low inclusions of HMC compared with WPCS in dairy cow diets dilutes the changes in HMC fatty acids and starch contribution to the diets which can explain the lack of changes in these simulations.

Generally, greater corn silage inclusion in the diet predicted greater milk fatty acids yield in all scenarios, which suggests that increased starch concentrations in the diet and increased starch intake are the main driver on these predictions. Yet, this is an exercise based on our prediction models, thus, nutritionists and farmers should still pay close attention when feeding high starch combined with high oil to diminish the risk of milk fat depression.

CONCLUSION

The objective of this study was to understand the associations of dietary starch and fatty acids on milk fat secretion and investigate the contributions of corn silage to milk fat secretion, therefore, it is not our intention to suggest that milk fat can be predict only by these factors. There were generally negative effects of unsaturated fatty acids on de novo, mixed and total milk fatty acids while dietary C16:0 and starch had positive effects on de novo and total milk fatty acids. These results suggest that energy contributions from starch and saturated fatty acids are important on milk fat synthesis predictions, while supporting unsaturated fatty acids role on milk fat depression described in the literature. Moreover, while predictions of milk fatty acids concentrations are useful, models that predict milk fatty acids yield had greater performance. The effects of management practices on WPCS and HMC fatty acids profile may affect rumen bacteria metabolism, further research is warranted to understand the effects of silage management practices on milk fat synthesis.

REFERENCES

Agarussi, M. C. N., V. P. Silva, E. M. Paula, D. Vyas, A. T. Adesogan, O. G. Pereira, and L. F. Ferraretto. 2020. Effects of ensiling of whole-plant corn on silage processing score and fermentation and long-chain fatty acid profiles. Appl. Anim. Sci. 36:167-171. https://doi.org/10.15232/aas.2019-01941.

Alrefai, R., T. G. Berke, and T. R. Rocheford. 1995. Quantitative trait locus analysis of fatty acid concentrations in maize. Genome 38:894–901. https://doi.org/10.1139/g95-118.

Alves, S.P., A. R. J. Cabrita, E. Jeronimo, R. J. B. Bessa, A. J. M. Fonseca. 2011. Effect of ensiling and silage additives on fatty acid composition of ryegrass and corn experimental silages. J. Anim. Sci. 89, 2537–2545. https://doi.org/10.2527/jas.2010-3128.

Baldin, M., Y. Ying, Y. Fan, G. Roth, D. P. Casper, K. J. Harvatine. 2018. Characterization of linoleic acid (C18:2) concentration in commercial corn silage and grain hybrids. J. Dairy Sci. 101: 222-232. https://doi.org/10.3168/jds.2017-12972.

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23:203–227. https://doi.org/10.1146/annurev.nutr.23.011702.073408.

Bauman D.E. and Lock A. L. 2006. Conjugated linoleic acid: biosynthesis and nutritional significance. In: Advanced dairy chemistry. Eds PF Fox, PHL McSweeney. pp. 93–136. https://doi.org/10.1007/0-387-28813-9_3.

Beadle, J. B., D. Just, R. Morgan, and R. Reiners. 1965. Composition of corn oil. J. Am. Oil Chem. Soc. 42:90–95. https://doi.org/10.1007/BF02545036.

Benchaar, C., G. A. Romero-Pérez, P. Y. Chouinard, F. Hassanat, M. Eugene, H. V. Petit, and C. Côrtes. 2012. Supplementation of increasing amounts of linseed oil to dairy cows fed total mixed rations: Effects on digestion, ruminal fermentation characteristics, protozoal

populations, and milk fatty acid composition. J. Dairy Sci. 95:4578-4590. http://dx.doi.org/10.3168/jds.2012-5455.

Burch, A. M., A. Pineda, and A. L. Lock. 2021. Effect of palmitic acid-enriched supplements containing stearic or oleic acid on nutrient digestibility and milk production of low- and high-producing dairy cows. J. Dairy Sci. 104:8673-8684. https://doi.org/10.3168/jds.2020-19913.

Chilliard, Y., C. Martin, J. Rouel, and M. Doreau. 2009. Milk fatty acids in dairy cows fed whole crude linseed, extruded linseed, or linseed oil, and their relationship with methane output. J. Dairy Sci. 92:5199-5211. https://doi.org/10.3168/jds.2009-2375.

Copelin, J. E., J. L. Firkins, M. T. Socha, and C. Lee. 2021. Effects of diet fermentability and supplementation of 2-hydroxy-4(methylthio)-butanoic acid and isoacids on milk fat depression: 1. Production, milk fatty acid profile, and nutrient digestibility. J. Dairy Sci. 104:1591-1603. https://doi.org/10.3168/jds.2020-18949.

Daley, V. L., L. E. Armentano, M. D. Hanigan. 2022. Models to predict milk fat concentration and yield of lactating dairy cows: A meta-analysis. J. Dairy Sci. 105:8016-8035. https://doi.org/10.3168/jds.2022-21777.

De Souza, J., and A. L. Lock. 2018b. Long-term palmitic acid supplementation interacts with parity in lactating dairy cows: Production responses, nutrient digestibility, and energy partitioning. J. Dairy Sci. 101:3044-3056. https://doi.org/10.3168/jds.2017-13946.

De Souza, J., and A. L. Lock. 2018c. Short communication: Comparison of a palmitic acid-enriched triglyceride supplement and calcium salts of palm fatty acids supplement on production responses of dairy cows. J. Dairy Sci. 101:3110-3117. https://doi.org/10.3168/jds.2017-13560.

De Souza, J., C. L. Preseault, and A. L. Lock. 2018a. Altering the ratio of dietary palmitic, stearic, and oleic acids in diets with or without whole cottonseed affects nutrient digestibility, energy partitioning, and production responses of dairy cows. J. Dairy Sci. 101:172-185. https://doi.org/10.3168/jds.2017-13460.

De Souza, J., C. L. Preseault, and A. L. Lock. 2016. Short communication: Lactational responses to palmitic acid supplementation when replacing soyhulls or dry ground corn. J. Dairy Sci. 99:1945-1950. http://dx.doi.org/10.3168/jds.2015-10367.

De Souza, J., J. L. Garver, C. L. Preseault, and A. L. Lock. 2017. Short communication: Effects of prill size of a palmitic acid–enriched fat supplement on the yield of milk and milk components, and nutrient digestibility of dairy cows. J. Dairy Sci. 100:379-384. https://doi.org/10.3168/jds.2016-11610.

Diepersloot, E. C., C. Heinzen Jr., B. A. Saylor, L. F. Ferraretto. 2022. Effect of cutting height, microbial inoculation, and storage length on fermentation profile and nutrient composition of whole-plant corn silage. Trans. Animal Sci. 6:1-10. https://doi.org/10.1093/tas/txac037.

Dorea, J. R. R., and L. E. Armentano. 2017. Effects of common dietary fatty acids on milk yield and concentrations of fat and fatty acids in dairy cattle. Anim. Prod. Sci. 57:2224–2236. https://doi.org/10.1071/AN17335.

Gardner, H. W. 1970. Sequential enzymes of linoleic acid oxidation in corn germ: Lipoxygenase and linoleate hydroperoxide isomerase. J. Lipid Res. 11:311–321. https://doi.org/10.1016/S0022-2275(20)42967-0.

Griinari, J. M., D. A. Dwyer, M. A. McGuire, D. E. Bauman, D. L. Palmquist, and K. V. Nurmela. 1998. Trans-octadecenoic acids and milk fat depression in lactating dairy cows. J. Dairy Sci. 81(5): 1251-1261. https://doi.org/10.3168/jds.S0022-0302(98)75686-3.

He, M., and L. Armentano. 2011. Effect of fatty acid profile in vegetable oils and antioxidant supplementation on dairy cattle performance and milk fat depression. J. Dairy Sci. 94:2481–2491. https://doi.org/10.3168/jds.2010-3755.

He, M., K. L. Perfield, H. B. Green, and L. E. Armentano. 2012. Effect of dietary fat blend enriched in oleic or linoleic acid and monensin supplementation on dairy cattle performance, milk fatty acid profiles, and milk fat depression. J. Dairy Sci. 95:1447-1461. http://dx.doi.org/ 10.3168/jds.2011-4635.

Khan, N., J. Cone, V. Fievez, and W. Hendriks. 2012. Causes of variation in fatty acid content and composition in grass and maize silages. Anim. Feed Sci. Technol. 174:36–45. https://doi.org/10.1016/j.anifeedsci.2012.02.006.

Lee, C., J. E. Copelin, T. Park, K. E. Mitchell, J. L. Firkins, M. T. Socha, and D. Luchini. 2021. Effects of diet fermentability and supplementation of 2-hydroxy-4(methylthio)-butanoic acid and isoacids on milk fat depression: 2. Ruminal fermentation, fatty acid, and bacterial community structure. J. Dairy Sci. 104:1604-1619. https://doi.org/10.3168/jds.2020-18950.

Lin, L. I. 1989. A concordance correlation coefficient to evaluate reproducibility. Biometrics 45:255-268. https://doi.org/10.2307/2532051.

Loften, J. R., J. G. Linn, J. K. Drackley, T. C. Jenkins, C. G. Soderholm, and A. F. Kertz. 2014. Invited review: Palmitic and stearic acid metabolism in lactating dairy cows. J. Dairy Sci. 97:4661–4674.https://doi.org/10.3168/jds.2014-7919.

Mannai, H., É. Charbonneau, L. Fadul-Pacheco, D. Pellerin, and P. Y. Chouinard. 2016. An appraisal of the concept of rumen unsaturated fatty acid load and its relation to milk fat concentration using data from commercial dairy farms. Prof. Anim. Sci. 32:665–671. https://doi.org/10.15232/pas.2016-01526.

Marques, J. A., T. A. Del Valle, L. G. Ghizzi, E. M. C. Zilio, L. S. Gheller, A. T. Nunes, T. B. P. Silva, M. S. S. Dias, N. T. S. Grigoletto, A. F. Koontz, G. G. da Silva, and F. P. Rennó. 2019. Increasing dietary levels of docosahexaenoic acid-rich microalgae: Ruminal fermentation, animal performance, and milk fatty acid profile of mid-lactating dairy cows. J. Dairy Sci. 102:5054–5065. https://doi.org/10.3168/jds.2018-16017.

Matamoros, C., R. N. Klopp, L. E. Moraes, and K. J. Harvantine. 2020. Meta-analysis of the relationship between milk trans-10 C18:1, milk fatty acids <C16, and milk fat production. J. Dairy Sci. 103:10195-10206. https://doi.org/10.3168/jds.2019-18129.

Molento, C. F. M., E. Block, R. I. Cue, and D. Petitclerc. 2002. Effects of insulin, recombinant bovine somatotropin, and their interaction on insulin-like growth factor-I secretion and milk protein production in dairy cows. J. Dairy Sci. 85:738–747.https://doi.org/10.3168/jds.S0022-0302(02)74131-3.

Oba, M., and M. S. Allen. 2003. Effects of corn grain conservation method on feeding behavior and productivity of lactating dairy cows at two dietary starch concentrations. J. Dairy Sci. 86:174–183. https://doi.org/10.3168/jds.S0022-0302(03)73598-X.

Palmquist, D. L. 2006. Milk Fat: Origin of fatty acids and influence of nutritional factors thereon. In: Advanced Dairy Chemistry, Volume 2: Lipids. Edited by P. F. Fox and P. L. H. McSweeney, Springer, New York.

Piantoni, P., A. L. Lock, and M. S. Allen. 2015. Milk production responses to dietary stearic acid vary by production level in dairy cattle. J. Dairy Sci. 98:1938–1949. https://doi.org/10.3168/jds.2014-8634.

Prom, C. M., and A. L. Lock. 2021. Replacing stearic acid with oleic acid in supplemental fat blends improves fatty acid digestibility of lactating dairy cows. J. Dairy Sci. 104:9956–9966. https://doi.org/10.3168/jds.2020-19985.

Prom, C. M., J. M. dos Santos Neto, and A. L. Lock. 2022. Abomasal infusion of different exogenous emulsifiers alters fatty acid digestibility and milk fat yield of lactating dairy cows. J. Dairy Sci. 105:3102–3112. https://doi.org/10.3168/jds.2021-21315.

Prom, C. M., J. M. dos Santos Neto, J. R. Newbold, and A. L. Lock. 2021. Abomasal infusion of oleic acid increases fatty acid digestibility and plasma insulin of lactating dairy cows. J. Dairy Sci. 104:12616–12627. https://doi.org/10.3168/jds.2021-20954.

Relling, A. E., and C. K. Reynolds. 2007. Feeding rumen-inert fats differing in their degree of saturation decreases intake and increases plasma concentrations of gut peptides in lactating dairy cows. J. Dairy Sci. 90:1506–1515. https://doi.org/10.3168/jds.S0022-0302(07)71636-3.

Rico, J. E., M. S. Allen, and A. L. Lock. 2014. Compared with stearic acids, palmitic acid increased the yield of milk fat and improved feed efficiency across production level of cows. J. Dairy Sci. 97:1057-1066. https://dx/doi.org/10.3168/jds.2013-7432.

Rigout, S., C. Hurtaud, S. Lemosquet, A. Bach, and H. Rulquin. 2003. Lactational Effect of Propionic Acid and Duodenal Glucose in Cows. J. Dairy Sci. 86:243–253. https://doi.org/10.3168/jds.S0022-0302(03)73603-0.

Saylor, B. A., C. L. McCary, E. C. Diepersloot, C. Heinzen Jr., M. R. Pupo, J. O. Gusmão, L. G. Ghizzi, H. Sultana, L. F. Ferraretto. 2021. Effect of forage processor roll gap width and storage length on fermentation profile, nutrient composition, kernel processing score, and starch disappearance of whole-plant maize silage harvested at three different maturities. Agriculture, 11, 574. https://doi.org/10.3390/agriculture11070574.

Shepardson, R. P., and K. J. Harvatine. 2021. Effects of fat supplements containing different levels of palmitic and stearic acid on milk production and fatty acid digestibility in lactating dairy cows. J. Dairy Sci. 104:7682–7695. https://doi.org/10.3168/jds.2020-19665.

Sinedino, L. D. P., P. M. H., L. R. L. Souza, A. L. Lock, M. P. B., C. R. Staples, W. W. Thatcher, and J. E. P. Santos. 2017. Effects of supplementation with docosahexaenoic acid on reproduction of dairy cows. Reproduction. 153(5):707-723. https://doi.org/10.1530/REP-16-0642.

Steele, W., and J. H. Moore. 1968. The effects of a series of saturated fatty acids in the diet on milk-fat secretion in the cow. J. Dairy Res. 35:361–370. https://doi.org/10.1017/S0022029900019099.

Steele, W. 1969. The effects of dietary palmitic and stearic acids on milk yield and composition in the cow. J. Dairy Res. 36:369–373. https://doi.org/10.1017/S0022029900012875.

Stoffel, C. M., P. M. Crump, and L. E. Armentano. 2015. Effect of dietary fatty acid supplements, varying in fatty acid composition, on milk fat secretion in dairy cattle fed diets supplemented to less than 3% total fatty acids. J. Dairy Sci. 98:431–442. https://doi.org/10.3168/jds.2014-8328.

Sukhija, P. S., and D. L. Palmquist. 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J. Agric. Food Chem. 36:1202–1206. https://doi.org/10.1021/jf00084a019.

Weiss, W. P., and J. M. Pinos-Rodríguez. 2009. Production responses of dairy cows when fed supplemental fat in low- and high-forage diets. J. Dairy Sci. 92:6144–6155. https://doi.org/10.3168/jds.2009-2558.

Weiss, W. P., J. M. Pinos-Rodríguez, D. J. Wyatt. 2011. The value of different fat supplements as sources of digestible energy for lactating dairy cows. J. Dairy Sci. 94:931–939. https://doi.org/10.3168/jds.2010-3745.

Western, M. M., J. S., and A. L. Lock. 2020. Effects of commercially available palmitic and stearic acid supplements on nutrient digestibility and production responses of lactating dairy cows. J. Dairy Sci. 103:5131–5142. https://doi.org/10.3168/jds.2019-17242.

TABLES

Table 4.1. Descriptive statistics for continuous variables used to investigate the relationship between dietary fatty acids and starch with milk fatty acids in lactating dairy cows (n = 23 studies).

Variable	Mean	Mode	SD	Minimum	Maximum
Diet					
C16:0, % DM	0.97	0.63	0.62	0.28	2.68
C18:0, % DM	0.38	0.16	0.57	0.05	2.68
C18:1, % DM	0.87	0.80	0.64	0.16	4.28
C18:2, % DM	1.48	1.52	0.65	0.29	4.45
C18:3, % DM	0.44	0.17	0.72	0.09	3.93
Total FA, g/100g	4.30	5.13	1.54	1.20	8.00
Starch, % DM	24.71	14.60	4.70	14.60	33.40
Forage, % DM	51.47	52.40	8.01	40.00	71.77
Intake					
Dry matter, kg/d	25.64	30.60	4.09	14.70	33.20
C16:0, g/d	275.07	NA	192.74	75.89	754.81
C18:0, g/d	93.11	25.96	154.80	0.50	651.01
C18:1, g/d	161.61	NA	137.41	12.60	950.16
C18:2, g/d	372.12	398.86	151.32	78.06	987.90
C18:3, g/d	99.74	30.60	137.64	27.36	788.10
Total FA, g/d	1076.79	NA	341.41	326.40	1897.20
Starch, kg/d	6.43	6.71	1.92	3.10	10.59
Forage, kg/d	13.12	13.10	2.71	8.85	20.89
Animal Performance[1]					
Days in milk	132	96.50	35	65	213
Milk, kg/d	38.63	44.80	7.63	18.90	49.70
Milk fat, %	3.54	3.25	0.42	2.46	4.54
De novo, % of FA	24.35	27.10	4.07	13.00	31.90
Mixed, % of FA	32.39	35.10	6.71	17.77	45.38
Preformed, % of FA	39.68	37.80	7.61	27.71	64.81
Milk fat, g/d	1367.91	1480.00	320.44	610.47	1986.60
De novo	331.95	NA	112.66	85.34	495.90
Mixed	449.20	NA	177.58	103.12	824.44
Preformed	525.85	534.28	89.58	318.60	711.49

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).

Table 4.2. Effects of dietary fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3% of DM), starch, and forage concentrations (% of DM) on milk fatty acids concentration(g/100g), n = 76 treatment means.

Item[1]	Intercept	Variable	Slope	*P*-value	RMSE	R^2	CCC
		Total milk fatty acids and summation by source (g/100 g)					
De novo	29.2	Diet C16:0	-2.13	0.001	1.48	0.87	0.87
		Diet C18:0	0.40	0.50			
		Diet C18:1	-1.69	0.001			
		Diet C18:2	-2.44	0.001			
		Diet C18:3	-2.06	0.001			
		Diet starch	0.16	0.22			
		Diet forage	-0.02	0.73			
Mixed	26.7	Diet C16:0	3.74	0.001	2.07	0.95	0.95
		Diet C18:0	-0.55	0.39			
		Diet C18:1	-1.58	0.001			
		Diet C18:2	-2.41	0.001			
		Diet C18:3	-1.97	0.001			
		Diet starch	0.56	0.01			
		Diet forage	-0.10	0.14			
Preformed	33.4	Diet C16:0	-1.04	0.16	4.19	0.82	0.83
		Diet C18:0	0.02	0.98			
		Diet C18:1	3.81	0.001			
		Diet C18:2	4.84	0.001			
		Diet C18:3	4.49	0.001			
		Diet starch	-0.21	0.18			
		Diet forage	0.00	0.99			
Total	2.5	Diet C16:0	0.15	0.001	0.18	0.82	0.88
		Diet C18:0	0.02	0.74			
		Diet C18:1	-0.11	0.01			
		Diet C18:2	-0.14	0.001			
		Diet C18:3	-0.09	0.06			
		Diet starch	0.02	0.09			
		Diet forage	0.01	0.22			

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).
RMSE, root mean-squared error of leave-one-out cross-validation; R^2, coefficient of determination; CCC, concordance correlation coefficient of leave-one-out cross-validation.

Table 4.3. Effects of dietary fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3% of DM) starch, and forage concentrations (% of DM) on milk fatty acids yield (g/d), n = 76 treatment means.

Item[1]	Intercept	Variable	Slope	*P*-value	RMSE	R^2	CCC
		Milk fatty acids and summation by source (g/d)					
De novo	326.0	Diet C16:0	13.18	0.18	49.14	0.91	0.89
		Diet C18:0	26.02	0.06			
		Diet C18:1	-17.39	0.06			
		Diet C18:2	-44.68	0.001			
		Diet C18:3	-39.12	0.001			
		Diet starch	7.78	0.02			
		Diet forage	-2.02	0.14			
Mixed	219.9	Diet C16:0	96.4	0.001	49.18	0.97	0.96
		Diet C18:0	12.81	0.38			
		Diet C18:1	-20.87	0.03			
		Diet C18:2	-30.58	0.001			
		Diet C18:3	-39.53	0.001			
		Diet starch	14.79	0.001			
		Diet forage	-2.60	0.11			
Preformed	410.2	Diet C16:0	19.96	0.02	34.86	0.91	0.91
		Diet C18:0	28.93	0.02			
		Diet C18:1	34.72	0.001			
		Diet C18:2	25.18	0.001			
		Diet C18:3	3.94	0.67			
		Diet starch	5.44	0.13			
		Diet forage	-2.09	0.13			
Total	859.09	Diet C16:0	99.48	0.001	91.14	0.96	0.96
		Diet C18:0	68.15	0.02			
		Diet C18:1	-19.77	0.27			
		Diet C18:2	-52.60	0.001			
		Diet C18:3	-68.12	0.001			
		Diet starch	32.12	0.001			
		Diet forage	-5.03	0.11			

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).

RMSE, root mean-squared error of leave-one-out cross-validation; R^2, coefficient of determination; CCC, concordance correlation coefficient of leave-one-out cross-validation.

Table 4.4. Effects of fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3 g/d), starch, and forage intake (kg/d) on milk fatty acids concentration (g/100g), n = 76 treatment means.

Item[1]	Intercept	Variable	Slope	*P*-value	RMSE	R^2	CCC
Milk fatty acids and summation by source (g/100 g)							
De novo	23.4	C16:0 intake	-0.007	0.001	1.68	0.83	0.89
		C18:0 intake	0.001	0.93			
		C18:1 intake	-0.007	0.01			
		C18:2 intake	-0.01	0.001			
		C18:3 intake	-0.008	0.01			
		Starch intake	1.23	0.01			
		Forage intake	0.05	0.85			
Mixed	24.4	C16:0 intake	0.01	0.001	1.86	0.93	0.96
		C18:0 intake	-0.001	0.63			
		C18:1 intake	-0.006	0.03			
		C18:2 intake	-0.01	0.001			
		C18:3 intake	-0.009	0.01			
		Starch intake	2.21	0.001			
		Forage intake	-0.25	0.42			
Preformed	41.0	C16:0 intake	-0.002	0.43	3.35	0.80	0.88
		C18:0 intake	-0.002	0.63			
		C18:1 intake	0.02	0.001			
		C18:2 intake	0.02	0.001			
		C18:3 intake	0.02	0.001			
		Starch intake	-1.84	0.001			
		Forage intake	-0.08	0.83			
Total	3.1	C16:0 intake	0.0004	0.01	0.18	0.80	0.88
		C18:0 intake	0.0001	0.52			
		C18:1 intake	-0.0005	0.06			
		C18:2 intake	-0.0007	0.001			
		C18:3 intake	-0.0004	0.09			
		Starch intake	0.05	0.31			
		Forage intake	0.01	0.60			

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).

RMSE, root mean-squared error of leave-one-out cross-validation; R^2, coefficient of determination; CCC, concordance correlation coefficient of leave-one-out cross-validation.

Table 4.5. Effects of fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3 g/d), starch, and forage intake (kg/d) on milk fatty acids yield (g/d), n = 76 treatment means.

Item[1]	Intercept	Variable	Slope	*P*-value	RMSE	R^2	CCC
Milk fatty acids and summation by source (g/d)							
De novo	184.7	C16:0 intake	0.02	0.61	34.21	0.90	0.95
		C18:0 intake	0.08	0.11			
		C18:1 intake	0.03	0.57			
		C18:2 intake	-0.18	0.001			
		C18:3 intake	-0.14	0.01			
		Starch intake	42.81	0.001			
		Forage intake	-4.68	0.38			
Mixed	119.9	C16:0 intake	0.29	0.001	36.80	0.96	0.98
		C18:0 intake	0.04	0.44			
		C18:1 intake	0.01	0.89			
		C18:2 intake	-0.12	0.01			
		C18:3 intake	-0.15	0.01			
		Starch intake	59.90	0.001			
		Forage intake	-5.03	0.46			
Preformed	360.1	C16:0 intake	0.06	0.02	28.03	0.91	0.95
		C18:0 intake	0.07	0.09			
		C18:1 intake	0.22	0.001			
		C18:2 intake	0.13	0.001			
		C18:3 intake	0.05	0.27			
		Starch intake	20.14	0.03			
		Forage intake	-5.30	0.31			
Total	705.8	C16:0 intake	0.31	0.001	61.98	0.96	0.97
		C18:0 intake	0.21	0.03			
		C18:1 intake	-0.01	0.88			
		C18:2 intake	-0.21	0.01			
		C18:3 intake	-0.25	0.01			
		Starch intake	116.11	0.001			
		Forage intake	-4.90	0.70			

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).

RMSE, root mean-squared error of leave-one-out cross-validation; R^2, coefficient of determination; CCC, concordance correlation coefficient of leave-one-out cross-validation.

Table 4.6. Effect of cutting height (regular and high cut) on individual fatty acid concentrations (g/100g of fat) and total fatty acids concentration (% of DM) of whole-plant corn silage.

	Cutting height			
Item	Regular	High	SEM	*P*-value
C12:0	0.16	0.16	0.00	0.42
C14:0	0.25	0.26	0.01	0.51
C15:0	0.03	0.02	0.00	0.01
C16:0	15.4	15.1	0.08	0.01
C16:1	0.11	0.11	0.00	0.06
C17:0	0.16	0.14	0.00	0.001
C18:0	1.73	1.67	0.02	0.01
C18:1	22.0	22.8	0.08	0.001
C18:2	51.7	52.8	0.15	0.001
C18:3	7.70	6.17	0.15	0.001
C22:0	0.29	0.29	0.00	0.45
C24:0	0.44	0.38	0.01	0.001
Total fatty acids	1.73	1.90	0.03	0.001

Table 4.7. Effect of storage length in days on individual fatty acid concentrations (g/100g of fat) and total fatty acids concentration (% of DM) of whole-plant corn silage.

	Storage length, d									*P*-values	
Item	1	3	5	7	14	28	56	90	SEM	Linear	Quadratic
C12:0	0.15	0.14	0.16	0.16	0.18	0.19	0.15	0.15	0.01	0.14	0.41
C14:0	0.24	0.25	0.26	0.26	0.29	0.28	0.24	0.24	0.01	0.24	0.81
C15:0	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.00	0.95	0.52
C16:0	15.4	15.3	15.3	15.2	15.7	15.4	15.0	14.8	0.16	0.52	0.98
C16:1	0.12	0.12	0.11	0.12	0.10	0.10	0.11	0.10	0.00	0.02	0.55
C17:0	0.15	0.16	0.15	0.15	0.15	0.15	0.14	0.13	0.01	0.60	0.36
C18:0	1.71	1.74	1.69	1.70	1.75	1.74	1.66	1.65	0.03	0.52	0.74
C18:1	22.8	22.5	22.3	22.3	22.0	21.9	22.8	22.5	0.16	0.02	0.27
C18:2	51.4	51.9	52.1	52.5	51.6	51.9	53.2	53.5	0.31	0.02	0.71
C18:3	7.25	7.14	7.06	6.88	7.44	7.54	5.96	6.18	0.29	0.37	0.90
C22:0	0.29	0.29	0.29	0.29	0.30	0.29	0.28	0.29	0.01	0.96	0.66
C24:0	0.41	0.42	0.41	0.41	0.43	0.43	0.39	0.38	0.02	0.73	0.73
Total fatty acids	1.71	1.78	1.97	1.85	1.78	1.66	1.90	1.86	0.06	0.03	0.13

[a,b]Means within the same row with different superscripts differ ($P \leq 0.05$).

Table 4.8. Effect of DM concentration (65 and 70% of as fed) on individual fatty acid concentrations (g/100g of fat) and total fatty acids concentration (% of DM) of high-moisture corn.

Item	Low DM	High DM	SEM	*P*-value
C14:0	0.03	0.03	0.00	0.06
C15:0	0.01	0.01	0.00	0.13
C16:0	11.99	12.67	0.04	0.001
C16:1	0.10	0.10	0.00	0.60
C17:0	0.08	0.07	0.00	0.23
C18:0	1.35	1.38	0.01	0.01
C18:1	28.53	28.81	0.08	0.03
C18:2	56.32	55.88	0.10	0.01
C18:3	1.07	1.05	0.01	0.19
C22:0	0.26	0.26	0.00	0.75
C24:0	0.15	0.15	0.00	0.86
Total fatty acids	2.91	2.84	0.08	0.55

[a,b]Means in the same row with different superscripts differ ($P \leq 0.05$).

Table 4.9. Effect of storage length in days on individual fatty acid concentrations (g/100g of fat) and total fatty acids concentration (% of DM) of high-moisture corn.

	Storage length, d					*P*-value	
Item	7 d	14 d	28 d	56 d	SEM	Linear	Quadratic
C14:0	0.03	0.03	0.03	0.03	0.00	0.10	0.95
C15:0	0.01	0.02	0.02	0.01	0.00	0.68	0.02
C16:0	12.42	12.36	11.80	11.73	0.05	0.001	0.92
C16:1	0.10	0.11	0.10	0.09	0.00	0.04	0.11
C17:0	0.07	0.07	0.08	0.07	0.00	0.83	0.76
C18:0	1.41	1.38	1.34	1.33	0.01	0.001	0.10
C18:1	29.07	28.77	28.51	28.33	0.12	0.001	0.63
C18:2	55.37	55.70	56.58	56.76	0.14	0.001	0.58
C18:3	1.00	1.06	1.05	1.12	0.02	0.001	0.55
C22:0	0.27	0.26	0.26	0.26	0.01	0.27	0.86
C24:0	0.15	0.15	0.15	0.15	0.01	0.33	0.68
Total fatty acids	2.79	2.76	3.03	2.92	0.12	0.21	0.74

[a,b]Means in the same row with different superscripts differ ($P \leq 0.05$).

Table 4.10. Predicted milk fatty acids as affected by dietary concentration or intake of fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage from diets varying in corn silage and high-moisture corn inclusion.

Item[1]	Diet 1	Diet 2	Diet 3	Diet 4
Model 1				
de novo, % FA	25.1	26.5	25.6	27.0
Mixed, % FA	28.5	33.1	31.0	35.6
Preformed, % FA	39.2	37.5	38.6	36.8
Total FA, g/100g	3.2	3.4	3.3	3.4
Model 2				
de novo, g/d	283.9	348.7	318.8	383.6
Mixed, g/d	329.3	454.0	399.9	524.6
Preformed, g/d	462.6	511.2	492.2	540.7
Total FA, g/d	1113.7	1389.8	1267.7	1543.9
Model 3				
de novo, % FA	23.5	27.3	25.3	29.2
Mixed, % FA	28.8	35.0	32.2	38.3
Preformed, % FA	42.5	37.6	40.3	35.4
Total FA, g/100g	3.2	3.3	3.3	3.4
Model 4				
de novo, g/d	252.9	381.3	322.3	450.7
Mixed, g/d	245.6	430.6	350.3	535.4
Preformed, g/d	444.0	538.8	502.5	597.3
Total FA, g/d	1136.4	1527.6	1353.3	1744.6

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).

Diets used for simulation consisted of Diet 1: (DM basis) regular cut (25 cm cutting height) corn silage (30%), alfalfa silage (22.2%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (16.2%) and concentrate (20%). Diet 2: (DM basis) regular cut corn silage (54%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (14.4%) and concentrate (20%). Diet 3: (DM basis) regular cut corn silage (30%), alfalfa silage (22.2%), high-moisture corn (14%), whole cottonseed (4.6%), soybean hulls (9.2%) and concentrate (20%). Diet 4: (DM basis) regular cut corn silage (54%), high-moisture corn (14%), whole cottonseed (4.6%), soybean hulls (7.4%) and concentrate (20%).

Model 1 and 2 inputs were dietary fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage expressed as % of DM. Model 3 and 4 inputs were dietary fatty acids intake (C16:0, C18:0, C18:1, C18:2, C18:3) expressed as g/d and starch and forage intake expressed as kg/d. Intakes was calculated multiplying dietary concentration by a set intake of 32 kg of DM per day.

Table 4.11. Predicted milk fatty acids as affected by dietary concentration or intake of fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch, and forage from different corn silage cutting heights.

	Diet 1		Diet 2	
Item[1]	Regular Cut	High Cut	Regular Cut	High Cut
Model 1				
de novo, % FA	25.1	25.2	26.5	26.7
Mixed, % FA	28.5	29.3	33.1	34.5
Preformed, % FA	39.2	39.0	37.5	37.2
Total FA, g/100g	3.2	3.2	3.4	3.4
Model 2				
de novo, g/d	283.9	294.4	348.7	367.5
Mixed, g/d	329.3	351.4	454.0	493.8
Preformed, g/d	462.6	472.5	511.2	528.8
Total FA, g/d	1113.7	1161.8	1389.8	1476.4
Model 3				
de novo, % FA	23.5	24.0	27.3	28.3
Mixed, % FA	28.8	29.8	35.0	36.8
Preformed, % FA	42.5	41.9	37.6	36.5
Total FA, g/100g	3.2	3.2	3.3	3.4
Model 4				
de novo, g/d	252.9	274.0	381.3	419.3
Mixed, g/d	245.6	278.3	430.6	489.6
Preformed, g/d	444.0	463.1	538.8	573.2
Total FA, g/d	1136.4	1204.0	1527.6	1649.3

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).

Diets used for simulation consisted of Diet 1: (DM basis) regular (25 cm) or high (65 cm) cutting height corn silage (30%), alfalfa silage (22.2%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (16.2%) and concentrate (20%). Diet 2: (DM basis) regular (25 cm) or high cutting height (65 cm) corn silage (54%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (14.4%) and concentrate (20%).

Model 1 and 2 inputs were dietary fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage expressed as % of DM. Model 3 and 4 inputs were dietary fatty acids intake (C16:0, C18:0, C18:1, C18:2, C18:3) expressed as g/d and starch and forage intake expressed as kg/d. Intakes was calculated multiplying dietary concentration by a set intake of 32 kg of DM per day.

Table 4.12. Predicted milk fatty acids as affected by dietary concentration or intake of fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch, and forage from corn silage (CS) harvested at different maturities.

	Diet 1		Diet 2	
Item[1]	Early CS	Late CS	Early CS	Late CS
Model 1				
de novo, % FA	24.3	24.2	25.1	25.0
Mixed, % FA	25.7	26.6	28.1	29.7
Preformed, % FA	40.0	40.3	38.9	39.4
Total FA, g/100g	3.1	3.1	3.2	3.2
Model 2				
de novo, g/d	245.5	256.3	279.5	299.6
Mixed, g/d	253.5	282.6	317.7	369.9
Preformed, g/d	432.3	449.8	456.5	488.1
Total FA, g/d	946.6	1010.6	1089.1	1204.3
Model 3				
de novo, % FA	21.3	21.6	23.4	24.0
Mixed, % FA	25.2	26.4	28.5	30.5
Preformed, % FA	45.2	45.0	42.4	42.2
Total FA, g/100g	3.1	3.1	3.2	3.2
Model 4				
de novo, g/d	178.0	204.3	246.5	293.7
Mixed, g/d	133.2	177.4	228.3	308.0
Preformed, g/d	383.6	416.7	430.2	489.7
Total FA, g/d	901.2	992.1	1104.3	1267.8

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).
Diets used for simulation consisted of Diet 1: (DM basis) corn silage harvested early (1/4 milk line) or late (3/4 milk line) (30%), alfalfa silage (22.2%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (16.2%) and concentrate (20%). Diet 2: (DM basis) corn silage harvested early (1/4 milk line) or late (3/4 milk line) (54%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (14.4%) and concentrate (20%).
Model 1 and 2 inputs were dietary fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage expressed as % of DM. Model 3 and 4 inputs were dietary fatty acids intake (C16:0, C18:0, C18:1, C18:2, C18:3) expressed as g/d and starch and forage intake expressed as kg/d. Intakes was calculated multiplying dietary concentration by a set intake of 32 kg of DM per day.

Table 4.13. Predicted milk fatty acids as affected by dietary concentration or intake of fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage from corn silage (CS) with different storage length.

	Diet 1		Diet 2	
Item[1]	CS 30 d	CS 240 d	CS 30 d	CS 240 d
Model 1				
de novo, % FA	23.9	24.1	24.4	24.6
Mixed, % FA	25.0	25.4	26.8	27.5
Preformed, % FA	40.6	40.4	40.1	39.7
Total FA, g/100g	3.1	3.1	3.1	3.1
Model 2				
de novo, g/d	234.9	240.1	260.4	269.9
Mixed, g/d	238.1	247.4	289.9	306.7
Preformed, g/d	430.3	433.2	453.0	458.3
Total FA, g/d	913.4	933.8	1029.4	1066.0
Model 3				
de novo, % FA	20.5	20.9	22.1	22.6
Mixed, % FA	24.3	24.7	26.8	27.7
Preformed, % FA	46.3	45.8	44.5	43.7
Total FA, g/100g	3.1	3.1	3.2	3.2
Model 4				
de novo, g/d	160.7	170.2	215.2	232.5
Mixed, g/d	111.4	125.0	189.0	213.5
Preformed, g/d	378.7	384.6	421.3	432.0
Total FA, g/d	855.5	883.9	1022.0	1073.1

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).
Diets used for simulation consisted of Diet 1: (DM basis) corn silage stored for 30 or 240 d (30%), alfalfa silage (22.2%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (16.2%) and concentrate (20%). Diet 2: (DM basis) corn silage stored for 30 or 240 d (54%), high-moisture corn (7%), whole cottonseed (4.6%), soybean hulls (14.4%) and concentrate (20%).
Model 1 and 2 inputs were dietary fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage expressed as % of DM. Model 3 and 4 inputs were dietary fatty acids intake (C16:0, C18:0, C18:1, C18:2, C18:3) expressed as g/d and starch and forage intake expressed as kg/d. Intakes was calculated multiplying dietary concentration by a set intake of 32 kg of DM per day.

Table 4.14. Predicted milk fatty acids as affected by dietary concentration or intake of fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage from high-moisture corn with different DM concentrations.

	Diet 1		Diet 3	
Item[1]	HMC 65% DM	HMC 70% DM	HMC 65% DM	HMC 70% DM
Model 1				
de novo, % FA	25.1	25.1	25.6	25.6
Mixed, % FA	28.3	28.5	31.0	31.1
Preformed, % FA	39.2	39.2	38.6	38.5
Total FA, g/100g	3.2	3.2	3.3	3.3
Model 2				
de novo, g/d	283.9	284.2	318.8	319.3
Mixed, g/d	329.3	329.8	399.8	400.9
Preformed, g/d	462.7	462.9	492.3	492.6
Total FA, g/d	1113.7	1114.5	1267.7	1269.9
Model 3				
de novo, % FA	23.5	23.5	25.3	25.3
Mixed, % FA	28.8	28.9	32.1	32.2
Preformed, % FA	42.5	42.5	40.3	40.3
Total FA, g/100g	3.2	3.2	3.3	3.3
Model 4				
de novo, g/d	252.9	253.4	322.3	323.3
Mixed, g/d	245.6	246.3	350.3	351.9
Preformed, g/d	444.1	444.4	502.6	503.3
Total FA, g/d	1136.4	1137.8	1353.3	1356.2

[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).

Diets used for simulation consisted of Diet 1: (DM basis) corn silage (30%), alfalfa silage (22.2%), high-moisture corn at 65 or 70% DM (7%), whole cottonseed (4.6%), soybean hulls (16.2%) and concentrate (20%). Diet 3: (DM basis) corn silage (30%), alfalfa silage (22.2%), high-moisture corn at 65 or 70% (14%), whole cottonseed (4.6%), soybean hulls (9.2%) and concentrate (20%).

Model 1 and 2 inputs were dietary fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage expressed as % of DM. Model 3 and 4 inputs were dietary fatty acids intake (C16:0, C18:0, C18:1, C18:2, C18:3) expressed as g/d and starch and forage intake expressed as kg/d. Intakes was calculated multiplying dietary concentration by a set intake of 32 kg of DM per day.

Table 4.15. Predicted milk fatty acids as affected by dietary concentration or intake of fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage from high-moisture corn with different storage length.

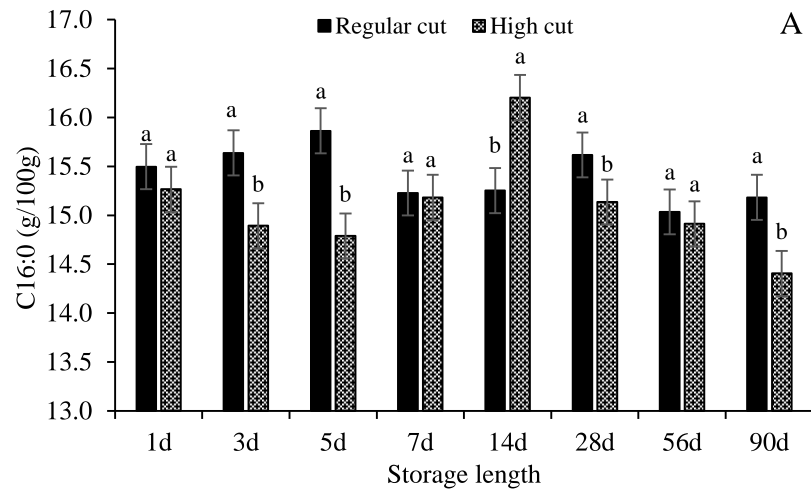
	Diet 1		Diet 3	
Item[1]	HMC 7 d	HMC 56 d	HMC 7 d	HMC 56 d
Model 1				
de novo, % FA	25.1	25.1	25.1	25.1
Mixed, % FA	28.5	28.5	28.5	28.5
Preformed, % FA	39.2	39.2	39.2	39.2
Total FA, g/100g	3.2	3.2	3.3	3.3
Model 2				
de novo, g/d	284.0	284.1	319.0	319.1
Mixed, g/d	329.5	329.6	400.3	400.5
Preformed, g/d	462.7	462.8	492.4	492.6
Total FA, g/d	1114.1	1114.5	1268.5	1269.3
Model 3				
de novo, % FA	23.5	23.5	25.3	25.3
Mixed, % FA	28.9	28.9	32.2	32.2
Preformed, % FA	42.5	42.5	40.3	40.3
Total FA, g/100g	3.2	3.2	3.3	3.3
Model 4				
de novo, g/d	253.1	253.3	322.8	323.0
Mixed, g/d	245.9	246.1	350.9	351.4
Preformed, g/d	444.2	444.4	502.8	503.2
Total FA, g/d	1136.9	1137.5	1354.4	1355.6

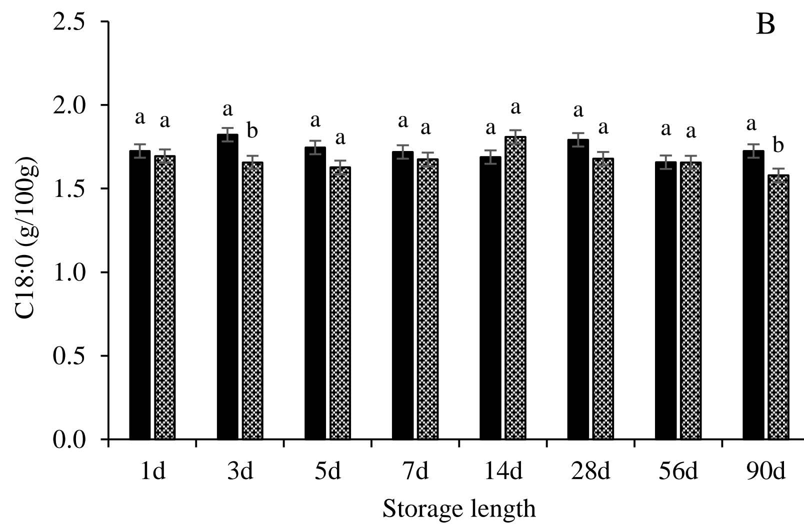
[1]De novo fatty acids originate from mammary de novo synthesis (<16 carbons), preformed fatty acids originate from plasma extraction (>16 carbons), and mixed fatty acids originate from both sources (sum of C16:0 and C16:1).

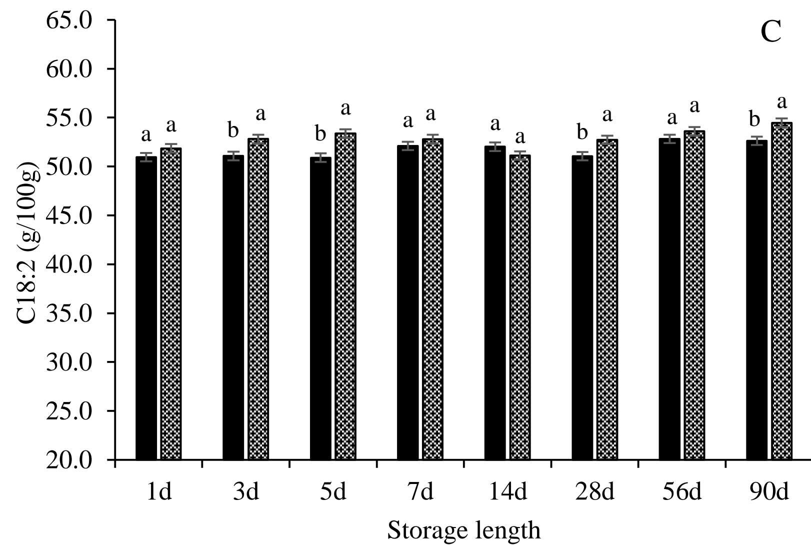
Diets used for simulation consisted of Diet 1: (DM basis) corn silage stored for 30 or 240 d (30%), alfalfa silage (22.2%), high-moisture corn stored for 7 or 56 d (7%), whole cottonseed (4.6%), soybean hulls (16.2%) and concentrate (20%). Diet 3: (DM basis) corn silage (30%), alfalfa silage (22.2%), high-moisture corn stored for 7 or 56 d (14%), whole cottonseed (4.6%), soybean hulls (9.2%) and concentrate (20%).

Model 1 and 2 inputs were dietary fatty acids (C16:0, C18:0, C18:1, C18:2, C18:3), starch and forage expressed as % of DM. Model 3 and 4 inputs were dietary fatty acids intake (C16:0, C18:0, C18:1, C18:2, C18:3) expressed as g/d and starch and forage intake expressed as kg/d. Intakes was calculated multiplying dietary concentration by a set intake of 32 kg of DM per day.

FIGURES







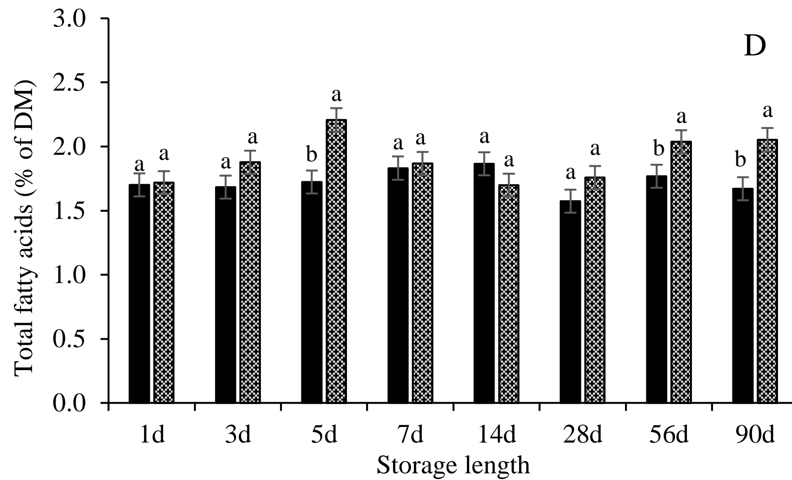


Figure 4.1. Effect of cutting height and storage length on C16:0 (Panel A; $P = 0.01$; SEM: 0.2), C18:0 (Panel B; $P = 0.04$; SEM: 0.04), C18:2 (Panel C; $P = 0.02$; SEM: 0.44) and total fatty acids (Panel D; $P = 0.01$; SEM: 0.08) concentrations in whole-plant corn silage. [a,b] Different superscripts within a day differ ($P < 0.01$).

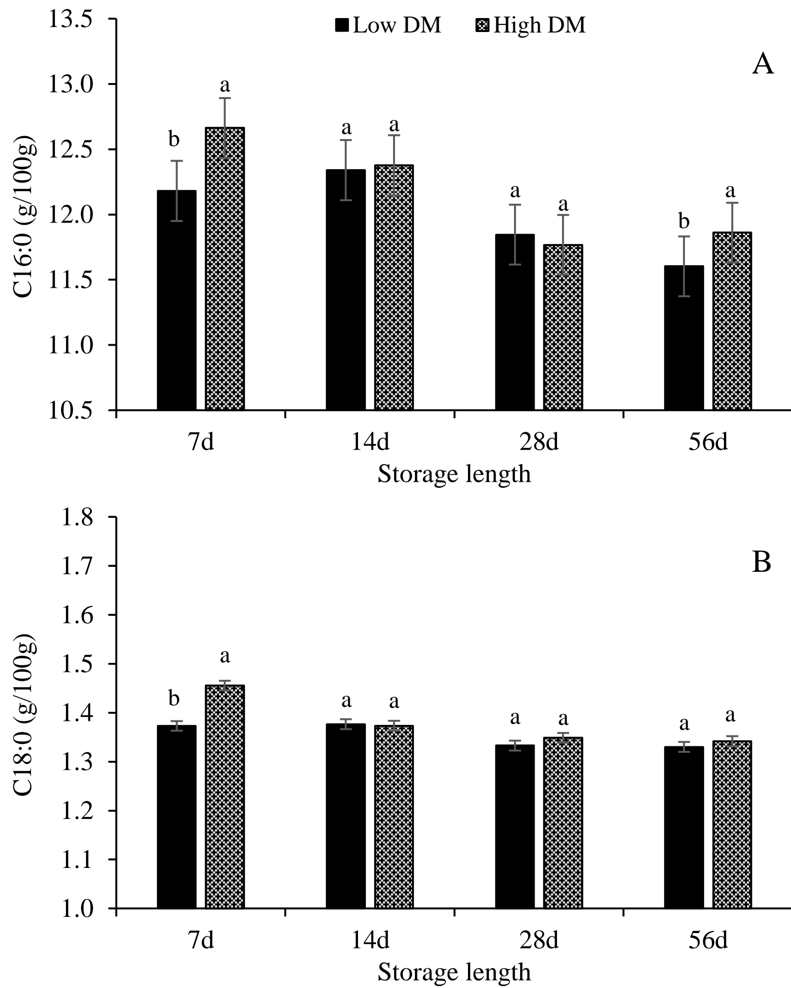


Figure 4.2. Effect of DM concentration and storage length on C16:0 (Panel A; $P = 0.001$; SEM: 0.07) and C18:0 (Panel B; $P = 0.03$; SEM: 0.01) concentrations in high-moisture corn. [a,b] Different superscripts within a day differ ($P < 0.01$).

APPENDIX

Table A1. Summary of the 23 studies (references) used in the meta-regression analysis of milk fatty acids responses to dietary fat, starch and forage.

Reference	n[1]	Fatty acids (% of DM)	Dietary starch (% of DM)[2]	Dietary forage (% of DM)
Benchaar et al., 2012	16	2.49, 4.28, 5.11, 5.77	19.7	48.7
Burch et al., 2021	72	2.53, 3.89, 4.02	32.0	60.1
Chilliard et al., 2009	32	2.30, 5.20, 5.70, 8.00	23.9	63.4
De Souza et al., 2018a	96	1.97, 3.47, 3.47, 3.46	27.4	45.2
De Souza et al., 2018b	40	3.36, 4.85	26.4	44.4
De Souza et al., 2018c	30	3.37, 4.82	26.0	42.0
De Souza et al., 2016	72	2.76, 4.15, 4.12	27.8	44.6
De Souza et al., 2017	96	2.90, 4.81, 4.82, 4.82	25.9	45.8
He and Armentano, 2011	216	2.71, 7.38, 7.65, 7.65, 7.77, 7.55	15.0	59.5
He et al., 2012	343	2.44, 5.28, 6.46, 6.51, 5.32, 4.12, 6.49	21.0	52.4
Marques et al., 2019	96	3.35, 3.38, 3.40, 3.43	26.3	48.0
Prom et al., 2021a	8	3.34	27.0	53.4
Prom et al., 2021b	32	1.83, 3.20, 3.21, 3.21	25.7	51.0
Prom et al., 2021c	8	3.32	25.8	51.8
Relling and Reynolds, 2007	16	2.34, 5.14, 5.15, 5.08	22.6	55.0
Rico et al., 2014	64	4.42, 4.42	27.1	40.1
Shepardson and Harvatine, 2021	48	3.26, 5.13, 5.13, 5.13	32.3	70.7

Sinedino et al., 2017	25	4.10	23.0	43.0
Stoffel et al., 2015	120	1.20, 2.80, 2.90, 2.80	18.4	44.3
Weiss and Pinos-Rodriguez, 2009	72	2.90, 5.00, 3.40, 5.50	27.3	50.0
Weiss et al., 2011	24	2.94, 6.57, 5.90	30.6	55.0
Western et al., 2020	64	4.41, 4.42	28.5	45.5
Western et al., 2021	72	2.71, 4.22, 4.29	26.9	42.8

[1]Number of experimental units used from the study in the analysis.
[2]Dietary starch and forage was averaged across all treatments used in trial.

CHAPTER FIVE: SUMMARY AND FUTURE DIRECTIONS

Feeding starch is a well-known strategy to increase milk production by dairy cows, and thus, corn became a large portion of modern dairy cow diets worldwide. Efficient utilization of starch is dependent upon its digestibility. Even though many strategies to improve corn silage quality exist, hybrid selection is one of the most important aspects of it, as it will affect feeding management during the whole year. Moreover, assessing starch digestibility and how diets affect milk components is a critical task to nutritionists. Therefore, the experiments described herein were conducted to understand a novel technology of corn silage hybrids and its effects on silage fermentation and starch digestibility, to better understand the sources of variation on starch digestibility assays in order to improve sample comparison and to assess the risks of feeding starch and fatty acids from corn silage in milk fat depression. Hopefully, our results will help field nutritionists, farmers, and other members of the dairy industry to deepen the understanding of dynamic processes of evaluating silage starch digestibility and starch feeding management.

The first experiment was conducted to evaluate the effects of ensiling a genetically-modified hybrid that express an alpha-amylase on the fermentation profile, nutrient composition and ruminal in situ starch disappearance of WPCS and earlage ensiled for 0, 30, 60, 90 and 120 d. Overall, minor effects of silage fermentation, microbial counts and aerobic stability were found between a corn hybrid with alpha-amylase and its isogenic counterpart, which suggests that this genetically-modified hybrid can be ensiled for longer periods without concerns of undesirable fermentation or greater loss. Our results, however, failed to support the hypothesis that the expression of an alpha-amylase in the grain would increase starch digestibility. Future research is warranted to understand the effects of feeding this hybrid WPCS and corn grain presented in recent literature.

The second article described a series of experiments to evaluate variation across time-points, sample grinding size and bag pore size related procedures used in ruminal digestibility assays that aimed to understand better starch digestibility assays. Our results showed large variation across laboratorial procedures in starch digestibility assays, however, variation within procedure is acceptable for unfermented samples, even though intrinsic rumen fluid variation is expected. Different grinding sizes and incubation time-points can change sample ranking, and thus, comparisons between samples should be used within the appropriate context of each procedure. Generally, shorter incubation time-points have large variation, and the results should be carefully considered. Further research is warranted to improve reproducibility of starch digestibility assays, by standardization of laboratorial procedures and lastly, research should focus on improve the understanding of starch washout fractions contributions to rumen fermentation.

The third experiment was conducted to investigate the influence of dietary concentrations of fatty acids and starch on milk fat synthesis through a meta-analysis, to evaluate the effect of common silage management practices in fatty acids profile of WPCS and HMC and, to investigate the contributions of fatty acids and starch derived from WPCS and HMC to milk fat secretion. Generally, dietary unsaturated fatty acids negatively affected de novo, mixed and total milk fatty acids, while dietary C16:0 and starch had positive effects on de novo and total milk fatty acids, which failed to support our hypothesis. Our models showed that energy contributions from starch and saturated fatty acids are critical in well-fed cows' milk fat synthesis. Moreover, minor effects of silage management practices on WPCS and HMC fatty acids profile were observed and that is unlikely to increase the risk of milk fat depression. Certainly, further investigation of the combination of factors that cause milk fat depression and its relationship with feed ingredients and nutrients will benefit the dairy industry.

Forage production is one of the most important activities in the dairy industry and production of high-quality corn silage is essential to improve the overall efficiency of dairy production. One of the main strategies to improve nutritional quality of corn is hybrid development, and different hybrids can address specific needs of dairy operations. As hybrid selection plays a major role in the year-round quality of corn silage, perhaps research should focus on developing hybrid technology for each scenario, particularly, genetically-modified hybrids that can increase nutrient digestibility. While greater digestibility of nutrients can be achieved by several different practices, the evaluation of starch digestibility and its relationship to in vivo starch digestion is a gap knowledge that future research should aim to breach. However, prior to standardizing the best laboratorial procedures that better mimic in vivo starch digestion, research should focus on understanding physical and chemical characteristics of individual feeds that affect in vitro and in situ starch digestibility assays. Moreover, external control practices should be implemented for starch digestibility assays to ensure the accuracy of the assay. For example, use of control samples, analyzing samples in triplicates, standardizing material (bags, chemicals and apparatus like grinders) and procedures, and training personnel regularly could help improve repeatability and reproducibility of the assays. Lastly, research of the contributions of silage starch and fatty acids to energy supply and milk fat synthesis should aim to delineate what are the dietary and feeding management conditions that in combination increase the risk of milk fat depression and dietary and feeding management conditions that can increase milk solids production efficiency.