Evaluating the Nutritive Value of Non-Genetically Modified Low-lignin Alfalfa at Multiple Physiological Stages

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ABSTRACT

Alfalfa (*Medicago sativa* L.) is a highly nutritious forage and most economical protein source in dairy cow's diet. However, with advancement in maturity, lignin accumulates in plant cell walls thereby reducing fiber digestibility and feed intake. In recent years, new alfalfa cultivars have been developed for improved digestibility by conventional breeding, but the optimal maturity stage corresponding to maximum fiber digestibility and milk production are still unknown. This study compared the nutritional values of two naturally bred, highly digestible alfalfa cultivars (Boost HG and Amina) with a standard alfalfa cultivar (control) when harvested every 4 days from mid vegetative to late pod maturity stages during two regrowth cycles per year (cuts 1 and 2) over 3 years (from 2020 to 2022). The study was conducted on two sites in Sainte-Anne-de-Bellevue. Nutritive values were determined using near-infrared spectroscopy (NIRS) and calibration equations were specifically developed for this study. Data were analyzed using a linear mixed model methodology and PROC Mixed procedure of SAS with fixed effects of cultivar, maturity stage, and cultivar x maturity interaction. Overall, nutritive values of alfalfa were mostly influenced by maturity stage than cultivar. Nevertheless, when compared to control, Boost HG had higher ($P < 0.0001$) concentrations of acid detergent lignin (ADL) (+6.8%) and neutral detergent fiber (NDF) (+5.3%) but lower ($P < 0.0001$) levels of total digestible nutrients (TDN) (-2%) and net energy of lactation (NEL) (-2.9%) in 2020 (cut 1) and 2021 (cut 2). However, fiber digestibility was not affected ($P < 0.93$). Nutritive values of Amina and control were similar throughout the study on both sites. In conclusion, the two highly digestible alfalfa cultivars (Boost HG and Amina) represent no additional nutritional benefits than conventional alfalfa for local dairy producers.

Key words: dairy cows, highly digestible alfalfa, low-lignin alfalfa, fiber digestibility

RÉSUMÉ

La luzerne (Medicago sativa L.) est un fourrage très nutritif et la source de protéines la plus économique dans l'alimentation des vaches laitières. Cependant, lorsque la plante mature, la lignine s'accumule dans les parois cellulaires, réduisant ainsi la digestibilité des fibres et la consommation alimentaire. Dans les dernières années, de nouveaux cultivars de luzerne ont été développés par sélection conventionnelle pour améliorer la digestibilité. Toutefois, le stade de maturité optimal correspondant à une digestibilité maximale des fibres et à une production laitière maximale est encore inconnu. Cette étude a comparé les valeurs nutritives de deux cultivars de luzerne hautement digestibles développés par sélection naturelle (Boost HG et Amina) avec un cultivar de luzerne standard (témoin). Toutes les luzernes ont été récoltées à tous les 4 jours sur une période de 3 ans (de 2020 à 2022) et 2 coupes par année, débutant du stade végétatif jusqu'au stade de développement des gousses. L'étude a été menée sur deux sites à Sainte-Anne-de-Bellevue. Les valeurs nutritives ont été déterminées par spectroscopie dans la proche infra-rouge (NIRS) avec des équations de calibrations spécifiquement développées pour cette étude. Les données ont été analysées en utilisant un modèle statistique de mixte linéaire et la procédure PROC Mixed de SAS avec des effets fixes du cultivar, du stade de maturité et de l'interaction cultivar x maturité. Les valeurs nutritives de la luzerne étaient plus influencées par le stade de maturité que par le cultivar. Néanmoins, par rapport au témoin, Boost HG avez des concentrations plus élevées ($P < 0.0001$) en lignine (+6.8 %) et de fibres de détergent neutre (+5.3 %) mais des niveaux plus faibles ($P < 0.0001$) en nutriments totaux digestibles (-2%) et d'énergie nette de lactation (-2.9%) en 2020 (coupe 1) et 2021 (coupe 2). Cependant, la digestibilité des fibres n'a pas été affectée ($P < 0.93$). Les valeurs nutritives d'Amina et du contrôle étaient similaires tout au long de l'étude sur les deux sites. En conclusion, comparé à la luzerne conventionnelle, les deux cultivars de luzerne hautement digestibles (Boost HG et Amina) n'offrent aucun avantage dans l'alimentation des vaches laitières.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

MSW	Main Stage Weight
DM	Dry Matter
DMD	Dry Matter Digestibility
NIR	Near Infrared Spectroscopy
NDF	Neutral Detergent Fiver
ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
CP	Crude Protein
NDFD	Neutral Detergent Fiber Digestibility
TDN	Total Digestible Nutriments
NEL	Net Energy of Lactation
SAS	Statistical Analysis Method
GM	Genetically Modified
C:F	Forage: Concentrate
TMR	Total Mixed Ration

CONTRIBUTION OF AUTHORS

This thesis has been written according to the “Guidelines for Manuscript-based Thesis preparation” which has been approved by the Faculty of Graduate Studies, McGill University. I conducted all the experimental procedures, analyzed statistical data and wrote the thesis. Dr. Raj Duggavathi and Dr. Shyam Bushansingh Baurhoo supervised and provided technical assistance throughout the study. Dr. Philippe Seguin provided help on experimental strategies. Dr. Shyam Bushansingh Baurhoo designed the experiments and provided funding throughout the project.

CHAPTER 1

1.1 GENERAL INTRODUCTION

In Canada, the total sale of food and beverage processing was $141.3 billion in 2021. The dairy industry accounted for 11.4 % of the national market and ranks second in the Canadian agricultural sector, just behind the meat industry (Agriculture and Agri-Food Canada, 2022). Approximately 82% of dairy farms are located in the provinces of Ontario and Quebec (Agriculture and Agri-food Canada, 2021).

Forages constitute a fundamental component at the base of the dietary pyramid for dairy cow (Tricarico, 2016), making it a crucial focus in the development of the dairy industry. As a primary source of nutrients for ruminants, forages play important role in maintaining animal health, supporting productivity, and ensuring milk quality (Barnes & Marten, 1979). Another vital component of dairy cow diet is concentrates, often referred to as supplemental feed or grain, which is a highly nutritious feedstuff that complements the nutritional profile of forage. Concentrates typically contain higher levels of energy and protein, while forage forms the foundation of the diet providing essential fiber for proper rumen functions and health. The ratio of concentrate to forage in dairy cow ration can vary depending on factors such as forage quality and nutritional requirements of cows.

Forages are often more cost-effective compared to concentrates, and increasing the proportion and utilization efficiency of forage may enhance the sustainability and profitability of the industry. The digestibility of forage is a key determinant of its quality (Eastridge, 2006). Higher digestibility means more efficient utilization of nutrients and higher feed intake, resulting in improved milk production and overall animal performance. To shift towards a higher forage-based diet, it is essential to understand and improve the quality of commonly used forages.

Alfalfa is regarded as "queen of forages" (Barnes & Marten, 1979), and holds a prominent position (Barros et al., 2014) in dairy cow rations due to its high nutritional value and palatability. This leguminous forage crop is rich in fiber, protein, minerals, and vitamins, making it an excellent source of essential nutrients for dairy cows (Annicchiarico et al., 2015). Additionally, alfalfa has deep root system which can improve soil fertility and health. Like other leguminous plants, alfalfa can fix nitrogen into the soil from the atmosphere. Therefore, the cultivation of leguminous forages may allow farmers to reduce utilization of synthetic nitrogen fertilizers, thereby lowering input costs and minimizing environmental impacts.

Lignin is a complex structural carbohydrate located in cell wall of plants. It is the indigestible portion of forage for dairy cow. Reduction in lignin content can be achieved by both traditional breeding methods and genetic engineering techniques (Sulc et al., 2016; Barros et al., 2019). Low-lignin alfalfa was developed to improve forage digestibility and nutrient availability for ruminants. This innovation of highly digestible alfalfa can potentially improve feed efficiency, milk production, and reduce concentrate levels in diets (Undersander et al., 2009). Many farmers and most importantly consumers are reticent to the use of genetically modified (**GM**) crops. To this end, non-GM or naturally selected alfalfa for high digestibility is a preferable option. However, there is relatively limited research on non-GM highly digestible alfalfa with regards to any improvement in nutritional values for feeding dairy cows that would eventually translate into higher feed intake and milk production.

1.2 Objectives

To compare a standard alfalfa with two different naturally-selected (non-GM) alfalfa cultivars for high digestibility over a range of physiological stages (i.e. from vegetative to pod).

The parameters evaluated were:

- Nutritive values (DM, CP, ether extract, NDF, ADF, ADL)
- In-vitro fiber digestibility at 12h, 30h, 48h and 240h
- Estimation of net energy of lactation (NEL) and total digestible nutrients (TDN)

1.3 Hypotheses

The hypothesis of this study was that, when compared to standard alfalfa, the two naturally-selected alfalfa for high digestibility shall improve forage quality (i.e. nutritive values) and fiber digestibility when harvested at same physiological stage thereby increasing feed intake and milk production when fed to dairy cows.

CHAPTER 2. LITERATURE REVIEW

2.1 Forage

Forages are plants consumed by grazing animals or used as feed for livestock. In Canada, dairy cows are mostly housed in barns or confined areas instead of grazing on pasture. Dairy cows obtain their required nutrients from TMR (total mixed ration), which typically consists of a combination of forages and concentrates (i.e. grains and soybean meal). In general, the ratio of forages to concentrates, known as the C:F ratio, should fall within the range of 40:60 to 60:40 (Mertens, 2009). Data from 350 dairy farms showed that TMR had an average forage to concentrate (F:C) ratio of 53% forage to 47% concentrate on a dry matter basis (Tricarico, 2016).

Forage fiber is necessary for proper rumen health and productivity of dairy cows. The inclusion of a minimum of 40% forage in TMR may reduce the risk of ruminal acidosis and improve palatability (Stelletta et al., 2007; Yang & Beauchemin, 2009). Dietary fiber promotes chewing, saliva secretion and stimulates rumination. During rumen fermentation, microbes break down fiber and yield microbial protein as a byproduct (Schwab & Roderick, 2017). This microbial protein serves as a major source of protein for dairy cow, and is recognized as high-quality protein due to its high digestibility and ideal amino acid profiles for milk synthesis. Two-thirds of the amino acids absorbed by ruminants are attributed to microbial protein (Pathak, 2008; Schwab & Roderick, 2017). Adequate amino acid supply through diets is required for milk production and animal health.

Forages serve as a basic feed component providing essential nutrients for dairy cows, whereas concentrates may serve to complement the nutritional profile of forage. By increasing the proportion of forage in the TMR, dairy producers can lower their reliance on more expensive concentrate feeds (Beauchemin & Rode, 2012). Therefore, maximizing forage utilization in dairy cow rations can promote animal health and reduce feed costs (Beauchemin & Rode, 2012).

2.2 Performance of dairy cow and feed quality

The productivity of dairy cows is influenced by various factors, but feed quality is the primary determinant. Feed quality significantly impacts both the digestibility of feed and the daily intake of dairy cows (Beauchemin & Rode, 2012). Dairy cows fed high-quality forage diet tend to exhibit improved resistance to parasites and diseases, as well as enhanced reproductive performance (Ball et al., 2001).

High-quality feed can provide sufficient nutrients for dairy cows, thereby contributing to the maintenance of health and supporting milk production (Barnes & Marten, 1979). The herd average milk yield has reached 12,500 kg per cow per lactation (Eastridge, 2006). With continuous improvement in animal productivity, higher Net Energy for Lactation (NEL) is needed to meet the nutritional requirements of dairy cows. However, feed intake is limited to 27 kg of dry matter per cow per day (Eastridge, 2006), principally due to rumen fill effect and limited rumen passage capacity. Consequently, improving feed quality is a viable option for improved intake and productivity in cows (Eastridge, 2006).

Forage quality can significantly impact milk production. Dairy cows fed a high-quality forage diet were found to yield more milk while requiring less concentrate supplementation compared to those fed low-quality forage (Linn & Kuehn, 1996). The same authors reported that lowering forage quality resulted in decreased milk production (Linn & Kuehn, 1996). Therefore, high-quality forage plays an important role in improving the productivity of dairy cows.

2.3 The importance of alfalfa

Alfalfa (*Medicago sativa* L.) is a perennial flowering plant in the legume family Fabaceae. It is the most cultivated forage legume with around 450 million tons grown on 30 million hectares of land around the world (Barros et al., 2014). In Canada, alfalfa ranks the fourth largest crop by area (Statistics Canada, 2022). It has the advantage of persisting over multiple growing seasons, providing reliable forage production and soil health benefits over time.

Alfalfa is regarded as the "queen of forages" (Barnes & Marten, 1979). It is well established as one of the most nutritious forage crops for dairy cows. The high nutritional quality of alfalfa can satisfy demands of ruminants efficiently. It contains 15-20% crude protein on dry matter basis (Beauchemin & Rode, 2012; Annicchiarico et al., 2015). Research conducted on composition of forages grown in Canada from 2010 to 2011 show that alfalfa hay contains 19.5% of protein, while alfalfa silage contains 17.9% of protein (Beauchemin & Rode, 2012); these values were higher than the protein content of barley silage and corn silage which were 11.5 and 8.4%, respectively (Beauchemin & Rode, 2012). Alfalfa, as protein-rich plant, can provide essential amino acids necessary for milk yield and the synthesis of milk proteins.

High-quality alfalfa enables ruminants to acquire adequate protein as well as fiber, minerals, and energy. Forage with higher dry matter digestibility (DMD) and higher neutral detergent fiber digestibility (NDFD) can provide more nutrients. In a study conducted by Gadeken & Casper (2017), alfalfa haylage possessed a dry matter digestibility (DMD) of 75.7% and a NDFD of 55.7% whereas DMD and NDFD of corn silage were 72.9% and 52.3%, respectively (Gadeken & Casper, 2017). Therefore, high-quality alfalfa had better nutrient contents compared to corn silage.

2.4 Forage accumulation and quality

2.4.1 Tradeoff between yield and quality

Alfalfa yield and quality are negatively correlated. On dairy farms, this tradeoff between higher forage yield and higher feed quality poses a great challenge for producers. Delaying harvest date from 30 days to 40 days resulted in 28% or 1.3 kg per ha higher DM yield (Grev et al., 2017). However, harvesting alfalfa at an advanced stage of maturity has been associated with lower nutritive value (Brink et al., 2010; Grev et al., 2020) especially during early summer harvests. The decline in NDFD is more pronounced with days during summer compared to spring (Sanderson and Wedin, 1988; Kallenbach et al., 2002; Brink et al., 2010;). Moreover, alfalfa maturity has major impact on milk production of high producing cows. In contrast to early-maturity alfalfa, feeding cows alfalfa harvested at mid and late maturity reduced milk production by 1.4 and 4.1 lb/day respectively (Linn & Kuehn, 1996).

Maturation of alfalfa has direct impacts on the levels of crude protein, NDF, ADF and digestibility of leaf and stem. The advancement of alfalfa maturity is accompanied with increased dry matter yield and fiber contents whereas crude protein levels and fiber digestibility are reduced (Grev et al., 2017; Barros et al., 2019; Grev et al., 2020). A study collected alfalfa samples at three distinct growth stages, which were at 21 days, 28 days, and 35 days, respectively. When harvesting date was delayed from 21 days to 35 days, crude protein contents were reduced from 20.8% to 17.3% and further to 17.0%, whereas the levels of indigestible NDF (add the values for iNDF here) and acid detergent lignin (6.3% to 6.9% and then to 7.3%) were increased (Palmonari et al. 2014). Based on the lower protein and energy levels, milk production was also expected to decrease by 2.8 kg and 1.4 kg per day from the 35-days harvesting interval, respectively (Palmonari et al. 2014).

2.4.2 Harvest window for alfalfa

The harvest window for alfalfa typically depends on several factors, including the stage of maturity, weather conditions and the use of the forage. Generally, the optimal time to harvest alfalfa is at pre-flowering stage for dairy producers. Alfalfa in pre-flowering stages (late-bud stage) accumulate dry matter while preserving its nutritive value and digestibility (Yu et al., 2003).

Weather condition is a factor that can impact the growth and development of alfalfa. Alfalfa quality tends to be lower during the summer period compared to spring-harvested alfalfa, although alfalfa is harvested at same stage of maturity. This is because higher temperatures accelerate the maturity process (Sanderson and Wedin, 1988;

Brink et al., 2010). Therefore, the harvest window for alfalfa may be shorter in some areas due to hot weather conditions.

Alfalfa may be harvested at earlier stage of maturity for higher forage quality containing more digestible nutrients from both leaf portion and stem portion. At early maturity stage (i.e. budding), alfalfa has higher crude protein concentration, lower Acid Detergent Lignin (ADL) content, and higher NDFD (Grev et al., 2020). However, opting for an early harvest may compromise dry matter yield. Therefore, producers must carefully balance between yield and quality when managing time of harvests and forage requirements for animal feeding.

2.5 Physiological and morphological changes as alfalfa mature

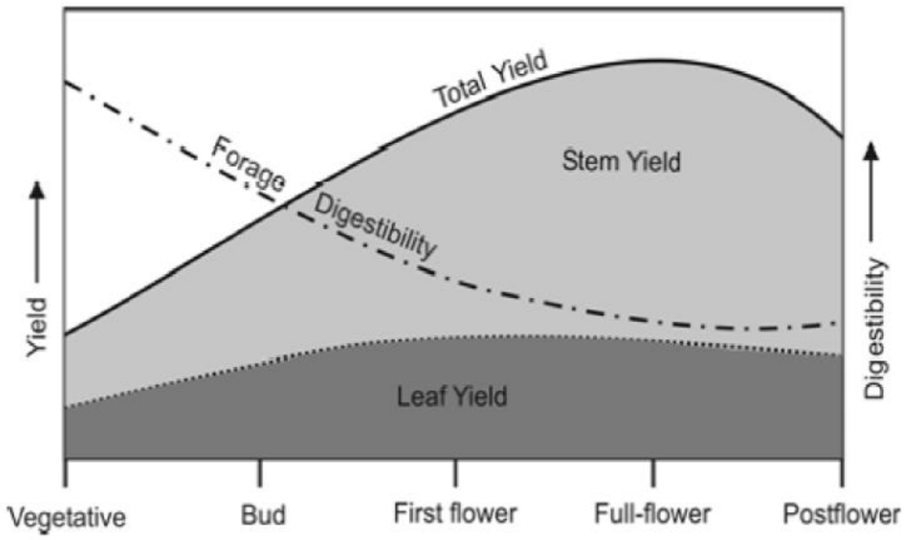
2.5.1 Physiological changes in leaf vs. stem

The leaf portion of alfalfa typically has higher nutritive value than stem fractions throughout the entire growth stage (Grev et al., 2020). Alfalfa leaves were found to have higher In Vitro Digestibility Coefficients of Dry Matter (IVDDM) and higher crude protein concentrations (Buxton et al., 1985; Fan et al., 2018). The higher digestibility and nutritional value associated with leaf fractions were linked to lower cell wall concentration and different cell wall components (Hatfield et al., 1999; Bhandari et al., 2023;). Conversely, the lower digestibility observed in stems was attributed to higher concentrations of cell wall polysaccharides and lignin (Grev et al., 2020). Cross-linking xylans to each other and to lignin in the cell wall leads to reduced degradability (Hatfield et al., 1999). However, these physiological changes are less pronounced within leaf fractions (Buxton et al., 1985; Buxton & Hornstein, 1986; Albrecht et al., 1987; Sheaffer et al., 2000; Marković et al., 2012; Grev et al., 2020). A more gradual physiological change in stem fraction results in a slower decline in the digestibility of the entire plant, thereby facilitating the accumulation of higher quality alfalfa forage.

2.5.2 Morphological changes as alfalfa mature

Morphological changes persist as alfalfa matures. Stem fractions have been shown to exert a stronger influence on forage quality (Jung & Lamb, 2006; Grev et al., 2020) due to significant increase in stem proportions as alfalfa matures (Sheaffer et al., 2000; Jung & Lamb, 2006). Stems make up 45% to 70% of the total forage biomass (Sheaffer et al., 2000; Lamb et al., 2003; Grev et al., 2020). Therefore, any loss in nutritive value of the stem fraction due to advancement in maturity will severely impact the overall forage quality of the entire plant. Additionally, as alfalfa matures, leaves undergo senescence and shedding, further diminishing the leaf-to-stem ratio (Buxton et al., 1985).

Figure 1. Relative forage yield and quality at different alfalfa growth stages.



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Source: Balancing Yield. Quality and Persistence. Steve Orloff and Dan Putnam 2004 Proceedings CA Alfalfa symposium

2.6 Quantifying morphological development of alfalfa

Growth stages of alfalfa have been described and standardized by Kalu and Fick in 1981. Their quantitative method for identifying the morphological stage of alfalfa has been widely acknowledged and adopted by researchers. Individual stem of alfalfa can be classified using the 10-stage numerical system ranging from stage 0 to 9 (Kalu & Fick, 1981) (Table 1).

Identification of the morphological differences, such as length of stems and the number of buds, flowers and pod, allows for classification of stems into different stages from early vegetative to ripe seedpod stages. To determine the average stage for a group of alfalfa plants, two methods can be employed: Mean Stage by Count (MSC) and Mean Stage by Weight (MSW) (Kalu & Fick, 1981).

Both methods quantify morphological development of alfalfa canopies with the consideration of anticipated effects of seasonal temperature. MSW calculates the average of individual stages present and weighted by the dry weight of alfalfa in each stage (Kalu & Fick, 1981). This method is superior in showing greater numerical differentiation and in distinguishing samples of diverse age and stage structure (Kalu & Fick, 1981).

Table 1. Definition of morphological stage of development for individual alfalfa stems

Stage number	Stage name	Stage definition
0	Early vegetative	Stem length ≤15 cm; no buds, flowers, or seed pods
1	Mid-vegetative	Sten length 16 to 30 cm; no buds, flowers, or seed pods
2	Late vegetative	Stem length, ≥ 31 cm; no buds, flowers, or seed pods
3	Early bud	1 to 2 nodes with buds; no flowers or seed pods
4	Late bud	≥ 3 nodes with buds; no flowers or seed pods
5	Early flower	One node with one open flower (standard open); no seed pods
6	Late flower	≥ 2 nodes with green seed pods
7	Early seed pod	1 to 3 nodes with green seed pods
8	Late seed pod	≥ 4 nodes with green seed pods
9	Ripe seed pod	Nodes with mostly brown mature seed pods

2.7 Lignin and lignification

Lignin is a phenolic polymer that is indigestible to rumen microbes. Approximately 40% to 50% of the forage yield is derived from cell walls which consist of cellulose, hemicellulose, lignin, pectin, and protein (Sheaffer & Undersander, 2015). The digestibility of cell walls varies and is unstable due to the different concentrations of these components. At advanced maturity stages, alfalfa contains higher lignin concentrations, which can hinder forage digestibility. Reducing lignin concentration may improve digestibility, thereby improving animal performance (Barros et al., 2018).

Lignin constitutes approximately 6-9% of the dry weight of alfalfa and accounts for about 20% of its cell walls (Hatfield et al., 2007; Sheaffer & Undersander, 2015). Cell walls serve as conduits in the vascular system, facilitating the transport of water and nutrients within the plant (Sheaffer & Undersander, 2015). In secondary cell walls, lignin is the second predominant component (Li et al., 2015; Grev et al., 2017). The accumulation of lignin within secondary cell walls provides strength and rigidity supporting plant structures and enabling plants to stand upright (Guo et al., 2001; Sheaffer & Undersander, 2015; Grev et al., 2017). For alfalfa, lignin types differ between leaf and stem fractions. Alfalfa leaves primarily contain guaiacyl-type lignin, while alfalfa stems predominantly contain guaiacyl-syringyl-type lignin (Kondo et al., 1998; Marković et al., 2012).

The lignification of alfalfa stems has significant impacts on its digestibility. In a study by Jung & Engels (2001), major discrepancies were found in the degradation characteristics among different cell wall tissues of alfalfa stems. Non-lignified cell walls were found to be completely degradable irrespective of tissue thickness (Jung & Engels, 2001). Conversely, lignified tissues exhibited diverse patterns of degradation, depending on the distribution of lignin within the tissues (Jung & Engels, 2001).

Lignification of cell walls is recognized as a primary factor that hinders the degradation of alfalfa (Kondo et al., 1998; Marković et al., 2012). It plays a important role in determining the IVDMD of harvested alfalfa forage (Grev et al., 2020). Alfalfa containing high concentration of lignin led to insufficient intake of nutrients which can negatively affect animal performance (Moore et al., 2001).

2.8 Low-lignin forages

Brown midrib (BMR) trait is a genetic characteristic discovered in some varieties of forage crops such as corn and sorghum. This trait results in reduced lignin content in the plant's cell walls. BMR forages are typically with higher digestibility (Oliver et al., 2005; Beck et al., 2007). Many studies indicated that BMR forages can improve dairy cow performance.

The higher fiber digestibility of BMR corn silage led to a faster rate of passage of particulate matter from the rumen, which could contribute to increase in DM intake (Eastridge, 1999). Crosses studies show that feeding BMR corn silage has generally resulted in higher DM intake compared to non-BMR corn silage. The average increase is approximate 3.6 lb/cow/day (Eastridge, 1999). The range of increase in DM intake varied from 0.9 to 7.3 lb/cow/day (Eastridge, 1999). Cows fed BMR corn silage produced 2.1 lb/day more milk compared to cows fed the control silage (Eastridge, 1999). Study by Gencoglu et al., cows fed bm3 corn silage consumed 1.2 kg more DM per day and produced 1.7 kg more milk per day (Gencoglu et al., 2008).

Bmr-18 and bmr-6 forage sorghum are low-lignin forages used to improve animal performance in dairy industry. Study by Oliver et al., (2005), The in situ extent of NDF digestion was 76.4% from bmr-6 sorghum diet, 73.1% from bmr-18 sorghum silage diet, and least from the conventional sorghum diet was 70.4% (Oliver et al., 2005). Among the different types of sorghum fed to the cows, those fed with bmr-6 sorghum produced the highest amount of 4% fat-corrected milk, the average was 33.7 kg/day. Cows fed with bmr-18 sorghum produced an average of 31.2 kg/day, while cows fed with conventional sorghum produced the lowest amount of an average of 29.1 kg/day (Oliver et al., 2005).

2.9 Low- lignin alfalfa

The development of low-lignin alfalfa cultivars aims to address the challenges associated with forage indigestibility, and more specifically stem digestibility.

The advantages of low-lignin alfalfa cultivars are lower degree of lignification within stems, higher leaf-to-stem ratio, and/or lower herbage maturity (Buxton et al., 1985; Sulc et al., 2016; Barros et al., 2019). Low-lignin alfalfa cultivars are produced either through genetic modification (Sulc et al., 2016; Barros et al., 2019) or traditional selective breeding approaches (Sulc et al., 2016).

The inclusion of genetically modified (**GM**) low-lignin alfalfa forage into cow's diet resulted in a 2.6 lbs/head/day higher milk yield than feeding conventional alfalfa forage (Undersander et al., 2009). Lately, non-GM low-lignin alfalfa cultivars have been developed with objective to increase feed intake and milk production. Data show a 7% to 10% reduction of lignin content from non-GM alfalfa which led to approximately 12% increase in total tract digestibility (by NIR analysis) (Alforex, 2014; Sheaffer & Undersander, 2015).

2.9.1 Genetically modified (GM) alfalfa

The synthesis of lignin in plants involves action of more than ten different enzymes (Getachew et al., 2018). By suppressing the activity of any of these enzymes or combinations, it is possible to alter the concentration and composition of lignin in genetically modified plants (Guo et al., 2001; Getachew et al., 2018). Genetic modifications targeting these enzymes successfully led to a decrease in lignin accumulation in alfalfa.

The lignin content was reduced by 13% in alfalfa with downregulated CCoAOMT and by 24% in lines with downregulated COMT, compared to their respective control groups (Getachew et al., 2011). The downregulation of COMT and CCoAOMT has been demonstrated to be effective in improving the digestibility of forage crops. Alfalfa with downregulated COMT exhibited a 16% increase in IVDMD, while downregulated

CCoAOMT showed a 4% increase in IVDMD, both compared to their respective control groups (Getachew et al., 2011).

2.9.2 Non-genetically modified low-lignin alfalfa

Non-genetically modified (non-GM) low-lignin alfalfa varieties with reduced lignin content have been developed through traditional breeding methods rather than genetic modification techniques. Non-genetically modified low-lignin alfalfa variety typically exhibits physical characteristics such as thinner stems, large leaves, higher leaf to stem ratio (Damiran et al., 2022), and improved resistance to leaf diseases and insect pests (Buxton et al., 1985; Juan et al., 1993). This combination of traits can contribute to the overall forage quality of alfalfa.

Non-genetically modified low lignin alfalfa varieties successfully exhibited reduced lignin concentrations compared to conventional varieties. A variety (Hi-Gest 360) showed a 4.2% reduction in lignin concentration (Damiran et al., 2022), while another variety (Hi-Gest) had 8.6% lower lignin (Min et al., 2016). These findings suggest that non-GM low-lignin alfalfa varieties have the potential to improve forage digestibility and overall nutritional quality.

2.9.3 Harvest window of low-lignin alfalfa

Compared to conventional alfalfa cultivars, low-lignin alfalfa offers a wider harvest window due to less intensive lignification with advancement of maturity. In alfalfa, lignification is negatively correlated with forage quality (Albrecht et al., 1987; Grev et al., 2020). Compared with conventional alfalfa cultivars at various maturity stage, GM low-lignin alfalfa can maintain 9, 10, and 12% greater level of Relative Forage Quality (RFQ) at 30-days, 35-days, and 40-days harvesting interval (Grev et al., 2017). Low-lignin alfalfa can be harvested later without losing significant nutritive value. Consequently, harvest management of low-lignin alfalfa is considered to be more flexible due to a wider harvest window.

Research by Grev et al. (2017) indicates that when harvest date of GM low-lignin alfalfa was delayed by 5 to 10 days (average 35 to 40 days between harvests), there was 20 % increased forage yields without any decrease in RFQ compared to conventional alfalfa which was under a 30-day harvesting management. According to Damiran et al. (2022), delaying the harvest date of GM low-lignin alfalfa by up to two weeks may yield the same or higher crude protein content while maintaining the same forage yield.

2.10 Nutritive values of forage

The determination of nutritive values of harvested forages is crucial for ration formulations on commercial farms. The keys parameters include dry matter, crude protein, ash, ether extract, NDF, ADF, ADL, and fiber digestibility. These parameters provide essential information about the nutritional composition and digestibility of the feed, which directly impacts the health, productivity and performance of the dairy cows.

Dry matter is the actual nutrient content in feed after removing moisture. By controlling the dry matter content in dairy ration, farmers can more accurately regulate the daily nutrient intake of cows, ensuring they receive sufficient energy, protein, and other essential nutrients. A mature dairy cow will consume DM of approximately 2.5-4.5% of her body weight (USDA, 2010). Adequate DM can stimulate rumen motility and saliva secretion in cows, facilitating digestion and nutrient absorption (Beauchemin et al., 2008; Castillo-Lopez et al., 2021), thereby maintaining digestive health and milk production performance in dairy cows.

NDF represents the total fiber content in feed including cellulose, hemicellulose and lignin. Higher levels of NDF indicate a greater proportion of fiber in the feed. Feed stays in the digestive system for a longer period as the level of NDF increases due to fiber has a filling effect in the rumen which can limit DMI (Allen, 2000; NRC, 2021). ADF is the least digestive portion of NDF, composed mainly of cellulose, lignin and a minor mineral. Degradation of cellulose is slow while ADL is indigestible. ADF determine digestibility of forage and NEL. The recommended total NDF content in the dairy ration range from 25% to 33% (DM basis) (Linn, 2003). Correspondingly, the forage NDF is advised to be within the range of 19% to 15%, while the ADF content should fall between 17% and 21% (Linn, 2003).

NDF content is positively correlated with rumination and chewing activity in cows. Appropriate levels of NDF are necessary to stimulate rumination, maintain rumen health, and support optimal milk production. Study by Beauchemin et al. (2008), ensalivation (g of saliva/g of DM) for straw, barley silage, alfalfa silage, and alfalfa hay are 7.23 4.15, 3.40, and 4.34 g/g of DM, respectively (Beauchemin et al., 2008). Cows ingest concentrate more rapidly than forages on a dry matter basis. The rate of consumption varies from 3 to 12 times faster. However, the insalivation of concentrate was 1.12 g/d of DM, which is significantly lower than for forages (Beauchemin et al., 2009). Appropriate NDF in feed can prevent insufficient saliva secretion, thereby reducing the risk of low rumen pH, which can lead to acidosis. High quality forage can provide sufficient NDF containing lower ADF/ADL, thereby contribute to rumen health and milk production.

Fat in the dairy ration has impact on both post-ruminal fat digestion and the digestibility of fatty acids, and there may be optimal levels of fat intake for maximizing digestibility. Forages typically contain relatively small amounts of fat compared to other components like fiber and proteins, analyzing the EE content in forages can still provide valuable information for formulating dairy rations. The apparent digestibility of fat increased when supplemental fat was increased from 0 to 3% of DM in the diet. However, when fat was further increased from 3 to 6 % DM, the apparent digestibility decreased (NRC, 2001). As the proportion of unsaturated fatty acids in the diet increases, the overall digestibility of the diet tends to improve. Unsaturated fatty acids are typically more easily broken down and utilized by rumen microbes, leading to better nutrient absorption. However, while increased unsaturation may enhance digestibility, it can also have adverse effects on rumen fermentation (NRC, 2001).

Dietary protein is typically measured as crude protein, which is calculated based on the nitrogen (N) content x 6.25 (NRC, 2001). There was a quadratic relationship between milk yield and dietary CP concentration. As the CP concentration in the diets increased, milk yield initially increased, the rate of increase slowed down and may have reached a plateau (NRC, 2001). Metabolizable protein, which is crucial for milk protein synthesis, is derived from microbial protein synthesized in the rumen, dietary rumen-undegradable protein, and endogenous protein produced by the animal's own tissues. These components collectively contribute to the pool of protein available for milk synthesis in animals (Clark et al., 1992). The importance of microbial CP, which contains a balanced profile of essential amino acids, in supporting milk and milk protein production in ruminant animals. It suggests that increasing the yield of microbial CP in the rumen can positively impact milk production and milk protein yield (NRC, 2001). Legume forages can provide sufficient nitrogen to rumen microbials, thereby improve microbial CP supply. However, excessive dietary protein levels beyond the requirement for milk production in dairy ration have detrimental effects on reproductive processes (NRC, 2001).

2.10.1 Fiber and in vitro fiber digestibility of alfalfa

Fiber can be separated into two categories, namely neutral and acid detergent fiber (Soest et al., 1991; Jung & Lamb, 2006). The fiber fractions which are insoluble in neutral detergent solution include cellulose, hemicellulose and lignin while the acid detergent insoluble fiber fractions include cellulose, lignin and ash (Ball et al., 2001). Acid Detergent lignin (ADL) is the indigestible component of ADF. Low-lignin alfalfa contain approximately 4% to 14% lower ADL in comparison with conventional alfalfa (Marita et al., 2003; Grev et al., 2020; Cherney et al., 2020; Damiran et al., 2022). In general, lower ADL concentration in forages is linked with higher fiber digestibility. Although NDF concentration between low-lignin and conventional alfalfa was found to be similar (Grev et al., 2017), low-lignin alfalfa had higher NDFD (Getachew et al., 2011; Grev et al., 2017, 2020; Sulc et al., 2020; Boucher et al., 2023). GM low-lignin alfalfa exhibited 8 to15% improvement of stem NDFD regardless of seeding year or production year (Grev et al., 2020). The study by Boucher et al. (2023) showed a 10%

higher NDFD in the entire GM low-lignin alfalfa plant compared to standard alfalfa. Therefore, GM low-lignin alfalfa can provide more digestible fiber to dairy cows thereby improving milk yields.

2.10.2 Protein contents of alfalfa

Studies have indicated similar concentrations of protein in both conventional and low-lignin alfalfa varieties (Getachew et al., 2011; Grev et al., 2017; Boucher et al., 2023). The proper harvesting management had profound effects on maintaining protein concentration. The progression of alfalfa maturation is often linked with a reduction in crude protein concentration (Buxton et al., 1985; Sheaffer et al., 2000; Marković et al., 2012; Grev et al., 2020). Both leaf and stem crude protein concentrations exhibit a negative correlation with maturity (Grev et al., 2019). Furthermore, leaf senescence and increased defoliation can exacerbate the decline in crude protein concentration (Albrecht et al., 1987; Sheaffer et al., 1988; Grev et al., 2017).

2.11 Near Infrared Spectroscopy (NIRS)

Near Infrared spectroscopy (NIRS) is a useful and economical technology that uses the infrared region of the electromagnetic spectrum (about 700 to 2500 nm) to investigate the physico-chemical properties of samples in a non-destructive way. The NIRS technique is widely used in animal agriculture to predict chemical compositions of forage or feed materials. Unlike wet-chemistry methods, NIRS technique is rapid, non-destructive, cost-effective and it can accurately predict a list of nutritive values at the same time.

For Near Infrared spectroscopy (NIRS) to produce accurate results, it requires robust calibration models which are developed using a large number of samples and including all variations of physical and/or chemical properties. In calibration phase, mathematical models or calibration equations are built using results from wet-chemistry laboratory analyzes and NIRS spectral data of respective samples (Marten et al., 1985;

Bastianelli, 2013). High-precision equations have been developed for several parameters (i.e. DM, NDF, CP, IVDMD) thereby contributing to the ruminant feeding systems (Andueza et al., 2001; Brogna et al., 2009; Zhang et al., 2021).

2.12 Critique of previous literature and the knowledge gap

Quebec and Nova Scotia produced the most certified organic milk in Canada. Yet, available data from the non-GM low-lignin alfalfa is lacking. Non-GM low-lignin alfalfa provides a viable option for farmers who prioritize non-GM forage or market demands that discourage the use of genetically modified crops. There is an abundance of data about GM low-lignin alfalfa. However, non-GM low-lignin alfalfa have received very little scientific investigations. In recent years, very few studies have evaluated non-GM low-lignin alfalfa at specific developmental stages (i.e. bud vs early flowering), and we believe that such evaluations are incomplete. Data pertaining to variations in nutritive values at different physiological stages of non-GM low-lignin alfalfa are still not available.

CHAPTER 3. MATERIALS AND METHODS

3.1 Experimental site and treatment descriptions

This experiment was established in Sainte-Anne-de-Bellevue, QC, Canada (45°25’33.5” N 73°55’52.9” W). Three cultivars of alfalfa were solo-seeded in 2019 and 2021, and harvested in 2020, 2021 and 2022. The three alfalfa treatments were: 1) standard alfalfa (cv. Acapella or CW 105023; **Control**); 2) naturally selected, highly digestible alfalfa (cv. Boost HiGest or CW 103009; **Boost HG**); 3) naturally selected, highly digestible alfalfa (cv. Amina or CW 104015). This experiment was conducted at two sites and in different soil types (clay loam in 2019 seeding and loam in 2021 seeding). Treatments were assigned to a randomized complete block design with split-plot restriction and each treatment being replicated four times. Plot dimensions are 5m x 1.3m each, 7 rows were sown for each treatment and spaced by 18 cm using a Fabro seeder (Swift Current, SK, Canada). Seeding rates for all treatments were 13kg/ha on a PLS (pure-live seed) basis and seeding depth was 1/2 inch. Plots were adequately fertilized prior to each seeding based on soil analysis results following local recommendations (CRAAQ, 2010). No herbicide or pesticide was applied for this experiment.

3.2 Field data collection

Plots were harvested twice during the seeding year to control weeds, but no data or sampling was performed. Sampling was performed for two subsequent post-seeding years in each of the two sites (i.e. 2019 and 2021 seedings). For each site, there were 2 adjacent sets of blocks – 4 blocks for first cut and 4 blocks for second cut. At each experimental site, plots (4 blocks for first cut) were hand-clipped (50cm × 50cm area) at soil surface every 4 days when alfalfa reached early vegetative physiological stage until late pod stage so to obtain a wide range of physiological maturity for each alfalfa type. Thereafter, the second set of blocks (second cut) was mowed and sampling was done on the regrowth only. Samplings were typically done between May to July for first cut and between July to September for second cut.

Harvested alfalfa stems were manually separated according to stage of development as per the guideline *Developmental stage of timothy and alfalfa* (Pomerleau-Lacasse et al., 2017). Separated alfalfa stems were then grouped by stage of development and dried in a forced-air oven at 55°C for 48 hours to determine the dry matter yield, and then used to calculate vegetation stage using mean stage by weight method (Kalu and Fick, 1981). All samples were subsequently ground through a 1 mm sieve using a Wiley mill (Standard model 4, Arthur H.Thomas Co., Philadelphia, PA) and kept in closed containers for later laboratory analyses.

3.3 Laboratory analyses

3.3.1 Chemical analysis of alfalfa

In this experiment, Near-Infrared Reflectance Spectroscopy (NIRS) technique was used to predict nutritive values of all samples. First all ground samples were scanned using a NIRS DS2500 monochromator instrument (Foss NIR System Inc., Eden Prairie, MN). The objective was to measure absorbance [log (1/R), where R is reflectance] in the visible and near-infrared regions between 400 and 2500 nm at 0.5-nm intervals. All spectral data were analyzed using the WinISI software (version 4.12, Foss) and a list of spectrally different samples was selected for chemical analysis. The calibration set consisted of samples collected in 2020 (n=119), 2021 (n=57), 2022 (n=9) and existing calibrations for alfalfa (n=300 to 770 depending on parameters).

Selected samples were analyzed for DM and ash followed standard protocols from the Association of Official Analytical Chemists (AOAC, 1990). Ash concentrations were estimated from burning samples in muffle furnace at 600°C for two hours. Crude protein (CP = N x 6.25) was analyzed using a LECO FP-828 combustion analyzer (Leco Nitrogen Analyzer Corp., St. Joseph, MI). Ether extract (EE) was analyzed using an AnkomXT15 Extractor (Ankom Technology Corp., Macedon, NY; XT4 filter bag) following standard procedures (AOAC, 1990). Neutral (NDF) and acid (ADF) detergent fiber concentrations were determined using an Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY; F57 filter bag) as previously described by AOAC (1990). Neutral detergent fiber was assayed with a heat stable α-amylase without the use of sodium sulfite (Van Soest et al., 1991). Acid detergent lignin (ADL) was determined from ADF residue soaked in 72% sulfuric acid. Acid (ADICP) and neutral (NDICP) detergent insoluble protein were determined by analyzing ADF and NDF residues for CP, respectively. Finally, total digestible nutrients (TDN; Weiss et al., 1992) and net energy of lactation (NEL; NRC, 2001) were calculated from chemical composition using standard equations.

3.3.2 In vitro NDF digestibility

In vitro NDF digestibility was determined using a DaisyII incubator (Ankom Technology, Fairport, NY). The rumen fluid was collected from a ruminally fistulated healthy cow in early lactation. Samples (0.25 or 0.5g) were heat-sealed in acetone pre-rinsed F57 filter bags and then incubated for 12, 30, 48 and 240h following recommended Ankom incubation procedures. All animal procedures were approved by the Animal Care Committee of McGill University.

3.3.3 NIRS calibrations and analysis

All wet-chemistry and spectral data were used to develop a set of calibration equations (n=1 per nutritional parameter) using the partial least squares regression method of the WinISI software. Calibration equations were considered reliable when values for the coefficient of determination R^2 equal to or greater than 0.9, combined with values of RPD equal to or greater than 3. Finally, all samples (n=764) were scanned using the NIRS DS2500 monochromator instrument and NIRS calibration equations to predict nutritive values of all samples.

The partial least squares regression (PLS) modeling technique was utilized to establish these calibration equations, which were subsequently validated and shown to accurately predict quality parameters of alfalfa samples. The coefficient of determination (R^2) was used to evaluate the accuracy of the models. A mathematical model was established to relate the infrared spectrum to the measured results. Subsequently, all samples were rescanned by NIR spectroscopy to generate the predicted results.

3.4 Statistics analyses

Data were analyzed by using PROC Mixed procedure of SAS, utilizing a linear mixed model methodology (SAS Institute, 2014), with a split plot arrangement of treatments for cutting management and repeated measures. The data were analyzed as a randomized complete block design and reported for each growth cycle sampling period separately. The ANOVA was used to analyze the effects of alfalfa maturity (MSW) on the variables of interest in this study. Each alfalfa maturity (MSW) was analyzed and reported separately. The fixed effects included alfalfa cultivar, alfalfa maturity (MSW), and their interactions. Subplot (quadrat in every four days) was the subject and regrowth duration within each cutting management. Random effect is the block. The compound symmetry structure was selected, and mean separations were performed using Tukey's HSD test with statistical significance at $P \leq 0.05$. Variables analyzed or calculated were DM, ADL, NDF, ADF, CP, ASH, FAT, ADICP, NDICP, NDFD (12, 30, 48 and 240h), TDN, and NEL.

CHAPTER 4. RESULTS AND DISCUSSION

This study compared the performance of two naturally-bred alfalfa cultivars for high digestibility with a control alfalfa cultivar. The primary objective was to compare variations in nutritive values when the three alfalfa types were harvested at multiple physiological maturity stages, and thereafter select which type and physiological maturity of alfalfa shall produce better forage quality to maximize the performance of dairy cows. To the best of our knowledge, this is the first study reporting nutritive values of alfalfa cultivars naturally-bred for high digestibility ranging from mid-vegetative to late pod physiological stages within two growth cycles. Alfalfa growth at the different experimental sites and years was within expected variations across the geographical area.

Our findings show that there were no major cultivar x MSW interactions on all measured nutritional parameters across years. Indeed, cultivar x MSW interactions were significant ($P < 0.05$) for TDN and NEL, and in growth cycle 2021-2 only (Table 2). There were only few significant ($P < 0.05$) main effects of cultivar on certain parameters in 2020-1 and 2021-2 and those are presented below. However, the main effect of MSW was consistently significant ($P < 0.001$) for most measured parameters. It is well documented that advancement in alfalfa maturity is negatively correlated with its nutritive values (Albrecht et al., 1987; Brink et al., 2010; Sulc et al., 2021). This negative effect of maturity advancement and nutritive value was similar between control and the two alfalfa cultivars naturally selected for high digestibility (data not shown). The effect of MSW on forge quality was not the primary focus of this study and shall therefore not be further discussed in this paper.

Over the 3 experimental years and 6 harvests, alfalfa cultivar had significant impacts on its nutritive values in growth cycles 2020-1 and 2021-2 only (Table 2). Boost-HG had higher ($P < 0.05$) ADL and aNDF concentrations but lower ($P < 0.05$) TDN and NEL levels than control (growth cycle 2020-1) or Amina (growth cycle 2021-2) (Table 3), indicating that Boost-HG may produce forage of inferior quality. Fiber digestibility (NDFD12h) was not different between alfalfa cultivars at all MSW.

However, NDFD30h, NDFD48h and NDFD240h were higher for Boost-HG compared to control in growth cycle 2021-1 whereas the same digestibility parameters were higher for control than Boost-HG in growth cycle 2022-1. In contrast, findings of previous report show no difference in nutritive values (i.e. CP, and, ADF, ADL, TDN) and NDF digestibility between several alfalfa cultivars naturally-bred for high digestibility and control cultivars (Damiran et al., 2021; Xu and Min, 2022; Boucher et al., 2023). However, NDF digestibility was improved for another alfalfa cultivar naturally-selected for high digestibility (i.e. Hi-Gest360) compared to a control (Damiran et al., 2021). Our findings together with those of Damiran et al. (2021), Xu and Min (2022) and Boucher et al. (2023) are in contradiction with data from the alfalfa breeder indicating that Boost-HG may improve fiber digestibility (5 to 10%) and crude protein contents (3 to 5%) through higher leaf:stem ratio (5 to 8% more leaves) (Alforex Seeds, 2024). In contrast, alfalfa cultivars genetically modified for reduced lignin consistently improved fiber digestibility by 8% to 24% (Grev et al., 2017, 2019; Arnold et al., 2019; Sulc et al., 2021; Boucher et al., 2023) due to lower lignin concentrations in plant stems (Grev et al., 2020) thereby offering the possibility of extending harvest intervals without compromising fiber digestibility or nutritive values. The lower ADL concentration was not due to morphological differences in alfalfa plant (i.e. higher leaf to stem ratio) but due to lower lignin deposition in alfalfa stems (Grev et al., 2020). According to previous studies, harvesting date of alfalfa cultivars genetically modified for reduced lignin could be delayed by 5 to 10 days (Arnold et al., 2019) or by 2 to 20 days (Sulc et al., 2021) without any loss in fiber digestibility which may represent major nutritional and financial benefits on dairy farms.

Fiber concentrations (ADL and aNDF) decreased ($P < 0.05$) whereas CP, TDN and NEL increased ($P < 0.05$) with advancing regrowth duration, which is typical for our local conditions considering that alfalfa is exposed to cooler temperatures and more precipitation during regrowth. It is well-established that nutritive values of alfalfa changes over time as plant grows.

Finally, the impact of cultivar x MSW interaction on alfalfa nutritive value as the most important evaluation of this study was significant only for TDN and NEL in growth cycle 2021-2 (Table 2). All remaining parameters (i.e. ADL, NDF, CP and NDFD12, 30, 48, and 240h) were not different in all growth cycles. Both TDN and NEL were lower ($P < 0.05$) for Boost-HG compared to Amina, but not different ($P > 0.05$) than control (Table 3). Following a regression analysis between MSW and the significant parameters (i.e. TDN and NEL), the rate of change (i.e. slope) was higher ($P < 0.05$) for Boost-HG than Amina but numerically less ($P > 0.05$) than control (Table 4; Figure 2). All of these findings indicate inferior quality of Boost-HG with advancement in physiological maturity compared to Amina and control. On the other hand, Boucher et al. (2023) reported no significant cultivar x cutting management effects on nutritive values when alfalfa cultivars naturally-selected for high digestibility were compared with standard alfalfa cultivars. When similar comparisons were made with GM reduced-lignin alfalfa cultivars, nutritive values were either not altered (Grev et al., 2020) or minimally altered over years (Sulc et al., 2020).

CHAPTER 5: GENERAL CONCLUSIONS

This is the first study reporting variations in nutritive values of alfalfa naturally-selected for high digestibility across a range of physiological maturity starting from mid vegetative to late pod stages. Our hypothesis that Boost-Hi-Gest and Amina would increase NDF digestibility which thereafter would increase feed intake and productivity of dairy cows was not validated in this study. However, our findings that alfalfa cultivars naturally bred for higher digestibility do not confer any advantage for nutritive values and NDF digestibility compared to conventional alfalfa are in agreement with other studies (Boucher et al., 2023). Conversely, NDF digestibility was consistently improved with alfalfa cultivars genetically modified for reduced lignin (Grev et al., 2017, 2020; Sulc et al., 2020; Boucher et al., 2023). Therefore, in light of current and previous findings, we may conclude that improvement in fiber digestibility and thereafter cow's productivity may not be feasible with alfalfa cultivars naturally selected for high digestibility. Finally, dairy producers are not recommended to cultivate Boost-Hi-Gest or Amina considering that these are usually more costly than standard alfalfa hybrids.

LIST of TABLES

Table 2. *P*-value from linear mixed-model analysis within growth cycles (year-growth cycle no.) for effects of cultivar, physiological maturity (MSW), and their interactions on the nutritive values of alfalfa

Parameter	Source of variation	Growth cycle[1] 2020-1	2020-2	2021-1	2021-2	2022-1	2022-2
ADL	Cultivar	.6936	.7347	.4424	.0101	.9541	.6936
	MSW	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Cultivar*MSW	.5800	.9400	.6283	.4003	.4810	.5800
NDF	Cultivar	.0609	.6427	.6712	.0084	.3262	.9591
	MSW	.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Cultivar*MSW	.1177	.8926	.9205	.0924	.5620	.7086
ADF	Cultivar	.0303	.6755	.5331	.0134	.5348	.8248
	MSW	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Cultivar*MSW	.3336	.8616	.7597	.1506	.2247	.7753
ASH	Cultivar	.0621	.5672	.5707	.3554	.4401	.3935
	MSW	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Cultivar*MSW	.4516	.9185	.1616	.3954	.3822	.7223
CP	Cultivar	.0942	.0981	.6923	.0086	.3060	.6107
	MSW	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Cultivar*MSW	.2965	.9604	.6342	.3086	.8320	.8057
FAT	Cultivar	.0031	.4178	.1053	.0993	.4279	.8563
	MSW	<.0001	.0673	<.0001	.0770	.0050	.0647
	Cultivar*MSW	.6282	.9825	.6223	.4178	.3820	.9301
ADICP	Cultivar	.2407	.7475	.4023	.0091	.3744	.3809
	MSW	<.0001	.0028	.0093	<.0001	.0032	.0007
	Cultivar*MSW	.7222	.1801	.9444	.4414	.5628	.7412
NDICP	Cultivar	.6016	.2465	.8890	.5439	.2871	.5571
	MSW	<.0001	.0806	.0007	<.0001	<.0001	.0003
	Cultivar*MSW	.4548	.8932	.5272	.4007	.2434	.6280
NDFD12H	Cultivar	.7802	.6977	.2952	.2073	.2984	.8136
	MSW	.9674	.5402	.7528	.7868	.5073	.9483
	Cultivar*MSW	.5684	.1391	.2690	.2003	.5987	.8708
NDFD30H	Cultivar	.8990	.4281	.1174	.8191	.1033	.3431
	MSW	.5830	.2160	.9563	.9103	.5009	.9905
	Cultivar*MSW	.6386	.2051	.7960	.5840	.9313	.7152
NDFD48H	Cultivar	.7670	.4235	.1187	.5471	.3586	.4412
	MSW	.5830	.2085	.8954	.5605	.1836	.0823
	Cultivar*MSW	.7285	.2051	.6949	.5308	.9387	.8955
NDFD240H	Cultivar	.7956	.3419	.1450	.4438	.1424	.2530
	MSW	.7592	.3130	.9483	.9914	.0667	.9238
	Cultivar*MSW	.6009	.4909	.9247	.6071	.7937	.5313
TDN	Cultivar	.0247	.7416	.4216	.0088	.6577	.8283
	MSW	.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Cultivar*MSW	.3986	.9709	.8756	.0312	.4695	.7431
NEL	Cultivar	.0259	.7435	.4234	.0090	.6545	.8309
	MSW	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Cultivar*MSW	.3995	.9721	.8771	.0325	.4691	.7460

[1]Growth cycle: year-growth cycle number within each year.

Table 3. Effects of cultivars across years and growth cycles on nutritive values of alfalfa

Parameter	Cultivar	Growth cycle[1]					
		2020-1	2020-2	2021-1	2021-2	2022-1	2022-2
ADL[4]	Acapella[2]	6.64^{b}	5.12	7.66	6.08^{ab}	7.42	6.78
	Boost HG[2]	7.13^{a}	5.31	7.75	6.38^{a}	7.44	6.91
	Amina[2]	6.85^{ab}	5.21	7.56	5.85^{b}	7.39	6.83
	SEM[3]	0.15	0.25	0.15	0.16	0.15	0.15
aNDF[4]	Acapella	36.5^{b}	29.7	41.4	25.2^{b}	41.2	35.3
	Boost HG	38.4^{a}	30.6	42.0	36.9^{a}	41.8	35.5
	Amina	37.4^{ab}	30.0	41.3	34.7^{b}	41.9	35.5
	SEM	0.79	0.96	0.90	0.67	0.70	0.69
CP[4]	Acapella	20.7	28.8	19.9	23.5^{a}	21.8	24.8
	Boost HG	19.9	27.3	20.6	22.0^{b}	21.3	24.3
	Amina	20.0	28.1	20.0	23.2^{a}	21.5	24.4
	SEM	0.40	0.63	0.82	0.47	0.60	0.58
NDFD12h[4]	Acapella	33.2	34.8	26.0	27.7	30.3	30.0
	Boost HG	33.9	34.5	28.2	26.3	29.1	29.2
	Amina	33.1	33.2	27.5	28.0	31.1	29.8
	SEM	0.98	1.2	1.0	27.7	0.95	0.78
NDFD30h[4]	Acapella	50.8	55.6	43.50^{b}	44.75	49.08^{a}	45.81
	Boost HG	51.9	52.3	49.14^{a}	44.42	44.05^{b}	48.89
	Amina	51.2	51.1	46.87^{ab}	45.62	48.62^{a}	48.51
	SEM	1.70	2.31	1.69	2.41	1.57	1.35
NDFD48h[4]	Acapella	56.7	62.8	47.9^{b}	49.8	54.8^{a}	51.0
	Boost HG	58.6	59.5	54.5^{a}	48.5	49.8^{b}	54.0
	Amina	57.7	57.7	51.5^{ab}	50.9	53.7^{ab}	53.6
	SEM	2.5	3.8	3.1	2.1	2.6	2.4
NDFD240h[4]	Acapella	63.3	69.0	56.3^{b}	57.26	62.03^{a}	57.1
	Boost HG	64.9	64.7	62.3^{a}	56.32	56.09^{b}	61.2
	Amina	64.1	63.9	59.3^{ab}	59.07	58.17^{ab}	59.9
	SEM	1.78	2.33	1.95	2.63	1.98	1.50
TDN[4]	Acapella	63.6^{a}	66.9	61.2	64.5^{ab}	60.6	63.3
	Boost HG	62.3^{b}	66.5	60.8	63.5^{b}	60.4	63.0
	Amina	63.0^{ab}	66.8	61.4	64.8^{a}	60.4	63.1
	SEM	0.44	0.62	0.42	0.39	0.42	0.36
NEL[4]	Acapella	1.43^{a}	1.52	1.38	1.46^{ab}	1.36	1.43
	Boost HG	1.40^{b}	1.50	1.37	1.43^{b}	1.36	1.42
	Amina	1.42^{ab}	1.51	1.38	1.46^{a}	1.36	1.42
	SEM	0.10	0.01	0.01	0.01	0.01	0.01

[a,b]Means within a column sharing different letter are statistically different by Tukey's HSD test at the 5% level of significance.

[1]Growth cycle: year-growth cycle number within each year.

[2]Acapella: control alfalfa; Boost HG and Amina: highly digestible alfalfa (naturally selected).

[3]SEM: Pooled standard error of the mean.

[4]ADL: acid detergent lignin; aNDF: neutral detergent fiber; CP: crude protein; NDFD: in-vitro NDF digestibility; TDN: total digestible nutrient; NEL: net energy of lactation.

Table 4. Regression analysis of cultivar x MSW interactions on significant (P < 0.05) nutritive values in year 2021 (growth cycle 2)

		2021-2	
Parameter	**Cultivar**	**Intercept**	**Slope**
TDN[3]	Acapella[1]	74.2	-2.15[ab]
	Boost HG[1]	75.7	-2.72[b]
	Amina[1]	74.3	-2.09[a]
	SEM[2]	1.7	0.38
NEL[3]	Acapella	1.69	-0.052[ab]
	Boost HG	1.73	-0.066[b]
	Amina	1.70	-0.051[a]
	SEM	0.43	0.009

[a,b]Means within a column sharing different letter are statistically different by Tukey's HSD test at the 5% level of significance.

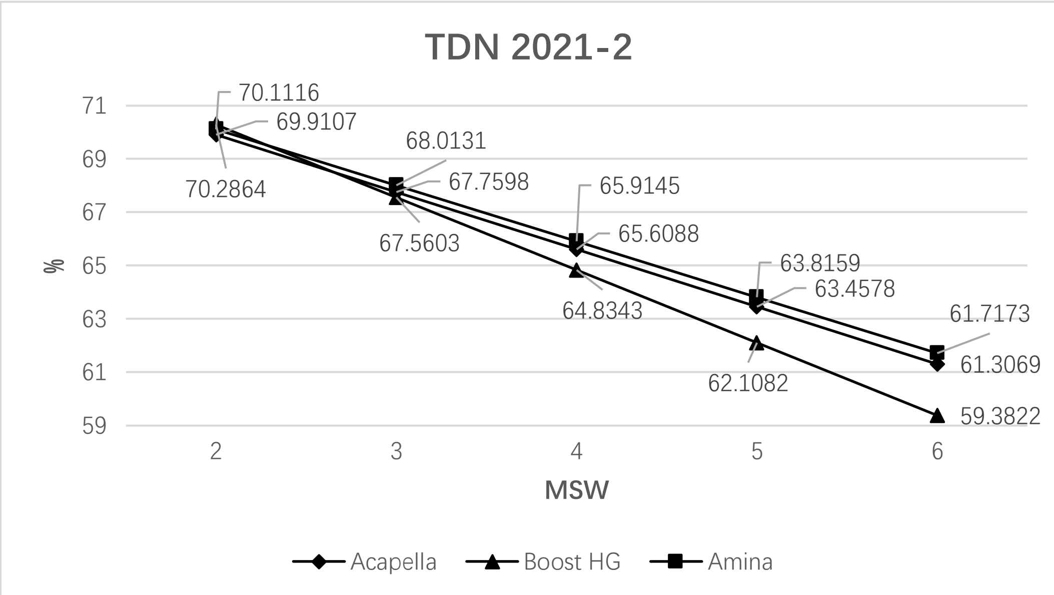
[1]Acapella: control alfalfa; Boost HG and Amina: highly digestible alfalfa (naturally selected).

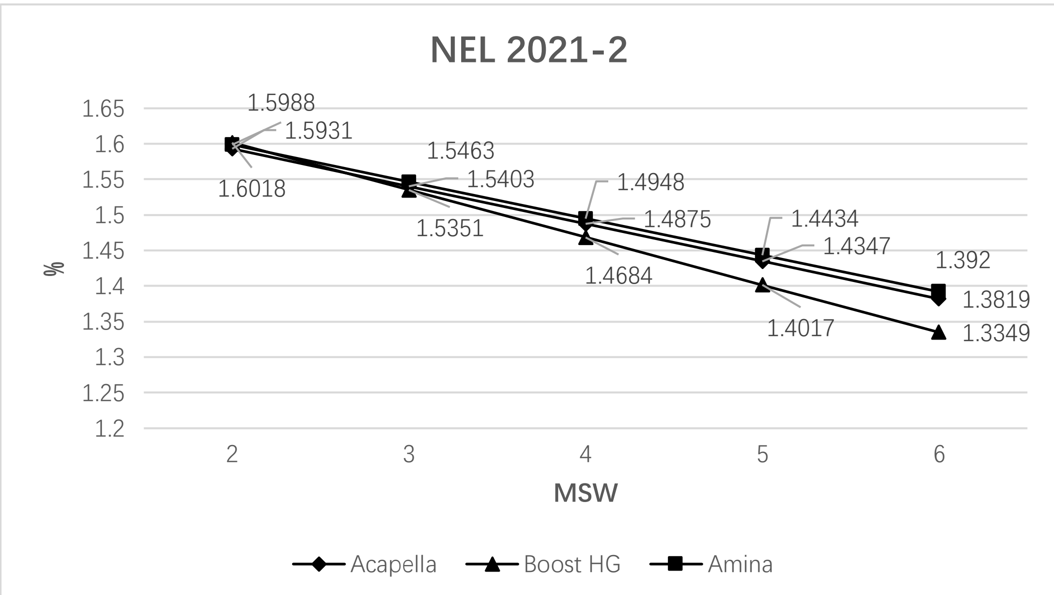
[2]SEM: Pooled standard error of the mean.

[3]TDN: total digestible nutrient; NEL: net energy of lactation.

LIST of FIGURES

Figure 2. Effect of MSW on total digestible nutrient (TDN) and net energy of lactation (NEL) in year 2021 (growth cycle 2) with a significant cultivar x MSW interaction (*P* < 0.05). Values are means across four replicates in each block. Regression coefficients are reported in Table 4.





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