The Diagnosis of Sub-Acute Ruminal Acidosis SARA on Commercial Farms Using Milk Fatty Acid Profile and Milk Amyloid A

By

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LIST OF ABBREVIATIONS

ARA: Acute ruminal acidosis

EL: Early lactation

HR: High risk

HREL: High risk, early lactation

HRLL: High risk, late lactation

HRML: High risk, mid-lactation

LCFAs: Long-chain fatty acids

LL: Late lactation

LPS: Lipopolysaccharide

LR: Low risk

LREL: Low risk, early lactation

LRLL: Low risk, late lactation

LRML: Low risk, mid-lactation

MAA: Milk amyloid A

MCFAs: Medium-chain fatty acids

ML: Mid lactation

MFD: Milk fat depression

PUFA: Polyunsaturated fatty acids

SARA: Sub-acute ruminal acidosis

SCFAs: Short-chain fatty acids

ABSTRACT

The objective of this research was to determine whether the fatty acid (FA) profile and the concentration of milk amyloid A (MAA) in milk from individual cows can be used to diagnose sub-acute ruminal acidosis (SARA) on commercial dairy farms. The use of the milk FA profile and milk amyloid A (MAA) to diagnose SARA has been validated in experimentally induced grain-based SARA challenges. A total of 320 milk samples from 24 commercial dairy farms in Quebec were tested for milk FA profile using gas chromatography method and for MAA using a commercial ELISA kit. Farms were divided into low SARA risk farms and high SARA Risk farms. High SARA Risk farms had a proportion of *de novo* FAs below 0.89 g/100 g of milk, a proportion of polyunsaturated FAs (PUFAs) greater than 3.40 g/100 g of total FAs, and a milk fat content below 4.00%, and a milk protein content above 3.05% in bulk tank milk. Low SARA Risk farms had a *de novo* FA content of milk higher than 1.07 g/100 g of milk. There were 12 blocks with each block containing a high SARA Risk farm and a low SARA Risk farm. On each farm, 7 early to mid-lactation (1 – 150 DIM) and 7 mid to late lactation cows (151 and more DIM) were randomly selected. Cows with a somatic cell count (SCC) of over 200,000 cells/ml were not included. Data were analyzed using SAS Proc Mixed with Cow risk of SARA and Farm Risk level of SARA as fixed factors, and Block as a random factor. The model for MAA also included somatic cell counts (SCC) and parity as covariates. The Farm Risk of SARA did not affect the milk fat proportions of FAs. The effects of Cow Risk of SARA and Farm Risk of SARA on MAA were not significant. The milk FA profile can contribute to the diagnosis of SARA, the identification of causes of milk fat depression, and the development of strategies to optimize the milk FA profile.

Keywords: Fatty acid (FA), MFAP, SARA, MAA.

Abbreviation key: Milk amyloid A (MAA), Subacute ruminal acidosis (SARA), milk fatty acid profile (MFAP).

FOREWORD

Parts of this thesis were presented virtually as poster presentations at the ASAS-CSAS Meeting in July 2020 and 2021. The reference format in this thesis is in APA format. This thesis is written in a manuscript format prepared during my master's degree program in animal science as listed below:

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1.0 GENERAL INTRODUCTION

Subacute ruminal acidosis (SARA), a metabolic disease during which the rumen pH is depressed, has been recognized as a concern in high-yielding dairy cows for many years (Kleen *et al.,* 2013; Danscher *et al.*, 2015; Plaizier *et al.,* 2018). Many studies have, therefore, been carried out to develop methods and ways to diagnose, treat, and prevent SARA (Enemark, 2008). The rumen is a fermentation vat that contains micro-organisms (Russell and Hespell, 1981; Steele *et al.,* 2011) that aid the breakdown of the complex components of feedstuffs, such as cellulose, hemicellulose, and starch (Zhang *et al.,* 2017). The populations and functionality of these microorganisms are affected by the diet of dairy cows, and SARA can greatly reduce this functionality (Khafipour *et al.,* 2009; Mao *et al.,* 2013; Plaizier *et al.,* 2018).

Early lactation dairy cows cannot consume enough feed to meet their energy requirements and rely on their body's energy reserves to meet their energy requirements (Morris *et al.,* 2009). To minimize loss in energy reserves and the accompanying loss in production and risk of metabolic diseases such as ketosis, fatty liver syndrome, high-energy diets must be fed (McArt *et al.,* 2012). This is achieved by large amounts of readily fermentable carbohydrates, such as starch (Zebeli *et al.,* 2012; Plaizier *et al.,* 2018). However, feeding these diets can result in the accumulation of volatile FAs (VFAs) and a reduction in buffering of the rumen that can reduce the rumen pH and lead to SARA (Jouany and Morgavi, 2007; Shingfield and Grinarii, 2007; Plaizier *et al.,* 2018).

When an animal is suffering from SARA, there are no distinctive clinical symptoms at the early stage (Plaizier *et al.,* 2008; Tajik and Nazifi, 2011). Conventional methods for the diagnosis of SARA involve invasive techniques that involve the collection and measuring of the pH in rumen fluid. They may not give accurate results because they are dependent on various factors, mainly the collection methods. This was discussed further in section 2.4 of the thesis. Therefore, various

authors have proposed different pH thresholds for SARA, depending on their research and methods of measuring rumen pH (Li *et al*., 2013; Abdela, 2016). To overcome the need to be dependent on these inaccurate and invasive methods, studies are being carried out to devise new methods, particularly the use of the milk fatty acid (FA) profile and acute-phase proteins, for the diagnosis of SARA (Fievez *et al.*, 2012; Patel *et al.,* 2013; Mitchell *et al.,* 2016).

The milk FA profile of dairy cows contains various classes of FAs, including short-chain FAs, medium-chain FAs, long-chain FAs, and odd- and branched-chain FAs (OBCFAs) (Fievez *et al.,* 2012). Some of these FAs are changed by dietary factors (increased grain to forage ratio) (Section 2.6). The concentration of OBCFAS will negatively alter the milk FA profile in the milk of cows (Bernard *et al.,* 2008). These FAs can be measured easily because they exist as stable compounds (Kenard, 1991). In addition, it has been reported that the concentrations of some OBCFAs (either at high or low levels) indicate the presence of SARA (Colman et *al.,* 2010; AlZahal *et al.,* 2011). Thus, these FAs could be used as non-invasive tools for researchers as well as a tool for farmers to assess the gut health of the cows.

So far, there are no suitable methods to sample ruminal fluid for pH measurement on commercial farms, and the currently available techniques give inconsistent results (Danscher *et al.,* 2015). This will be discussed further in this thesis (sections 2.1.1 and 2.4). The concentration of milk amyloid A (MAA) in milk may be a non-invasive tool for diagnosing SARA. The concentration in milk has been shown to mirror that of serum amyloid A (SAA), which is an acute-phase protein associated with the acute phase response that is triggered during SARA (Tothova *et al.,* 2014). Therefore, since the milk FA profile and acute phase proteins (SAA and MAA) are discussed and recommended from previous studies, it may be possible to diagnose SARA on commercial dairy farms using the FA profile and MAA content of milk. The use of milk parameters to diagnose

SARA will be an efficient way for producers to keep track of their herd health, especially as milk is already being tested for other problems like antibiotics, mastitis, bacterial count, and pregnancy. The use of milk for diagnostic purposes will certainly prevent any obstruction in the activities of the farm since milking is already a normal farm activity.

Using milk as a biological material with which SARA is diagnosed will ensure that animals do not undergo stress during this diagnosis. Also, if this technology is found effective, this diagnosis may complement other diagnoses on diseases and disorders in milk that are already in place. For instance, using milk somatic cell count (SCC) for the detection of mastitis, amyloid A for the detection of inflammation, and other indicators for the diagnosis of pregnancy. This research aims to validate the use of the milk FA profile and MAA as an indicator of rumen pH on commercial farms. This will be a useful tool to determine the presence of SARA in a dairy herd. This study will also verify specific FAs that indicate SARA, as suggested by previous research, and stipulate the use of biomarkers in milk as an alternative to conventional invasive diagnostic methods.

2.0 LITERATURE REVIEW

2.1 Definition of sub-acute ruminal acidosis (SARA)

Rumen acidosis in dairy cows can be classified into 4 groups, namely: Mild, Subacute, Acute, and Peracute, in order of increasing severity (Duffield *et al.,* 2004). This paper will focus on subacute ruminal acidosis (SARA), which is a common metabolic disease in high-yielding dairy cattle (Gakhar *et al.,* 2008). It is a condition during which the rumen pH falls below 5.6 for more than 3 hours per day (Gozho *et al.,* 2007; Steele *et al.,* 2012). The disease occurs when the rumen pH drops due to excessive amounts of readily fermentable carbohydrates in the diet (Kleen *et al.,* 2003). Subacute ruminal acidosis is a syndrome because it is characterized by a series of symptoms (Steele *et al.,* 2012). These symptoms are not overt (Plaizier *et al.,* 2008). Therefore, SARA is not easily diagnosed in cows and is, as a result, often not treated (Enjalbert *et al.,* 2008; Li *et al.,* 2012).

While this can be attributed to a lack of overt clinical signs, it is also attributed to the fluctuations in rumen pH throughout the day, and the inability to collect rumen fluid samples that accurately represent the rumen environment (Li *et al.,* 2012). The pH fluctuations increase the inaccuracy of rumen pH as a biomarker for the diagnosis of SARA. Until recently, the pH of the rumen was the only confirmatory factor to determine the presence of SARA (Li *et al.,* 2012). Rumen fluid can be collected with cannula, oral probes, and rumenocentesis (Plaizier *et al.,* 2018). To determine the rumen pH, there is a need for the collection of rumen fluid and measuring the pH of this fluid. The methods involved in collecting rumen fluid from animals are invasive and require considerable expertise (section 2.4). These methods cannot be used on commercial dairy farms, since producers need the animals to function optimally with minimal stress. For this reason, alternative methods to

diagnose SARA that can be used on commercial farms are being developed to replace the existing invasive methods.

These methods may involve the measurement of the FA profile and the concentration of MAA in milk (Enemark, 2008; Danscher *et al.*, 2015; Plaizier *et al.*, 2018). There is existing evidence to show that the rumen pH is not a sufficiently accurate indicator of SARA (Steele *et al.*, 2012). Therefore, ruminal pH alone should not be used to diagnose SARA in dairy cows, and other biomarkers are required for this diagnosis (Steele *et al.*, 2012; Danscher *et al.*, 2015). Despite the search for alternative biomarkers, the term "ruminal acidosis" is still derived from a depressed rumen pH. Subacute ruminal acidosis is quite common in dairy herds. It is reported to have had a prevalence of between 19% and 26% in early and peak lactation dairy cows, respectively (Mitchell *et al.*, 2016). Steele *et al.* (2012) estimated that about 20% of lactating cows will experience SARA, whereas Danscher *et al.* (2015) reported a prevalence of up to 40% in some dairy herds.

Several non-rumen pH-related methods for the diagnosis of SARA exist, including measuring the lipopolysaccharide (LPS) concentration in feces, the milk FAs profile, and the concentrations of acute-phase proteins in the blood and milk (Li *et al.*, 2010; Plaizier *et al.*, 2008; Fievez *et al.*, 2012; Tothova *et al.*, 2014). The acidity of the rumen has been linked to a rise in the LPS content in the rumen, because of the lysis of gram-negative bacteria (Emmanuel *et al.*, 2008; Plaizier *et al.*, 2012). This LPS may translocate into the interior circulation, where it triggers an acute phase response that may progress into inflammation (Plaizier *et al.*, 2012). The reason for this translocation is likely because SARA reduces the barrier function of the rumen epithelium (Plaizier *et al.*, 2008).

The reduction of the rumen pH during SARA is caused by an accumulation of volatile fatty acids (VFAs), including acetate, butyrate, and propionate in the rumen (Kleen *et al.,* 2003; Gott, 2011; Plaizier *et al.,* 2018). According to Gott (2011), VFAs have the following characteristics. They are:

a. Organic.

b. Short-chain fatty acids (SCFAs).

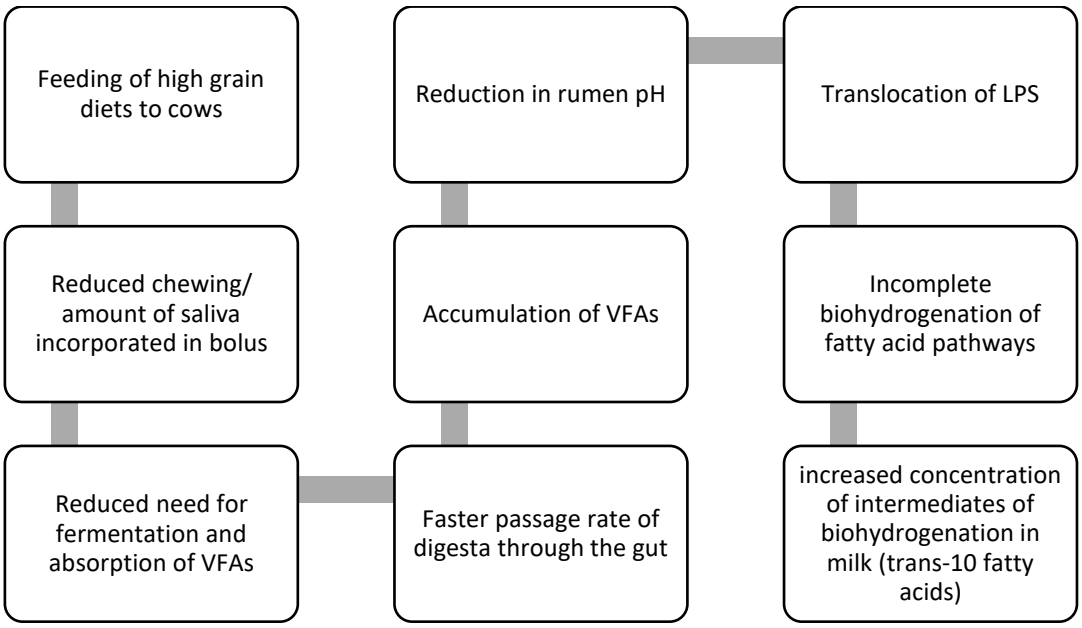
c. End products of microbial fermentation.

2.1.1 Low pH and pH thresholds that define SARA.

High-yielding dairy cows receive highly fermentable high starch diets that increase the production of VFA in the rumen. and reduce the amount of saliva incorporated into the feed during chewing, resulting in insufficient buffering for the acids produced in the rumen during fermentation (Gott, 2011). As a result, the production of VFAs will exceed the capacity of the rumen to absorb them, and the rumen pH will drop. (Danscher *et al.,* 2015).

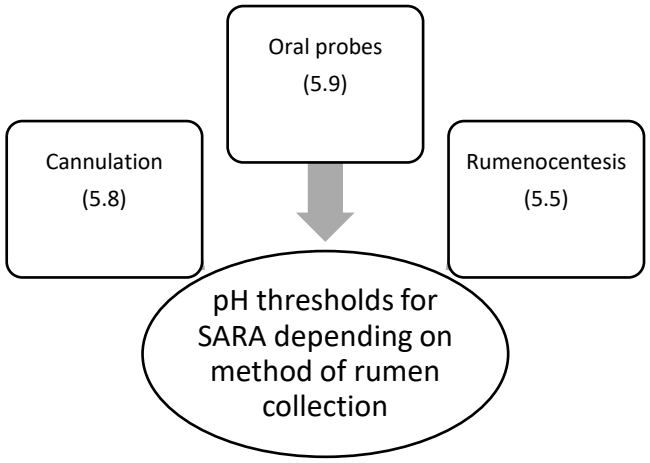
The relationship between feed, SARA, and milk FA profile is shown in figure 2.1. The process is well documented by several authors, with each phase acting as an antecedent for the next.

Figure 2.1: The relationship between feed, subacute ruminal acidosis and milk FA profile (Khafipour *et al.*, 2009; Gott, 2011; Li *et al.*, 2013; Plaizier *et al.*, 2017).



Abdela (2016) reported that the method used to obtain ruminal fluid influences the acceptable range of the rumen pH as shown in figure 2.1. The rumen fluid was obtained from animals in which SARA was induced. The SARA thresholds for different methods of rumen fluid collection are given in figure 2.2.

Figure 2.2: The method of collection of rumen fluid and pH ranges (Gakhar *et al.,* 2008; Li *et al.,* 2013; Abdela, 2016).



2.2 Causes of SARA: risk factors.

To accurately diagnose SARA, it is important to understand its causes. The main cause of SARA is the large amount of readily fermentable carbohydrates that are incorporated in the animal's diet to meet their high energy requirements, and the rapid increases in the dietary contents of these carbohydrates. These readily fermented carbohydrates are often fed in the form of high starch grains (Aschenbach *et al.,* 2010). This pattern of feeding sets in motion several processes that lead to SARA. The severity of SARA is determined by the amounts of highly degradable carbohydrates, as well as the lack of coarse forages that are incorporated into the diet (Plaizier *et al.*, 2017). Feeding such diets can lead to insufficient rumen buffering, accumulation of VFAs in the rumen and hindgut, and reduction of rumen pH (Plaizier *et al.,* 2017). One of the first reactions of cows to SARA is reduced feed intake. If the cows reduce their feed intake long enough, then SARA can be reversed and the cows may recover (Oetzel, 2007; Khafipour *et al.,* 2009). Generally, the causes of SARA include dietary risk factors (section 2.2 and section 2.4.2.3), inadequate saliva buffering

and excessive incorporation of readily fermented carbohydrates in the cow's diet (Gott, 2011; Li *et al.,* 2013). Subacute ruminal acidosis can be explained in terms of each of these causes. However, these causes are all interrelated.

In this paper, the factors that increase the susceptibility of animals to SARA are referred to as the risk factors for SARA. Risk factors for SARA include breed, feed formulation and strategies, management, season, stage of lactation, geographical location, presence of other metabolic disorders, supplementation of fats, and genetics (Mansonn, 2008; Coppa *et al.,* 2012, Jing *et al.,* 2018). The stage of lactation affects the milk fat composition as well as a cow's risk of SARA (Palmquist *et al.,* 1993). The animals that are prone to SARA include cows in early lactation (transition animals), cows in mid-lactation (high dry matter intake (DMI) animals), grazing cows, cows fed rapidly fermentable grasses, and primiparous cows (Stone, 2004; Enemark, 2008; Li *et al*., 2013). Cows transitioning from the pregnant and dry to the lactating stage, cows having high dry matter intakes, which is required for energy needed for milk production, cows subjected to higher variability in ration and meal patterns are at the risk of SARA (Stone, 2004). In previous studies, it has been reported that the abundance of some of the *de novo* FAs (C6:0 to C14:0) were highest in the third month of lactation, while C18:0 had the lowest abundance for both Norwegian cows and Holstein cows (Stoop *et al.,* 2009).

Several authors have concluded that SARA is not solely the result of a depressed rumen pH, and that feed intake, milk yield, and milk-based biomarkers must be considered to diagnose SARA (Li *et al.,* 2012). Housing and management are also risk factors for SARA. Jing *et al.* (2018) identified the intensive dairy management system (which is a common practice in Canada) as an indicator of animals' risk to SARA. This is because in intensive dairy management systems high amounts of starch are incorporated into the diets of the animals to supply the energy required by

dairy cows to produce large amounts of milk (Villot *et al.,* 2018). Also, cows in free stalls were reported to have an increased risk of SARA than cows kept in bedded packs, because the stocking density in pens was not appropriate (Kitkas *et al.,* 2013). This was attributed to shared access to the feed. The author reported that the SARA herds had errors in ration formulation and that the particle size of total mixed rations (TMR) was not insufficient. Therefore, the housing and feeding system affect the risk of SARA greatly. Hence, it is important to note that management can affect the risk of animals to SARA.

While milk fat depression can be used to diagnose SARA, SARA is not the only reason for a low milk fat content (Shingfield and Grinarii, 2007; Steele *et al.,* 2012). The identification of risk factors is the main method currently utilized in identifying the susceptibility to SARA, especially on commercial dairy farms, where the pH of the rumen cannot be measured. In several prior studies, the pH of the rumen fluid has been used to diagnose SARA (Plaizier *et al.,* 2017). Therefore, the significance of the pH of the rumen to assess the severity and the impact of SARA is well understood. However, information on the rumen pH is not easily available. The milk fat percentage at normal and depressed levels of the breeds of dairy cows is shown in table 2.1.

Table 2.1: The milk fat percentage and milk fat depression of breeds of dairy cows (Oetzel, 2007).

Breed	**Milk fat percentage (%)**	**Milk fat depression (%)**
Holstein, Ayrshire, and Milking Shorthorn	3.4-4.0	<3.2
Jerseys	4.2-5.0	<3.4
Brown Swiss	3.6-4.2	<4.2
Guernsey	4.0-4.8	<4.0

High-yielding dairy cows are fed high grain and low forage diets to meet their high energy requirements (Krause and Oetzel, 2006). However, feeding such diets can induce SARA (Kleen and Cannizzo, 2012; Li *et al.,* 2013; Abdela, 2016). When high amounts of starch are incorporated into the diets of dairy cows, this leads to decreased saliva in the bolus and causes insufficient saliva

secretion, which acts as a buffer in the rumen, and hence, increases acidity (Beauchemin, 2018). The occurrence of SARA in dairy herds can result in a drastic reduction in milk production and lead to large financial losses for the farmer (section 2.5) (Enemark, 2008).

SARA can lead to the following:

(a) Reduction in chewing time, and hence inadequate saliva production to act as a buffer for the rumen (Gott, 2011).

(b) Accumulation of VFAs due to reduced absorption of fermentation acids. This causes a change in the ruminal microbes due to the changed availabilities of substrates for fermentation (Li *et al*., 2013).

(c) Injury or inflammation of the rumen epithelium (Plaizier *et al.*, 2008).

(d) Translocation of lipopolysaccharides from the rumen and the hindgut to the interior circulation (Plaizier *et al.*, 2012).

(e) Clinical symptoms, including reduced and erratic feed intake, laminitis, and milk fat depression (Enemark, 2008).

(f) Economic losses (section 2.5) (Enemark, 2008).

2.3 Symptoms of SARA.

Subacute ruminal acidosis does not have any marked or overt symptoms (Mitchell *et al.*, 2016). However, previous authors have suggested symptoms that could suggest the presence of SARA. Most of these symptoms indicate that the condition is clinical. Symptoms of SARA include: 1) Decreased bodyweight 2) Diarrhea 3) Reduced milk fat content 4) Decrease in milk production efficiency 5) Reduced fiber digestion 6) Acute phase response 7) Low and erratic feed intake 8) Inflammation 9) Laminitis (Enemark, 2008; Tajik and Nazifi, 2011; Li *et al.*, 2012), 10) poor body

condition,11) Dehydration 12) increase in rumen motility (Duffield et al., 2004) 13) Liver abscesses and 14) Increases in acute-phase proteins in the blood circulation (Plaizier et al., 2008).

2.4 Techniques for diagnosing SARA in dairy cows.

These techniques are divided into two groups, i.e., conventional, and unconventional. Conventional techniques are techniques that involve the collection of rumen fluid. These methods are invasive. Non-conventional methods are methods that do not require rumen fluid for diagnosis, meaning that they are non-invasive. Rumen pH is the conventional biomarker for SARA (Li *et al.,* 2012). Limitations to the use of the measurement of the pH of rumen fluid for the diagnosis of SARA exist (Kovacs *et al.,* 2020). These limitations include invasiveness, health problems for cows, diurnal variation of rumen pH, and the inability to obtain good representative samples of the rumen fluid (Mitchell *et al.,* 2016). It is recommended that rumen fluid for animals fed with total mixed rations is collected 5 to 8 hours after feeding, while for those fed concentrates and forages separately, rumen fluid should be collected after 2 to 5 hours (Duffield *et al.*, 2004). At this time, the pH of the rumen is at its lowest daily point (nadir), making it a good time to assess the challenge to the rumen (Duffield *et al.,* 2004).

2.4.1 Conventional methods for diagnosing SARA in dairy cows.

Some of these include the measurement of the pH of the rumen collected using rumenocentesis, oral stomach tubes, rumen sensors, and cannula. These are not the only methods available to diagnose SARA. The focus of this paper, however, is on the unconventional methods listed below and explained further in section 2.4.

2.4.1.1 Use of rumenocentesis and oral stomach tubes

The two methods commonly used for the collection of rumen fluid are rumenocentesis and oral stomach tubing (Li *et al.,* 2012). Oral stomach tubes can give inaccurate results because of saliva

contamination (Duffield *et al.,* 2004). Also, these techniques are not ideal because of the stress they might cause animals. These stresses could be in the form of abscesses, abrasions, damage to the mouth and/or esophagus, or alteration of pH due to saliva contamination when stomach tubes are used (Li *et al.,* 2012). Rumenocentesis, according to Panousis *et al.* (2018), is the best invasive technique, because the negative impact on the animal is less when compared to stomach tubing.

2.4.1.2 Use of rumen sensors:

Recent rumen measuring diagnostic tools, such as rumen sensors, have been developed, but they are not durable and are expensive (Panousis *et al.,* 2018). They can be inserted surgically through a rumen cannula or administered through the esophagus (Castro-Costa *et al*., 2015). Hence, these indwelling sensors and wireless sensors can be placed in both cannulated and non-cannulated animals (Dijikstra *et al.,* 2020). These sensors are still undergoing technological advancements (Panousis *et al.,* 2018). They are suitable for obtaining data from individual animals (Dijikstra *et al.,* 2020). These tools have been able to resolve the invasive, stressful characteristics of the conventional rumen fluid collection methods because they can measure the rumen pH without rumen fluid collection. Limitations exist when it comes to calibration and controlling the position of sensors in the reticulorumen, and that they are not easily retrieved (pH varies with location in the rumen) (Dijkstra *et al.,* 2020). These factors, as well as variations among individual animal characteristics (section 2.1), need to be accounted for, for the accurate diagnosis of SARA (Villot *et al.,* 2018).

2.4.1.3 Use of cannula:

Permanent cannulation of cows has been used for a long time in laboratory animals. They enable the collection of rumen fluid, which can be further used for *in-vitro* rumen fermentation studies, and a pH meter can be used to measure the rumen pH directly Duffield *et al.* (2004) identified four

sites of the rumen that can be accessed when animals are cannulated: cranial ventral, caudal ventral, central, cranial dorsal regions. Garret *et al.* (1999) reported that the central region and a combination of various sites will help improve the sample's representation of the rumen.

2.4.1.4 Use of lipopolysaccharide (LPS) concentrations:

LPS, in its free form, are endotoxins that trigger the immune response in the host animal when they enter the interior circulation (Plaizier *et al.,* 2012). SARA has been reported to increase the LPS concentration in both the rumen and feces (Li *et al.,* 2010). LPS is released after the lysis of the gram-negative bacteria or because the enzymes aid the growth phase of bacteria (Gozho *et al.,* 2007). LPS in the rumen and large intestine is proposed to originate from the lysis of gram-negative bacteria within the large intestine or the rumen (Li *et al.,* 2010). The concentration of LPS in the rumen is increased when dairy cows suffer from SARA (Zhao *et al.,* 2018). Earlier studies reported contradictory results, however recent studies have shown that LPS increases when animals have SARA (Plaizier *et al.,* 2012). This increase has been attributed to the increased fermentation of starch, causing increased concentrations of free endotoxins in the digesta found in the large intestine (Li *et al.,* 2010). This may cause a change in the microbial populations of the rumen, particularly an increase in gram-negative bacteria that shed LPS (Plaizier et al., 2012). Some symptoms and sequels of SARA, such as laminitis, sudden death, acidosis, and rumenitis have been linked to the release of LPS into the interior circulation (Gozho *et al.,* 2007). The liver plays a major role in reducing the negative impact of free LPS (Gozho *et al.,* 2007). Li *et al.* (2010) reported that bile acids detoxify free endotoxins, although it is unknown if they will detoxify all the endotoxins from the rumen. Li *et al.* (2010) also reported that there is also a relationship between days in milk (DIM) and fecal endotoxin due to the low feed intake, low fermentation in the rumen and large intestine when animals are not prone to SARA (during late lactation stage).

2.4.2 Unconventional methods of diagnosing SARA in dairy cows:

Other methods mentioned below are not invasive. and have also been partially validated in studies during which SARA was experimentally induced. These methods are useful for occasions where clear symptoms of SARA are not noticed, and assessment of rumen fluid is not possible, such as commercial farms. The latter is the focus of this study. These methods include:

1. Use of milk FA profile (section 2.6)
2. Use of acute-phase proteins (section 2.7)
3. Observation of risk factors (section 2.2)

2.5 Impact of SARA

Subacute ruminal acidosis can lead to economic losses for farmers. These losses are estimated to have an annual range of 500 million to 1 billion dollars, and a daily cost of 1.12 dollars per cow in the United States (Enemark, 2008). These losses are due to reduced milk and milk fat yields, as well as increased culling rates and veterinary costs. (Gakhar *et al.,* 2008; Tajik and Nazifi, 2011; Li *et al.,* 2012; Plaizier *et al.,* 2012). The increased culling is mainly due to increased risk of lameness and sudden death (Gozho *et al.,* 2007; Enemark, 2008). Subacute ruminal acidosis impacts animal welfare, which is a rising public concern (Oetzel, 2007; Panousis *et al.,* 2018). Impacts of SARA also include laminitis, rumenitis, inflammation, and liver abscesses (Steele *et al.,* 2012).

One of the symptoms of SARA is laminitis. Laminitis occurs due to the swelling of sensitive portions, including capillaries, of the hooves of cows (Nordlund *et al.,* 2004). This condition has been reported to affect up to 55% of animals in dairy herds in North America, United Kingdom, and Scandinavia (Cook *et al.,* 2004). Lameness can impact milk yield due to an animal's inability to move around and may be a symptom of a chronic metabolic condition (Nordlund *et al.,* 2004).

The risk of lameness can be affected by housing factors, parturition, lactation stage, and metabolic diseases such as SARA, as SARA can cause inflammation of hooves (Fiore *et al.,* 2019). Lameness may not be solely a nutrition-related symptom but may also occur due to poor housing and flooring (Nordlund *et al.,* 2004; Cook *et al.,* 2004). Depending on the floor structure, trimming the hooves of dairy cows can help prevent lameness, provided it is discovered early (Fiore *et al.,* 2019). Also, plastic or sandy floors are preferred to slippery cement floors, as they reduce the need for hoof trimming. These preventive measures will only be useful for cows that do not have laminitis, which is defined as an inflammation of the hoof. Subacute ruminal acidosis may cause laminitis in dairy cows (Cook *et al.,* 2004; Nouri and Dezfulian, 2014). Cook *et al.* (2004) suggested that several theories may explain this. Firstly, gelatin proteases elongate collagen fibers and loosen connective tissues of the hooves and can cause laminitis in dairy cows. Secondly, bacterial LPS endotoxins may inflame capillaries in the hooves, and a link is assumed between hoof disease and *S. bovis,* a bacterium that thrives during SARA (Cook *et al.,* 2004).

Another sequel to SARA is acute ruminal acidosis (ARA). This is a metabolic condition characterized by a decreased blood pH (Hernandez *et al.,* 2014). During ARA, marked clinical symptoms are present and the ruminal pH is lower than during SARA (Enemark, 2008). Another common sequel to SARA is inflammation of the rumen epithelium referred to as ruminitis, as well as systemic inflammation due to LPS translocation (Oetzel, 2007; Enemark, 2008; Zhao *et al.,* 2018). While the relationship between rumenitis, inflammation, and SARA is not fully understood, they are suggested to be linked by translocation of LPS (Zhao *et al.,* 2018). Finally, abscesses in the liver are also common in animals that have suffered from SARA (Krause and Oetzel, 2006). The formation of abscesses is associated with rumenitis because it occurs after animals have acidosis or rumenitis and bacteria from the digestive tract enter the liver (Enemark,

2008; Amachawadi and Nagaraja, 2016). According to Amachawadi and Nagaraja (2016), this is called the acidosis-rumenitis-liver abscess complex. Generally, liver abscesses are associated with the gram-negative anaerobe bacteria, with *Fusobacterium necrophorum* being the main causative agent with *Trueperella pyogenes* following closely (Amachawadi *et al.,* 2016).

2.5.1 Disparity between cows affected by SARA and those that are not:

Early lactation cows are prone to have acidosis from up to 120 days in milk (Oetzel et al., 1999), with the highest risk particularly during the first 30 to 35 days in milk (Amachawadi *et al.,* 2016). Therefore, it is conclusive that early and mid-lactation cows have a higher risk of SARA than late lactation cows (Enemark, 2008; Li *et al.,* 2013). This is because cows in early lactation are fed diets that contain comparatively high amounts of grains, as they have a comparatively low dry matter intake (Enemark, 2008). Also, the absorption capacity of the rumen for VFAs may not be at its maximum in early lactation cows (Plaizier *et al.,* 2018).

2.5.2 Animal's response to SARA

In describing the animal's response to SARA, two concepts are important, including the prevalence and the incidence of SARA. Kleen and Cannizo (2012), distinguish these concepts and reported that prevalence refers to the number of cows that have SARA within the herd at a particular time. Incidence refers to the number of animals expected to have SARA within a period. It is assumed that the impact of SARA is increased greatly when it is accompanied by other metabolic disorders, such as laminitis, fatty liver, ruminitis, and ketosis (Kleen and Cannizo, 2012). Previously, SARA was usually determined herd-wise, and not by individual animal diagnosis (Enemark, 2008). That may not have been efficient and accurate, because animals with this condition are not easily identified. Therefore, it is preferred to consider SARA in terms of individual dairy cows, instead of relying entirely on herd-based conclusions (Kleen and Cannizo, 2012). If symptoms of SARA

are observed early, then the diet and management can be modified to help animals recover (Enemark, 2008). Therefore, if a herd has SARA, the feed intake, milk yield, and milk fat yield will be significantly reduced. This will enable the producer to aim towards the prevention of progression to ARA that will be prevented (Enemark, 2008; Kleen and Cannizo, 2012). While SARA is known to affect animal performance, welfare and health, there is a need for more information about how dairy cows adjust when SARA occurs (Steele *et al.*, 2012).

2.5.3 Preventive measures of SARA

As previously mentioned, SARA can impact a producer substantially by causing financial losses (Enemark, 2008). Therefore, preventive measures are necessary to reduce the number of cases of SARA on the farm per year. The use of a feeding regime that enhances adequate adaptation of epithelia, the functionality of microbiota, and buffering in the rumen will prevent SARA (Krause and Oetzel, 2006, Enemark, 2008; Plaizier *et al.*, 2018). This includes gradual transition in dietary changes, frequent feed delivery, reduction of large inclusion rates of rapidly degradable grains, and incorporation of sufficient dietary coarse fiber (Krause and Oetzel, 2006, Enemark, 2008). These practices will ensure that there is a balance between the production and absorption of VFAs, without which SARA occurs (Beauchemin, 2007). It is reported that about 30 to 50% of ruminal VFAs are buffered by saliva from chewing, whereas only a small proportion, approximately 10% of VFAs, are washed out to the small intestine (Hernandez *et al.*, 2014). This could mean that it is quite common for VFAs to accumulate in the rumen when the production of VFA exceeds their absorption.

By using a feeding regime that will minimize the accumulation of VFAs, one important practice is the incorporation of physically effective fiber into the animal's diet, which encourages chewing and in turn, increases saliva production and buffering (NRC, 2001). Physically effective neutral

detergent fiber (peNDF) refers to the physical characteristics of fiber (primarily particle size) that stimulate chewing activity (Allen, 1997). The use of NDF in feed assessment relates to the entire fiber fraction. Animals sort their feed by selecting only the grain fraction and small particles in the feed, and this can lead to SARA due to reduced chewing, saliva production, and rumen buffering (Kleen and Cannizo, 2012). Most dairy producers use total mixed rations (TMR) because this ensures the balance between non-structural carbohydrates and fiber, thereby preventing excessive intake of non-structural carbohydrates (NRC, 2001). Also, most producers use the Penn State Particle Separator on the farm to check if the dietary particle size distribution and dietary peNDF are adequate (Heinrichs and Kononoff, 2002). Hence. the preventive measures of SARA include the incorporation of peNDF, buffering (artificial buffers), proper housing structure for easy access to feed, supplement, and forage ratio (to minimize sorting), and administration of a TMR (Kleen and Cannizo, 2012).

2.6 Milk FA profile - an alternative biomarker to rumen pH for diagnosing SARA.

Changes in the FA profile of milk occur due to changes in the biohydrogenation pathway of dietary FAs. When this biohydrogenation process is incomplete, it leads to the formation of intermediates, such as isomers of conjugated linoleic acid (CLA) (Zened *et al.*, 2012). Rumenic acid (C18:2 *cis*-9, *trans*-11) is the main precursor of CLA. It is produced by rumen bacteria from dietary linoleic acid by the enzyme delta 9- desaturase from the 18:1 *trans*-11 (Rego *et al.*, 2009). After the isomerization of cis-9, *cis*12 C18:2, *trans* C18:1 is formed (Zened *et al.*, 2012). Vaccenic acid (*trans* 11, C18:1) is the main *trans* 18:1 isomer in milk fat. It constitutes, on average, 2.7% of the total FA content in milk. The proportion of this FA is particularly affected by season (Mansonn, 2008). CLAs are a group of isomers resulting from the biohydrogenation of unsaturated FAs, that are found in milk fat. Rumenic acid is the main CLA, accounting for 75 to 90% of total CLA in

milk fat. It is synthesized by the action of mammary Δ-desaturase on Vaccenic acid (*trans* 11, C18:1) (Mansonn, 2008). Feeding a high starch diet causes increases in the proportion of *trans*10 C18:1 instead of *trans*11 C18:1 as well as C18:2 *(n-6)* and total *n-6* FA in milk (Coppa *et al.*, 2012).

Trans 10 and 11 FAs are found in beef and milk because their formation is associated with incomplete biohydrogenation pathways of dietary polyunsaturated FAs (PUFAs), which are strongly influenced by the cow's diet (Zened *et al.*, 2012). *Trans*11 isomers are the major intermediates of this biohydrogenation (Zened *et al.*, 2012). *Trans*10 FA is the second most abundant isomer, and includes *trans*10, *cis*12 CLA, whose increase is associated with the reduced mammary synthesis of *de novo* FAs (short and medium-chain FAs), which causes milk fat depression (MFD) (Bauman and Grinarii, 2003; Shingfield and Grinarii, 2007). *Trans*10 C18:1 has been linked to high starch diets and rumen pH depression and reduces the milk fat content in milk of dairy cows (Bernard *et al.*, 2008). Increases in the proportion of PUFAs in milk fat have been reported to be due to several factors. When the forage to grain ratio is increased in the diets of dairy cows, there will be an increase in the PUFAs and *n-3* FAs proportions in milk, and a corresponding decrease in the proportions of saturated fatty acids (SFAs) (Coppa *et al.*, 2012). There will also be an increase in the proportion of *trans*11 C18:1, *cis*9 *trans*11 CLA, and C18:3 *(n-3)* in milk fat (Coppa *et al.*, 2012). Odd and branch-chain fatty acids (OBCFAs) have also been proposed as biomarkers for SARA (Fievez *et al.*, 2012; Mitchell *et al.*, 2016). The presence of *Iso* C13:0 and *iso* C16:0 may also indicate SARA (Colman *et al.*, 2010; AlZahal *et al.*, 2011). The milk of dairy cows contains the following OBCFAs that may be biomarkers for SARA: isomers of tridecanoic acid (*iso* C13:0), tetradecanoic acid (*iso* C14:0), pentadecanoic acid

(C15:0, *iso* C15:0 and *anteiso* C15:0), hexadecanoic acid (*iso* C16:0) and heptadecanoic acid (C17:0, *iso* C17:0 and *anteiso* C17:0) (Fievez *et al.*, 2012).

2.6.1 Milk fatty acid (FA) profile.

For this paper, the milk FA profile is a comprehensive quantification of all the FAs in the milk fat of dairy cows. This profile was described by Mansonn (2008), Fievez *et al.* (2012), and Liu *et al.* (2016). From previous studies, various responses (either increased or decreased) to a low rumen pH on FAs have been observed in animals in which SARA was induced (section 2.6). Hence, the FA profile may be able to diagnose SARA (Fievez *et al.*, 2012; Patel *et al.*, 2013; Mitchell *et al.*, 2016). The importance of having alternative diagnostic tools for SARA is due to the existing inability to diagnose SARA without the use of costly and invasive methods in commercial dairy cow farms has directed the current research towards the development of alternative ways to diagnose this disease (Plaizier *et al.*, 2008, Fievez *et al.*, 2012). This has given rise to the use of the milk FA profile as a biomarker for the diagnosis of SARA (Fievez *et al.*, 2012). The composition of milk fat is affected by diet and the resulting conditions in the rumen, as well as by the stage of lactation (Palmquist *et al.*, 1993). This is closely related to the shifts in the fatty acid composition of the feed, biohydrogenation pathways (section 2.6.4), and the energy status of the cows (Stoop *et al.*, 2009). The proportion of *de novo* short-chain FAs is lower during the beginning of lactation and continues to increase until the 8th to 10th week of lactation (Palmquist *et al.*, 1993). Rumen health and milk fat depression are linked to the milk FA profile as described by the conceptual theory of Bauman and Grinarii (2003). This theory is described in section 2.6.4.

The reactions of specific FAs to experimentally induced SARA have been well documented, and findings include:

- an increase in *trans*10, C18:1 in the rumen, blood, and milk of dairy cows is strongly associated with milk fat depression (MFD) (Bauman and Grinarii, 2003).
- Propionate produced during fermentation is responsible for the synthesis of C15:0 and C17:0 (Cabrita *et al.*, 2007).
- An increase in the concentration of C17:0 and C17:1 *cis*9 and a decrease in the concentration of *Iso* C14:0 are potential SARA indicators (Fievez *et al.*, 2012).
- The concentration of unsaturated C18 FAs decreases with milk fat concentration, while C16 increases with milk fat concentration (Stoop *et al.*, 2008).
- Odd and branched-chain FAs (OBCFAs) originate from bacteria leaving the rumen (Fievez *et al.*, 2012). According to Fievez *et al*. (2012) and Patel *et al.* (2013), they are either increased or decreased by SARA as they indicate the pattern of rumen fermentation (aiding with the prediction of the ruminal microbiome) and the duodenal flow of microbial protein during washout from the rumen.

Enjalbert *et al.* (2008) revealed that the mean ruminal pH has a direct relationship with the milk fat content and the proportion of odd-chain FAs and an indirect relationship with the ratio of *trans*10 C18:1/*trans*11 C18:1 in milk. This agreed with other studies which also indicated that there is a direct relationship between the nutritional components of the animal's feed and the fat content in milk (Chilliard *et al.*, 2007; Fievez *et al.*, 2012; Patel *et al.*, 2013; Michelle *et al.*, 2016).

In conclusion, the starch content of the diet will cause the rumen pH to be depressed, which in turn, will alter the FA profile of the milk (discussed further in sub-sections). The diagnosis of SARA is important because marked and clear clinical symptoms of this disease are not present (Mutsvangwa *et al.*, 2002; Krause and Oetzel, 2006). This is the main reason why milk biomarkers

that have been shown to respond distinctly to rumen pH, might be the preferred alternatives to invasive techniques involving rumen fluid collection on commercial dairy farms.

2.6.2 Reasons why milk biomarkers are relevant alternatives to conventional SARA diagnostic tools.

The use of milk FA profile as a biomarker for the diagnosis of SARA would be beneficial because it is less invasive and will not have adverse long-term effects, unlike the conventional methods which involve the collection of rumen fluids. Milk biomarkers are already used for the diagnosis of other subclinical diseases and disorders, such as mastitis using SCC; therefore, it will be an efficient tool for indicating SARA (DHI) (Oetzel, 2007). Research has been carried out to assess the possibility of using milk FA profile and amyloid A to diagnose the incidence of SARA, but that research has not been carried out in the field (Colman *et al.*, 2013; Plaizier *et al.*, 2018). Conventional methods for the diagnosis of SARA that involve the measurement of the pH of rumen fluid, the direct indicator of rumen acidity, have existed for several years, but there have been disagreements about whether these methods affect the health and welfare of the animal (Panousis *et al.*, 2018). Therefore, these methods are considered not to be useful on commercial farms. This is quite different from the indirect relationships researched upon to diagnose SARA, without using rumen fluid, such as milk biomarkers, conducted by several authors including Colman *et al.* (2010, 2013); Fievez *et al.* (2012); Patel *et al.* (2013). Such relationships were introduced earlier and are discussed later in this paper.

2.6.3 Milk FA profile: the composition of milk and milk fat

From an economic standpoint, milk fat is an important component of milk for dairy producers (Shingfield and Grinarii, 2007). The milk of dairy cows contains over 400 different FAs present in small quantities, making it a complex compound (Manson, 2008). The components of milk are

shown in Table 2.2 below. Milk FAs are either obtained from the feed, the ruminal microbes (C15:0 and C17:0) in dairy cows, or by *de novo* milk fat synthesis from VFAs in the mammary gland (Mansonn, 2008). The *de novo* synthesis of FAs involves the production of the total short and medium-chain FAs and 50% percent of C16 (acids having between 4 and 16 carbon chains) (Mansonn, 2008). These accounts for about 45% (weight basis) of the total FAs (Mansonn, 2008). Dietary fats are responsible for between 40 and 60% % of the total (MacGibbon and Taylor, 2006; Palmquist, 2006). This makes diet composition a very prominent factor in the milk fat composition of dairy cows. The FAs that are obtained from dietary fat include the other 50% of C16 and long-chain FAs (Mansonn, 2008). Proportions of unsaturated FAs can be altered by the process of desaturation in the mammary gland to form monosaturated acids (Mansonn, 2008). The composition of milk FAs is shown in Table 2.2.

Table 2.2: Composition of milk fat and FAs.

Components	Milk and its components Percentage composition
OBCFAs	2 to 3% (Patel *et al.,* 2013) of total milk fat
SFAs	66% -70% by weight (Mansonn, 2008) of total FAs
Monounsaturated FAs	25 - 30% (Mansonn, 2008) of milk FAs
PUFAs	2.3% (Mansonn, 2008) of milk FAs
***Trans* FAs**	2.7% (Mansonn, 2008) of milk FAs
Free FAs	0.1% (Mansonn, 2008) of milk fat
Cholesterol	Less than 0.5% (Mansonn, 2008) of milk fat
Diacylglycerol	2% (Mansonn, 2008) of milk fat
Triglycerides	98% (Mansonn, 2008) of milk fat
Moisture	87% (Randojic *et al.,* 2019) of milk
Lactose	4.6% (Randojic *et al.,* 2019) of milk
Protein	3.4% (Randojic *et al.,* 2019) of milk
Fat	4.2% (Randojic *et al.,* 2019) of milk
Vitamins	0.1% (Randojic *et al.,* 2019) of milk
Minerals	0.8% (Randojic *et al.,* 2019) of milk

2.6.4 Synthesis of milk fat

Milk FAs have 4 major pathways of origin and synthesis. These are dietary fatty acids, *de novo*, body fat reserves, ruminal biohydrogenation of dietary fatty acids. And bacterial degradation in the rumen (Stoop *et al.,* 2009). Liu *et al.* (2016) reported that in the process of increasing milk fat and milk yield, high-quality forage should not be replaced with lower quality forage and grains.

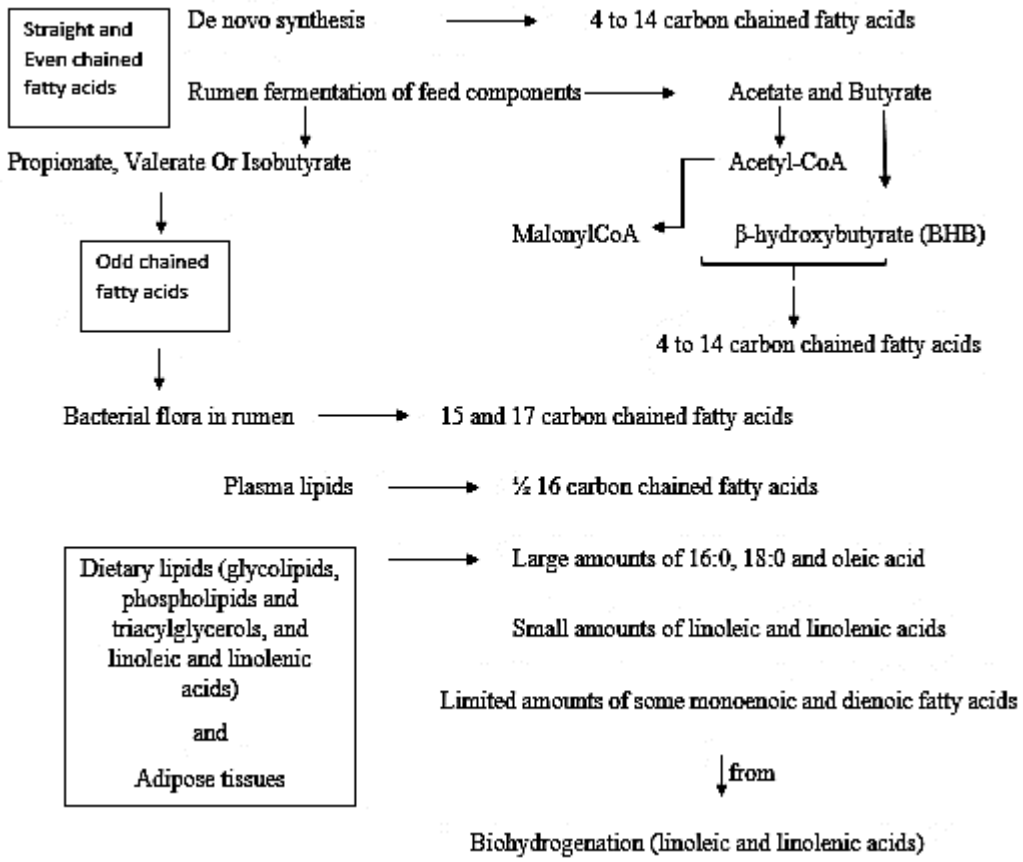
To categorize milk FAs,

- Short-chain FAs (SCFAs) contain 4 to 8 carbons (Liu *et al.,* 2016). Mansonn (2008) categorized short-chain FAs to include 10 carbon chains.
- Medium-chain FAs (MCFAs) contain between 10 to 14 carbons (Liu *et al.,* 2016). Mansonn (2008) categorized medium-chain FAs to range from 12 to 14 carbon chains.

- Long-chain FAs (LCFAs) contain 16 or more carbon (Liu *et al.*, 2016).
- Odd and branched-chain FAs (OBCFAs) (Fievez *et al.*, 2012)
- Polyunsaturated FAs (PUFAs) (Coppa *et al.*, 2012)

Milk fat synthesis and milk fat depression are affected by the composition of the diet of dairy cows (see section 2.2 above as well as the description below) (Shingfield and Grinarii, 2002). The synthesis of milk fat is shown in figure 2.3. Milk FA synthesis has been studied extensively, therefore the underlying causes of milk fat depression (bulk tank or specific FAs) could be understood.

Figure 2.3: Milk FA synthesis (MacGibbon and Taylor, 2006; Mansonn, 2008).



To understand the synthesis of milk fat, it is important to understand the different theories of milk fat depression (MFD) (Bauman and Grinarii, 2003). They are either related to animal

characteristics (*de novo* synthesis) or diets that cause incomplete biohydrogenation of dietary unsaturated fatty acids. This might explain why milk fat depression is not always SARA-related (diet-induced). There are four theories of milk fat synthesis proposed by Bauman and Grinarii (2003). Of these four, only two of these theories can be used to explain SARA. They are:

(a) The relationship between *trans* FAs and milk fat:

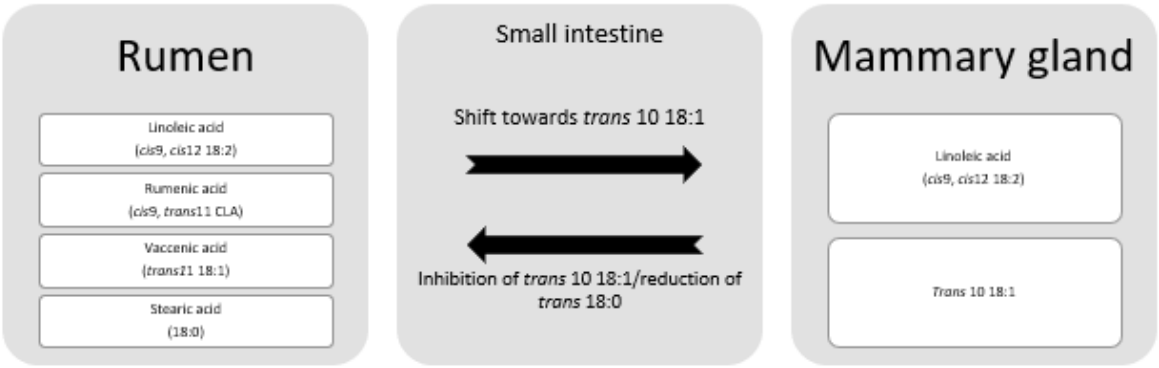
This theory assumes that an increase in *trans* octadecenoic acids indicates that the biohydrogenation of FAs in the rumen is incomplete. Because of inconsistencies in results, researchers carried out studies that identified that *trans*10, C18:1 (without sufficient evidence because it is hardly obtained as its pure form) and *trans*10, *cis*12 CLA (showed from previous studies) was the fatty acid major isomer associated with MFD, not *trans*11, C18:1 (which was initially thought to be the isomer responsible for MFD) (Bauman and Grinarii, 2003). Coppa *et al.* (2012) reported that an increased concentration of *trans*11, C18:1 was associated with MFD due to the feeding of forages, while increased *trans*10, C18:1 was related to high grain feeding (hence, closely related to SARA).

(b) The relationship between dietary concepts and milk fat: (also see section 2.6.1)

Rumen biohydrogenation converts unsaturated FAs into saturated FAs (Thanh, 2014). Bauman and Grinarii (2003) reported that ruminal incomplete biohydrogenation (Figure 2.4) of FAs is related to dietary conditions, such as feeding unsaturated FAs and high starch feeds. The rumen plays an important role in the flow of unsaturated FAs to the small intestine, where their absorption occurs. This is because of either the change in ruminal microbial processes or the incorporation of unsaturated FAs in the diets of dairy cows. The first is closely related to VFA patterns and low rumen pH affects the microbes and, hence, the biohydrogenation pathways. The second is associated with milk fat depression, which can occur when unsaturated FAs feed sources are fed

to cows. Therefore, it can be said that there is no consistency in the biohydrogenation reactions in the rumen (Beam et al., 2000). In previous research carried out by Stone (2004), the percentage of milk fat increased from 3.2 to 3.8% when there was an increase in the rumen pH from 5.4 to 6.25. This increase in rumen pH was due to the feeding of dairy cows with coarse instead of fine hay (Stone, 2004). This is the theory upon which the current study is based. Changes in biohydrogenation of FAs due to a reduction in rumen pH occur through pathways that give rise to increases of isomers of *trans* octadecanoic acid (partial biohydrogenation pathway). This will impact milk FAs profile in the mammary gland (Enjalbert *et al.*, 2003; Plaizier *et al.*, 2008). This principle is based on the theory by Bauman and Grinarii (2003), which identifies SARA as a diet-induced metabolic condition. MFD is associated with the incomplete biohydrogenation of FAs in the rumen (Oetzel, 2007). After absorption in the small intestine, the *trans* octadecanoic FAs are retrieved by the mammary gland where they inhibit the synthesis of fat (Oetzel, 2007).

Figure 2.4: The biohydrogenation pathway of linoleic acid (Bauman and Grinarii, 2003).



Other theories not directly related to diets; hence, SARA are:

(c) The relationship between ruminal acetate and butyrate and milk fat:

There is a relationship between changes in the concentrations and type of VFA and MFD. The reduction of acetate and butyrate (which serve as substrates for *de novo* milk fat synthesis) is

associated with the increase in propionate (which is mainly used for gluconeogenesis). If an altered rumen biohydrogenation is the cause of MFD, that is unrelated to the limited production of acetate and butyrate. (Bauman and Grinarii, 2003).

(d) The relationship between insulin and milk fat:

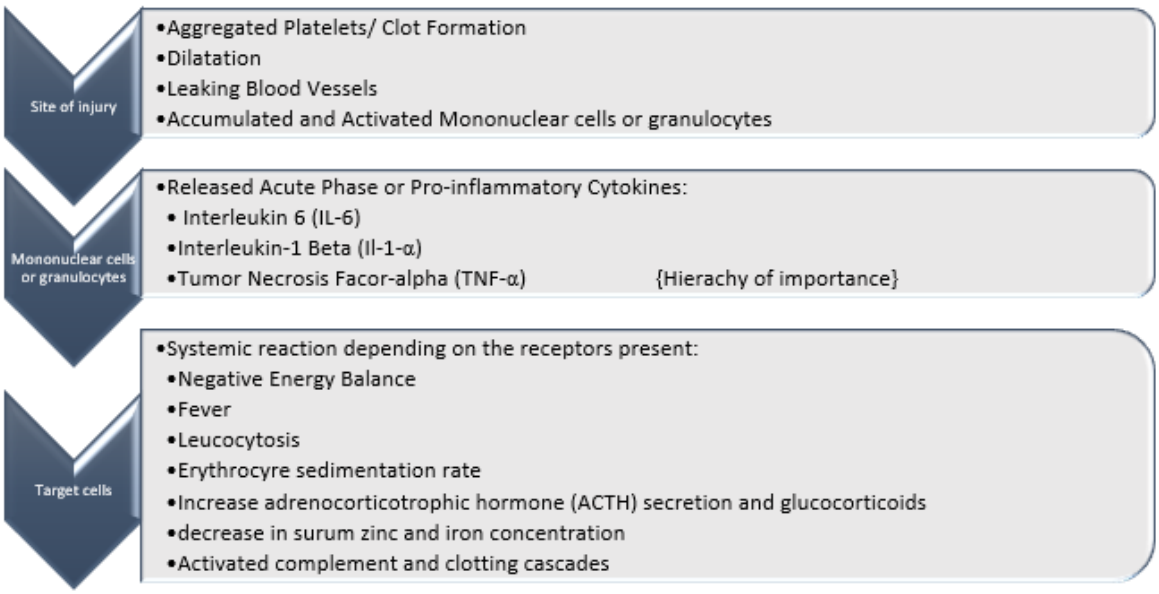
While insulin is useful for the regulation of the homeostasis of glucose in ruminants (which have only glucose transporters), it also helps in mammary cell function. Insulin regulates the rate of lipogenesis and lipolysis in adipose tissues; therefore, it can indirectly affect the nutrients supplied to the mammary gland. This theory states that insulin will affect how nutrients are utilized in the mammary gland by increasing the utilization of acetate by the adipose tissue and decreasing the mobilization of long-chain FAs from body fat reserves. Hence, it cannot be a factor involved in diet induced MFD (Bauman and Grinarii, 2003).

2.7 Definition of acute-phase response

The acute phase response is a process that helps to repair damaged tissues by mitigating continuous damage and ensuring that infectious agents, harmful molecules, and released residues do not multiply (Basbug *et al.,* 2020). During this process, the concentrations of acute-phase proteins (APPs) that are synthesized in the liver are altered. These proteins are released in the interior circulation, enabling their use as diagnostic biomarkers for infections, since they can be measured easily (Basbug *et al.,* 2020). It is a defense mechanism that restores homeostasis after infections and trauma (Basbug *et al.,* 2020). The acute phase response is a systemic reaction to an imbalance in homeostasis due to infection, tissue damage, immunological disorders, and neoplastic growth (Paulina and Tadeusz, 2011). The acute phase response represents a group of physiological processes, as shown in figure 2.5 below, that occur soon after the onset of infection, injury, trauma, and inflammatory processes, and are closely related to infectious diseases (Tothova *et al.*, 2014).

Acute-phase proteins (APPs) are formed when the acute phase response occurs (Tothova *et al.*, 2014). The release of APPs is associated with inflammation, such as systemic inflammation, rumenitis, metritis, and mastitis (Oetzel, 2007; Enemark, 2008; Zhao *et al.*, 2018). The liver is the main site of the synthesis of APPs (Paulina and Tadeusz, 2011). Previous research has reported that SAA does not significantly impact milk amyloid A (MAA) (O'Mahony *et al.*, 2006). According to Tothova *et al.* (2014), however, the concentration of MAA mirrors the concentration of SAA in blood.

Figure 2.5: The synthesis of acute phase proteins (Paulina and Tadeusz, 2011).



2.7.1 Definition and examples of acute-phase proteins

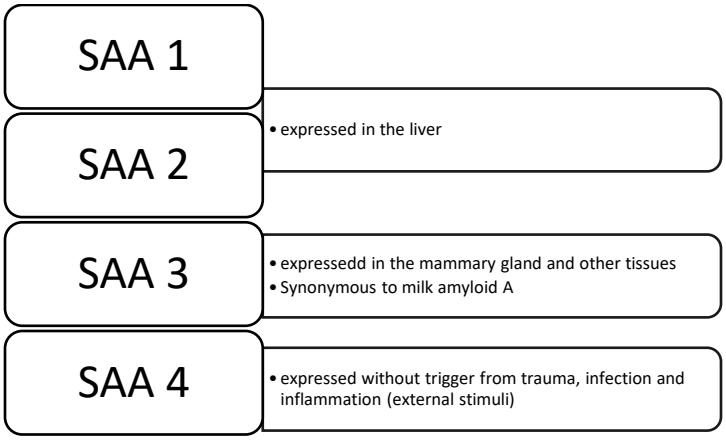
Acute phase proteins are plasma proteins that can act as biomarkers for early diagnosis of inflammation and infection in ruminant animals, especially in animals that do not show clinical signs of metabolic disorders (Paulina and Tadeusz, 2011). Milk Amyloid A (also called M-SAA or SAA3) is an acute-phase protein that is present in milk. It is an isotype of SAA expressed in the mammary gland (Kovac *et al.*, 2011). Experimentally induced SARA has been reported to increase

the concentration of APPs such as SAA, LBP, and haptoglobin (Hp) in blood plasma (Gozho *et al.,* 2007). Hence, SAA, Hp, and SAA and a mammary-associated SAA3 are biomarkers that can indicate SARA-related inflammation in dairy cows. However, increases in the concentrations of these APPs may not be related to SARA, as they indicate the presence of inflammation, but not necessarily the cause of the inflammation (Eckershall and Bell, 2010; Tothova *et al.*, 2014). Animals with inflammation have higher concentrations of APPs in blood and milk, but this may not necessarily indicate that the inflammation is related to SARA (Tothova *et al.,* 2014).

The APPs are, therefore, used to differentiate animals that need further examination for subclinical inflammatory disorders from those that do not (Karreman *et al*., 2000). Subacute ruminal acidosis was reported to trigger an acute phase response in cows (Plaizier *et al.*, 2018). Hence the premise that APPs might be released and can be measured to diagnose SARA (section 2.7.2).

The sites of expression of SAA are shown in figure 2.6.

Figure 2.6: SAA and sites of expression (Tothova *et al.,* 2014).



2.7.2 The synthesis of APPs and their relationship with SARA

The presence of SARA is associated with the release and translocation of endotoxic cell wall components (LPS) of gram-negative bacteria in the rumen digesta (Zebeli and Ametaj, 2009; Plaizier *et al.,* 2018). These endotoxins stimulate macrophages and other leukocytes to release pro-

inflammatory cytokines that activate hepatocytic receptors. These reactions are the pre-requisites for the synthesis of APPs (Zebeli and Ametaj, 2009). During SARA, the rumen pH is depressed for several hours per day, and this depression can cause injury and a reduction of the barrier function of the rumen epithelium. The LPS produced by gram-negative bacteria in the digestive tract bacteria are then translocated giving rise to an inflammatory response (Gozho *et al.*, 2005), which includes an acute phase response (Tothova *et al.*, 2014). Because blood concentrations of APPs increase during most inflammations, this response may not always indicate translocation of LPS. (Tothova *et al.*, 2014). An increase in APPs can also be caused by rumenitis, which can also occur due to SARA (Oetzel, 2007; Enemark, 2008; Zhao *et al.*, 2018). The functions of APPs are summarized in Table 2.3 below:

Table 2.3: The functions of Acute phase proteins (Tothova *et al.*, 2014).

S/N	Acute Phase Proteins	Functions	+/-
1	MAA	Prevents bacterial colonisation by inducing mucin production in intestinal cells	+
2	SAA	Involves the reverse transport of cholesterol from tissue to hepatocytes	
3	Haptoglobin	Reduces the availability of haemoglobin residue for bacterial growth by binding free haemoglobin.	+
4	Fibrinogen	It is involved in homeostasis	+
5	Alpha-1 acid glycoprotein	Acts as an anti-inflammatory and immunomodulatory agent	+
6	Alpha-1 antitrypsin	Limits host tissue injury by proteases at the site of inflammation	+
7	Transferrin	Reduces viral, bacterial, and fungal organisms' growth and multiplication by limiting their access to iron	-
8	Albumin	Amino acids source, carrier protein morbidity and mortality indicator	-
9	Transthyretin ('Prealbumin')	Transports thyroid hormones and vitamin A in plasma	-
10	Ceruloplasmin	Plays a role in Iron Metabolism and Copper Homeostasis	+
11	Lactoferrin	Affects the growth and proliferation of many infectious agents	+

2.7.3 Inflammation and APPs

The acute phase response is a process that helps to repair tissues that have been damaged by inflammation and infections by mitigating damage and ensuring that infectious agents, harmful molecules, and released residues do not multiply (Basbug *et al.*, 2020). During this process, the concentrations of APPs, synthesized in the liver, are altered by either increasing or decreasing their

concentrations in blood. These concentrations could, therefore, aid in the diagnosis of inflammation (Basbug *et al.,* 2020).

The following is a description of the roles and concentrations of cytokines and the APPs that are affected by them.

Interleukins (IL):

IL-4: This is lower in the serum and milk of cows with mastitis due to *Staphylococcus sp*. (86.1 and 123.17 pg/mL) compared with animals without mastitis (413.5 and 670.2 pg/mL) (Bochniarz *et al.,* 2017).

IL-10: It is higher in the milk of cows without mastitis than in cows with mastitis (39.78 and 22.5 pg/mL, respectively) (Bochniarz *et al.,* 2017).

Haptoglobin (Hp):

This is associated with lower total protein and casein levels (Akerstedt *et al.,* 2008). It can also alter milk composition (i.e., protein content) (Akerstedt *et al.,* 2008).

Serum amyloid A (SAA) and milk amyloid (MAA):

Serum amyloid A is associated with lower casein and lactose levels (Akerstedt *et al.,* 2008). It can also alter the milk protein content (Akerstedt *et al.,* 2008). It has been reported that MAA, but not SAA, is affected by mastitis (Gerardi *et al.,* 2009). Hence, the reliability of SAA for the diagnosis of mastitis is unclear.

2.7.4 APPs and other inflammatory biomarkers

Interactions between APPs and other inflammatory biomarkers like somatic cell counts (SCC) have been studied. It has been reported that APPs affect the SCC in milk (Akerstedt *et al.,* 2008). The SCC is a well-recognized inflammatory biomarker that indicates the state of health of the mammary gland (Gerardi *et al.,* 2009). It is known to be a fast and cost-efficient diagnostic tool

(Gerardi *et al.,* 2009). However, it is not suitable for differentiating between clinical and subclinical mastitis (Gerardi *et al.,* 2009). The correlation between MAA and SCC was reported in Gerardi *et al.* (2009). In another study, amyloid A (both SAA and MAA) were significantly higher in the milk of cows with mastitis when compared with cows without subclinical mastitis (1134.25 ng/mL and 324.50 ng/mL (Bochniarz *et al.,* 2017). When comparing the relationship between MAA and the causal organism of mastitis, several interactions have been reported. Cows with Mastitis caused by *Strep. agalactiae* and *Strep. Uberis* had the highest concentrations of MAA (3882.50 ng/mL, 2587.75 ng/mL respectively), whereas the milk of cows with mastitis was caused by *Strep. dysgalactiae* had low concentrations of MAA (812.00 ng/mL) (Bochniarz *et al.,* 2017).

2.8 Summary

Lactating dairy cows have high nutrient requirements to sustain them during lactation. One of these requirements is energy. Negative energy balance is common in transitioning and high-yielding cows when increases in feed intake lag the increases in milk yield. To meet the energy requirements, the energy (grain) content of diets is increased, which reduces the rumen pH and can induce SARA. Low rumen pH is the conventional indicator of SARA. There are no overt external signs or symptoms of SARA. Therefore, there is a need to collect rumen fluid to measure its pH. Rumen fluid collection methods are invasive. Hence, they cannot be used in commercial dairy farms. Therefore, there is a need for alternative methods and tools for the diagnosis of SARA. Milk FA profile may be useful in diagnosing SARA. This is because of the FA isomers that are formed due to the incomplete biohydrogenation of unsaturated FAs when the rumen pH is depressed. When these concentrations are monitored, SARA may be diagnosed non-invasively. Another milk-based biomarker that is promising for the diagnosis of SARA is MAA. This is an acute-phase

protein that is released during infection, inflammation, and trauma during a process called the acute phase or immune response. This response occurs to restore homeostasis. SARA is associated with inflammatory responses, such as systemic inflammation, inflammation of the rumen, and inflammation of the hooves. Therefore, the concentrations of these proteins in the interior circulation (serum) and milk of the affected cows will change, making it an easily accessible diagnostic tool for SARA. If these biomarkers can diagnose SARA in commercial farms, then they can be easily implemented and help monitor and treat this condition.

3.0 RESEARCH HYPOTHESIS AND OBJECTIVES

3.1 HYPOTHESES

1. SARA increases MAA concentrations in commercial lactating dairy cows.
2. SARA will reduce de novo fatty acids in commercial lactating dairy cows.
3. SARA will increase polyunsaturated fatty acids in commercial lactating dairy cows.
4. SARA will change concentrations of odd and branched chain fatty acids in commercial lactating dairy cows (*iso* C14:0 and *iso* C15:0 will increase with acetate and decrease with propionate; C15:0 will increase with decrease in C17:0; SARA increases C17:0 + C17:1 *cis*-9; SARA decreases *iso* C14:0 (Fievez *et al.*, 2012)).

3.2 OBJECTIVES

1. To determine if MAA is an accurate tool for the diagnosis of SARA in dairy cows.
2. To validate the diagnosis of SARA using milk FA profile from samples collected on dairy farms. This will involve the assessment of SARA in individual cows based on their milk FA profile. Common practice is herd-based assessment of SARA.

4.0 MANUSCRIPT

THE DIAGNOSIS OF SUBACUTE RUMINAL ACIDOSIS (SARA) USING THE MILK FATTY ACID PROFILE (MFAP) AND MILK AMYLOID A (MAA) CONCENTRATIONS ON DAIRY FARMS.

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4.1 ABSTRACT

The study was conducted to determine if the fatty acid (FA) profile and the milk amyloid A (MAA) content of milk of individual cows can be used as biomarkers to diagnose subacute ruminal acidosis (SARA) in commercial dairy farms. A total of 320 milk samples from individual cows on 24 commercial dairy farms in Quebec were tested. Farms were paired in blocks based on geographical location and management. Blocks included a high Risk of SARA farm and a Low Risk of SARA farm. High SARA Risk farms had a proportion of *de novo* FAs below 0.88 g/100 g of milk, a proportion of polyunsaturated FAs (PUFAs) greater than 3.58 g/100 g of total FAs, a milk fat content below 4.01%, and a milk protein content above 3.04% in bulk tank milk. Low SARA Risk farms had a *de novo* FA content of milk higher than 1.12 g/100 g of milk. On each farm, 7 early to mid-lactation (1 – 150 days in milk, DIM) and 7 mid to late lactation cows (151 and more DIM) were randomly selected. The early to mid-lactation cows were high SARA Risk cows, whereas the mid-to-late lactation cows were low SARA Risk cows. Cows with a somatic cell count (SCC) of over 200,000 cells/ml were not included. Farm Risk of SARA did not affect the milk fat proportions of fatty acids, except for *trans* 10 *cis* 12 C18:2, which was higher in high SARA Risk Farms. Low SARA Risk Cows had a higher milk fat content and higher milk fat proportions of *de novo*, C16 fatty, and odd and branch-chain fatty acids compared to high SARA Risk cows. The effects of Farm Risk of SARA and Cow Risk of SARA on MAA were not significant, but MAA was correlated with SCC. The milk fatty acid profile can contribute to the diagnosis of SARA in individual cows, but other measures are needed for an accurate diagnosis, as non-SARA-related animal and dietary factors also affect this profile.

Keywords: MAA, SARA, Dairy cows, Milk biomarkers, milk fatty acids.

Abbreviation key: Milk amyloid A (MAA), Subacute ruminal acidosis (SARA).

4.2 INTRODUCTION

To meet the high energy requirements of high-yielding dairy cows, they are commonly fed high grain diets. As these diets are highly fermentable and contain low amounts of coarse fiber, these diets can cause an accumulation of fermentation acids in the rumen, and a reduction of rumen buffering, leading to depressions of the rumen pH for extended periods each day (Kleen *et al.*, 2003; Krause and Oetzel, 2006; Plaizier *et al.*, 2018). These pH depressions can cause gut health disorders, such as subacute ruminal acidosis (SARA) (Kleen *et al.*, 2013; Krause and Oetzel, 2006; Plaizier *et al.*, 2018). This disorder affects the production and health of dairy cows by decreasing milk fat production, nutrient utilization, the functionality of the rumen epithelium, and feed intake, as well as by causing inflammation, laminitis, and diarrhea (Li *et al.*, 2016; Plaizier *et al.*, 2018). If diagnosed early, these impacts can be limited (Enemark, 2008; Plaizier *et al.*, 2018). However, the current tools for the diagnosis of SARA that are based on the measurement of the rumen fluid pH are inaccurate, high cost, invasive, and can cause health problems to the cow (Enemark, 2008; Steele *et al.*, 2012; Plaizier *et al.*, 2018). Hence, SARA is commonly not diagnosed, and non-invasive and more accurate tools for this diagnosis are needed. Among the options for these, biomarkers of SARA in milk would be useful, as they do not require invasive sampling and cause little stress (Enemark, 2008; Plaizier *et al.*, 2018). In addition, as milk samples from the bulk tank and individual cows are analyzed regularly, these biomarkers could be used for routine monitoring of herds and cows.

Rumen pH depression is associated with milk fat depression and changes in the milk fatty acid (FA) profile (Kadegowda *et al.*, 2008; Bauman and Griinari, 2003; Plaizier *et al.*, 2018). These effects are likely due to changes in the biohydrogenation of unsaturated fat in the reticulorumen resulting from the rumen pH depression and increases the concentrations of *trans* octadecenoic

acids that inhibit *de novo* milk fat synthesis (Bauman and Griinari, 2003; Coppa *et al.*, 2012; Zened *et al.*, 2012). SARA can reduce milk fat content, as well as the proportion of *de novo* FAs (Kadegowda *et al.*, 2008; Colman *et al.*, 2013; Mitchell *et al.*, 2016). Studies on experimentally grain-induced SARA have shown that this induction alters the proportions of odd- and branch-chain FAs (Fievez *et al.*, 2012. Colman *et al.*, 2013; Mitchell *et al.*, 2016). As a result, diagnostic SARA based on the proportions of short, odd, and branch chain FAs may be possible. However, many feed and animal factors, including ones that are not related to SARA, such as genetics and stage of lactation, affect the milk FA profile (Kay *et al.*, 2005; Palmquist *et al.*, 2013; Bilal *et al.*, 2014).

Experimental grain-induced SARA was associated with an acute phase response, including an increase in the blood concentration of SAA (Emmanuel *et al.*, .2008; Enemark, 2008; Plaizier *et al.*, 2018). The concentration of Milk Amyloid A (MAA) mirrors that of SAA in the blood (Gerardi *et al.*, 2009; Tothova *et al.*, 2014), which offers perspectives for the diagnosis of SARA based on the acute phase response. However, many infectious diseases in dairy cows, including mastitis, also increase the concentrations of MAA (Gerardi *et al.*, 2009; Wollowski *et al.*, 2021), which may affect the accuracy of MAA for the diagnosis of SARA. In earlier studies in which the effects of SARA on milk FA profile and MAA concentrations were examined, SARA was experimentally induced, predominantly by high grain feeding (Enjalbert *et al.*, 2008; Colman *et al.*, 2013; Plaizier *et al.*, 2019). This examination also needs to be conducted on commercial dairy farms, where naturally occurring SARA may be present, as the etiology of naturally occurring SARA and experimentally induced SARA may differ (Plaizier *et al.*, 2018).

Dairy herd improvement (DHI) organizations already test milk from individual cows for a variety of components and biomarkers, such as fat, protein, lactose, milk urea nitrogen, beta-

hydroxybutyrate, and somatic cell counts (SCC) (Lactanet, 2020). If the diagnosis of SARA through milk biomarkers is effective, then this can complement the other DHI tests, and provide a more comprehensive assessment of the production and health of dairy cows. We hypothesize that cows on farms with a High Risk of SARA and early- to mid-lactation that are at a high Cow Risk of SARA have a higher concentration of MAA and a different milk FA profile, including lower proportions of short and medium-chain FAs and a higher proportion of odd- and branch chain FAs, compared to cows on low-risk SARA farms. The main objective of this research is to evaluate the use of the milk FA profile and MAA as diagnostic tools of SARA on commercial farms and to identify FAs that are biomarkers for SARA.

4.3 MATERIALS AND METHODS

4.3.1 Experimental design

A total of 336 Lactating Holstein dairy cows on Quebec dairy farms were used in a Randomized Complete Block (RCB) design with a Split-Plot arrangement. The main plot was the farm, and the stage of lactation (early to mid-lactation with 1 to 150 days in milk (DIM) and mid to late lactation with 151 and more DIM was the subplot. Cows with 150 DIM were considered to be at high risk of SARA, and cows with 150 or more DIM were considered to be at low risk of SARA. Farms were paired in 12 blocks according to their management and geographical location. Each block consisted of a High Risk of SARA farm and a Low Risk of SARA farm. As a result, there were 4 animal groups: high-risk cows on high-risk farms (HH), low-risk cows on high-risk farms (HL), high-risk cows on low-risk farms (LH), and low risk cows on low-risk farms (LL). The risk level of SARA was determined based on the content and composition of far in the bulk tank of farms that were averaged for the month before the visit. High-risk farms had to meet the following criteria: a low proportion of *de novo* FAs (below 0.88 g/100 g of milk fat), a high proportion of

polyunsaturated FAs on a total FA basis PUFA (greater than 3.58 g/100 g of total FAs), a low milk fat content (below 4.01%), and sufficient milk protein content (higher than 3.04%). Low-risk farms had a high *de novo* FA content of milk (higher than 1.12 g/100 g of milk fat). Farms that fed cows substantial amounts of fat supplements, such as palm oil, were not included in the study. On each farm 7 cows with a DIM between 1 and 150 days, and 7 cows with a DIM greater than 150 days were randomly selected. Cows with an SCC greater than 200,000 were excluded. Milk samples from selected individual cows were collected by Lactanet (Sainte-Anne-de-Bellevue, QC) on regular DHI test days during January 2020, and stored at -18°C until further analysis.

4.3.2 Milk fat analysis

Frozen samples were transferred to a cooling room (4^{o}C) to thaw the night before analysis. On the analysis day, each sample was left to thaw in the water bath (40^{o}C). Milk fat extraction was carried out using the mini Röse-Gottlieb method (Chouinard *et al.*, 1999). This method involved adding ammonium solution (25%), ethanol, diethyl ether, and petroleum ether to the milk sample during intermittent mixing with a vortex mixer. The newly formed compound was left to stand until there were two distinct phases, i.e., an upper part and a lower part. The upper part containing the extracted fat was taken out to be methylated. Methylation was carried out using the method of Stefanov *et al.* (2010). In this method, hexane, methyl acetate the methylation reagent (methanol and sodium methoxide solution), termination reagent (oxalic acid and diethyl ether), and calcium chloride were added to the milk fat extract during intermittent vortex mixing. Subsequently, the newly formed methylated extract was removed for gas chromatography (GC) analysis. The FAMEs were analyzed by split mode injection (ratio 44.5:1) and 1uL of the sample was injected into a Perkin Elmer 580 gas chromatograph (GC) with a flame ionization detector (FID). The gas

was equipped with a Zebron capillary GC column (30m x 0.25 mm ID x 0.2 µm film thickness (Phenomenex, Torrance, CA) with helium as carrier gas at 13.6 psi and GC-FID.

The oven temperature was held at 100 ºC for 1.35 min, then programmed to increase at 4.5 ºC/min to 250 ºC and maintained at this temperature for 10 minutes. The FID temperature was 250ºC at H2 flow of 40 mL/min and airflow of 450 mL/min. FA methyl esters (FAMEs) were identified by comparison of retention times of the FAMEs from milk samples with retention times of commercial standards (Sigma-Aldrich and MJS BioLynx.inc). TotalChrom (Perkin Elmer, Waltham, MA) was used for data analysis. After the FID was ignited, a 4-6 hour wait time was used to achieve the conditions of the FID, including temperature, split, and pressure, suitable for analysis. This wait time was required for the signal of the FID to become constant. The wash vial was filled with hexane. Vials with samples were placed in an auto-sampler. The 37 components of the FAME mix were run on the GC. The software used for data analysis was TotalChrom (Perkin Elmer, Waltham, MA). The peak area of each peak in the chromatogram was calculated by manual integration, during which the peak height or area is integrated manually by setting the baseline using the chromatographic software.

The peaks in the chromatogram were identified by comparison with the retention time of standards, including C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, *iso* C13:0, *anteiso* C13:0, *iso* C14:0, a*nteiso* C15:0/*iso* C15:0, C15:0, *cis*10 C15:1/*iso* C16:0, *iso* C17:0, a*nteiso* 17:0, C17:0, C21:0, C23:0, C16:0, C18:0, C20:0, C22:0, C24:0, *trans*10 *cis*12, C18:2, *Cis*9 *trans*11 C18:2, C18:3 (n-6), C18:3 *(n-3)* (C18:3 *cis* 9, 12, 15), C20:3 *(n-6)*, C20:4 *(n-6)*, C20:3 *(n-3)*, C20:5 *(n-3)*, *cis*9 c*is*12 C18:2, *cis*11 *cis*14 20:2, *cis* 9 14:1, *cis* 9 C16:1, *cis* 11 C18:1, *cis* 11 20:1, *cis* 15 24:1, *trans*9 C16:1, and *trans* 9 C18:1, *cis* 9 18:1/*trans* 11 C18:1 from Sigma Aldrich (St. Louis, MO) and MJS BioLynx. Inc. (Brockville, ON, Canada).

4.3.3 MAA analysis

The MAA in milk samples was analyzed using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) (Tridelta Development, Maynooth, Ireland) following the manufacturer's instructions. Optical densities (ODs) were read using a microplate plate reader (Biotek 800, Winooski, VT) at 450 nm and a reference at 630 nm. Milk samples were either diluted in the ratio of 1:25 or 1:20. The sensitivity of the assay was 0.1µg/ml. The working range for the assay was 0.468 – 7.5 µg/ml. The concentration of the diluted sample needed to be within the range of the curve for the sample to be considered valid. This range was from 9.4 to 150 ng/ml. If a sample was out of the range, then the sample was diluted further and analyzed samples again. Samples were analyzed in duplicates.

4.3.4 Feed analysis

Forage samples used in lactating cow rations were collected on the day of the visit by the Lactanet farm advisor and were analyzed by near-infrared spectroscopy (NIRS) using models and equations developed by Cumberland Valley Analytical Services (Waynesboro, PA).

4.3.5 Statistical analysis

The MAA concentrations and the proportions of milk FAs were analyzed using Proc Mixed (SAS Institute, Cary, NC) using the model:

$Yijk = \mu + Li + Rj + (L \times R)ij + Bk + \beta 1Pl + \beta 2Sijkl + eijkl$

Where:

Yijkl = the dependent variable (MAA concentration, proportion of individual milk FA),

µ = overall mean,

Li = effect Cow Risk of SARA/Lactation stage (i = 1, 2)

Rj = effect of Farm Risk of SARA (j = 1, 2)

$(L \times R)ij$ = effect of Cow Risk × Fam Risk (ij = 1, 2, 3, 4)

Bk = effect of Block (k = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12)

$\beta 1–2$ = coefficients associated with each covariate

Pijkl = effect of parity

Sijkl = effect SCC

eijkl = random residual error

The model included SCC and parity as covariates. The effects of Cow and Farm Risk of SARA were considered as fixed effects, while Block was considered as a random effect.

4.4 RESULTS

4.4.1 Diet composition

The average diet composition for experimental groups is presented in Table 4.1. There were significant ($p<0.05$) differences in the dietary contents of CP, NDF, ADF, NFC, and CFAT among these groups. The effect of the interaction between Risk level and Stage was significant for the dietary CP, ADF, and CFAT contents, whereas this interaction tended to be significant for NDF. The dietary CP content was lower for the LL group than for the HH and LH groups. The dietary NDF content was the highest for the LL group, whereas the dietary ADF contents were lower for the HH and HL groups than for the LH and LL groups. Dietary NFC and CFAT contents were the lowest for the LL group.

Table 4.1: Comparison of diet composition among experimental groups.

	HH	HL	LH	LL	SE	Farm Risk	Cow Risk	Farm Risk*Cow risk
CP, % DM	17.0[a]	16.7[ab]	17.1[a]	16.3[b]	0.29	0.05	<0.01	0.02
NDF, % DM	35.3[b]	35.4[b]	36.1[b]	39.1[a]	1.20	<0.01	<0.01	0.02
ADF, % DM	21.6[c]	21.7[c]	23.5[b]	25.7[a]	0.87	<0.01	<0.01	0.05
NFC, % DM	39.3[a]	39.3[a]	38.5[a]	36.9[b]	0.99	<0.05	<0.05	0.05
CFAT, % DM	3.63[a]	3.58[ab]	3.68[a]	3.44[b]	0.10	0.43	<0.05	0.12

HH: High risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

HL: High risk farm, low risk cows (mid to late-stage animals (> 150 DIM))

LH: Low risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

LL: Low risk farm, low risk cows (mid to late lactation stage animals (> 150 DIM))

SE= Standard error

4.4.2 MAA and milk production

The effects of the Risk level of SARA and Stage of lactation, SCC, and parity on milk production and MAA are given in Table 4.2. This table shows that this risk did not affect lactation milk yield, milk composition, and MAA. Late lactation cows had higher milk fat and milk protein contents compared to earlier lactation cows. The risk level of SARA and Stage of lactation did not affect MAA. Both SCC and PAR affected milk yield, milk composition, and MAA. Higher SCC was associated with lower lactation milk yields and higher MAA. The effects of PAR on milk yield, milk composition, and MAA were quadratic, with higher parities being associated with higher milk yields and the milk contents of fat, protein, and MAA.

Table 4.2: Effects of farm Risk of SARA (High = H, low = L) and cow risk of SARA (H = ≤ 150 DIM, L = > 150 DIM) on milk production and milk amyloid A (MAA)

						Significance, P -values					
	HH	HL	LH	LL	SE	Farm Risk	Cow risk	Farm Risk*Cow risk	SCC	PAR	SCC*PAR
Milk, kg/305 d	10015[b]	10787[a]	11147[a]	11040[a]	299.7	N.S	<0.01	N.S	<0.01	<0.01	<0.01
Milk fat, %	3.94[b]	4.22[ab]	4.02[b]	4.45[a]	0.16	N.S	<0.01	N.S	N.S	<0.01	<0.01
Milk protein, %	3.33[b]	3.56[a]	3.31[b]	3.67[a]	0.11	N.S	<0.01	N.S	N.S	<0.01	<0.01
MAA, µg/ml	360.4[a]	328.8[ab]	398.0[ab]	256.8[b]	50.5	N.S	N.S	N.S	<0.01	<0.01	0.04

HH: High risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

HL: High risk farm, low risk cows (mid to late-stage animals (> 150 DIM))

LH: Low risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

LL: Low risk farm, low risk cows (mid to late lactation stage animals (> 150 DIM))

SE= Standard error

SCC = Somatic cells counts

PAR = Parity

SE= Standard error

4.4.3 Effects of Risk level of SARA and Stage of lactation on proportions of *de novo* milk FAs and C16 FAs

The risk level of SARA and the interaction of Risk level of SARA and Stage of lactation did not affect the proportions of the *de novo* milk FAs (Table 4.3). In contrast, the Stage of lactation affected the proportions of C4:0, C6:0, C12:0, C14:0, and C14:1. Of these, the proportions of C4:0 and C6:0 were lower in later lactation cows than in earlier lactation cows. The proportions of C12:0, C14:0, and C14:1 were higher in later lactation cows than in earlier lactation cows. Across all *de novo* milk FAs, the proportion of these FAs was the highest in later lactation cows. The effects of SCC and PAR on the proportions of *de novo* milk FAs were not significant.

The risk level of SARA and the interaction of Risk level of SARA and Stage of lactation did not affect the proportions of C16 milk FAs (Table 4.4), except for a trend towards a higher proportion of *cis*9 C16:1 in low SARA Risk farms. The effects of SCC and PAR on the proportions of C16 milk FAs were not significant. The interaction of Risk level of SARA and Stage of lactation was significant for C16:1, *trans*9 C16:1, and Total C16, with later lactation cows in the LB group having the highest proportions of C16:0, *cis*9 C16:1, and total C16, and having the lowest proportion of *trans*9 C16:1. The effects of SCC and PAR on the proportions of C16 milk FAs were not significant.

Table 4.3: Effects of farm Risk of SARA (High = H, low = L) and cow risk of SARA (H = ≤ 150 DIM, L = > 150 DIM) on *de novo* milk fatty acids proportions (g/100 g of FA)

						Significance, P -values					
	HH	HL	LH	LL	SE	Farm Risk	Cow risk	Farm Risk*Cow risk	SCC	PAR	SCC*PAR
C4:0	3.16[a]	2.96[b]	3.24[a]	2.93[b]	0.06	N.S	<0.01	N.S	N.S	N.S	N.S
C6:0	2.28[a]	2.17[b]	2.29[a]	2.16[b]	0.03	N.S	<0.01	N.S	N.S	N.S	N.S
C8:0	1.40	1.36	1.41	1.36	0.02	N.S	N.S	N.S	N.S	N.S	N.S
C10:0	3.26	3.26	3.68	4.04	0.09	N.S	N.S	N.S	N.S	N.S	N.S
C12:0	3.70[b]	3.90[b]	3.68[b]	4.40[a]	0.12	N.S	<0.01	N.S	N.S	N.S	N.S
C14:0	11.9[b]	12.4[a]	11.8[b]	12.6[a]	0.22	N.S	<0.01	N.S	N.S	N.S	N.S
***Cis*9 C14:1**	1.17[b]	1.47[a]	1.14[b]	1.48[a]	0.04	N.S	<0.01	N.S	N.S	N.S	N.S
Total *de novo*	26.9[b]	27.6[a]	26.8[b]	27.9[a]	0.44	N.S	0.03	N.S	N.S	N.S	N.S

HH: High risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

HL: High risk farm, low risk cows (mid to late-stage animals (> 150 DIM))

LH: Low risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

LL: Low risk farm, low risk cows (mid to late lactation stage animals (> 150 DIM))

SCC = Somatic cells counts

PAR = Parity

SE= Standard error

Table 4.4 Effects of farm Risk of SARA (High = H, low = L) and cow risk of SARA (H = ≤ 150 DIM, L = > 150 DIM) on C16 milk fatty acids proportions (g/100 g of FA)

						Significance, P -values					
	HH	HL	LH	LL	SE	Farm Risk	Cow risk	Farm Risk*Cow risk	SCC	PAR	SCC*PAR
C16:0	32.6[b]	33.1[b]	32.1[b]	34.5[a]	0.67	N.S	<0.01	<0.01	N.S	N.S	N.S
Cis C16:1	1.34[c]	1.49[b]	1.41[b]	1.64[a]	0.07	N.S	<0.01	N.S	N.S	N.S	N.S
Trans C16:1	0.097[a]	0.085[b]	0.099[a]	0.081[c]	0.005	N.S	<0.01	0.02	N.S	N.S	N.S
Total C16	31.5[a]	32.1[b]	31.2[b]	33.7[a]	0.71	N.S	<0.01	<0.01	N.S	N.S	N.S

HH: High risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

HL: High risk farm, low risk cows (mid to late-stage animals (> 150 DIM))

LH: Low risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

LL: Low risk farm, low risk cows (mid to late lactation stage animals (> 150 DIM))

SCC = Somatic cells counts

PAR = Parity

SE= Standard error

4.4.4 Effects of Risk level of SARA and Stage of lactation on proportions of odd and branch chain milk FAs

The risk level of SARA and the interaction of Risk level of SARA and Stage of lactation did not affect the proportions of odd and branch chain FAs (Table 4.5). The stage of lactation affected the proportion of all odd and branch chain FAs, except for *iso* C17:0, *anteiso* C17:0, and C21:0. Of all the odd and branch chain FAs that were affected, the proportions were higher in later lactation cows than in earlier lactation cows. The exception to this was C17, where the opposite was observed. The effects of SCC and PAR on the proportions odd and branch chain FAs were not significant.

Table 4.5. Effects of farm Risk of SARA (High = H, low = L) and cow risk of SARA (H = ≤ 150 DIM, L = > 150 DIM) on odd and branch chain milk fatty acids proportions (g/100 g of FA)

						Significance, P -values					
	HH	HL	LH	LL	SE	Farm Risk	Cow risk	Farm Risk*Cow risk	SCC	PAR	SCC*PAR
***Iso* C13:0**	0.07[b]	0.09[b]	0.06[b]	0.09[a]	0.003	N.S	<0.01	N.S	N.S	N.S	N.S
***Anteiso* C13:0**	0.09[b]	0.11[a]	0.09[b]	0.12[b]	0.005	N.S	<0.01	N.S	N.S	N.S	N.S
***Iso* C14:0**	0.10[b]	0.12[a]	0.10[b]	0.12[a]	0.005	N.S	<0.01	N.S	N.S	N.S	N.S
***Anteiso* C15:0/*Iso* C15:0**	0.40[b]	0.45[a]	0.39[b]	0.44[a]	0.02	N.S	<0.01	N.S	N.S	N.S	N.S
C15:0	1.16[b]	1.25[a]	1.07[b]	1.25[a]	0.04	N.S	<0.01	N.S	N.S	N.S	N.S
Cis 10 C15:1/*Iso* C16:0	0.23[b]	0.27[a]	0.23[b]	0.27[a]	0.01	N.S	<0.01	N.S	N.S	N.S	N.S
C17:0	0.58[a]	0.54[b]	0.60[a]	0.55[b]	0.01	N.S	<0.01	N.S	N.S	N.S	N.S
C23:0	0.020[b]	0.022[a]	0.020[b]	0.022[a]	0.0002	N.S	<0.01	N.S	N.S	N.S	N.S
Total odd and branch chain	3.30[b]	3.52[a]	3.19[b]	3.50[a]	0.07	N.S	<0.01	N.S	N.S	N.S	N.S

HH: High risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

HL: High risk farm, low risk cows (mid to late-stage animals (> 150 DIM))

LH: Low risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

LL: Low risk farm, low risk cows (mid to late lactation stage animals (> 150 DIM))

SCC = Somatic cells counts

PAR = Parity

SE= Standard error

4.4.5 Effects of Risk level of SARA and Stage of lactation on the proportion of long-chain milk FAs

The risk level of SARA and the interaction of Risk level of SARA and Stage of lactation did not affect the proportions of saturated long-chain FAs (Table 4.5). Stages of lactation affected the proportions of C18:0, C24:0, and total long-chain saturated FAs, with later lactation cows having lower proportions of C18 and total saturated long-chain FAs and a higher proportion of C24:0 than earlier lactation cows. The effects of SCC and PAR on the proportions of saturated long-chain FAs were not significant.

The risk level of SARA did not affect the proportions of mono-unsaturated long-chain FAs. Stage of lactation affected the proportions of c*is*11 C18:1/ *trans*11 C18:1. The effects of the interaction between the Risk level of SARA and the Stage of lactation were significant for *cis*11 C20:1 and *cis*11 C18:1/ *trans*11 C18:1. The proportion of *cis*11 C18:1 was lower in the LL group than in the HH group. The proportion of *cis*11 C18:1 was lower in later lactation cows than in earlier lactation cows. The proportion of *cis*11 C18:1/ *trans*11 C18:1 was lower in the LL group than in the other groups. The effects of SCC and PAR on the proportion of *cis*11 C20:1 were significant, with higher SCC and PAR being associated with a higher proportion of this FA.

The risk level of SARA and the interaction of Risk of SARA and Stage of lactation did not affect the proportions of poly-unsaturated long-chain FAs. Stage of lactation affected the proportions of C20:3 *(n-6)*, *trans*10 c*is*12 C18:2, *cis*9 *trans*11 C18:2, and *cis*9 *cis*11 C18:2. These proportions were higher in later lactation cows compared to earlier lactation cows. The proportion of total polyunsaturated long-chain FAs tended to be lower in the later lactation cows on the low SARA Risk farms. Higher SCC was associated with higher proportions of C18:3 *(n-3)* and C20:5 *(n-3)*. Parity affected the proportions of C20:4 *(n-6)* and *cis*9 *trans*11 C18:2 quadratically.

Table 4.6: Effects of farm Risk of SARA (High = H, low = L) and Cow risk of SARA (H = ≤ 150 DIM, L = > 150 DIM) on long chain milk fatty acids proportions (g/100 g of FA)

						Significance, P -values					
	HH	HL	LH	LL	SE	Farm Risk	Cow risk	Farm Risk*Cow risk	SCC	PAR	SCC*PAR
C18:0	10.1[a]	8.9[b]	9.7[a]	8.2[b]	0.37	N.S	<0.01	N.S	N.S	N.S	N.S
C24:0	0.028[b]	0.031[a]	0.027[b]	0.031[a]	0.002	N.S	0.02	N.S	N.S	N.S	N.S
Total saturated	10.3[a]	9.1[b]	9.9[b]	8.4[c]	0.38	N.S	<0.01	N.S	N.S	N.S	N.S
Cis 11 C18:1	0.44[a]	0.41[ab]	0.41[ab]	0.37[b]	0.02	N.S	0.03	N.S	N.S	N.S	N.S
Cis 11 C20:1	0.022[a]	0.016[b]	0.025[a]	0.015[b]	0.0002	N.S	<0.01	N.S	N.S	0.05	N.S
***Cis* 9 C18:1/*Trans* 11 C18:1**	18.6[a]	18.2[a]	19.5[a]	17.2[b]	0.58	N.S	0.05	N.S	N.S	N.S	N.S
C20:3(n-6)	0.08[b]	0.09[a]	0.07[b]	0.09[a]	0.003	N.S	<0.01	N.S	N.S	N.S	N.S
C20:5(n-3)	0.046[a]	0.045[b]	0.051[a]	0.047[a]	0.004	N.S	<0.01	N.S	<0.01	N.S	N.S
Trans 10, Cis 12 C18:2	0.076[b]	0.087[a]	0.067[c]	0.081[a]	0.003	N.S	<0.01	N.S	N.S	N.S	N.S
Cis 9, Trans 11 C18:2	0.48[b]	0.57[a]	0.53[ab]	0.59[a]	0.06	N.S	0.01	N.S	N.S	0.02	N.S
Cis 9, Cis 12 C18:2	1.88	1.74	1.87	1.64	0.07	N.S	<0.01	N.S	N.S	N.S	N.S

HH: High risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

HL: High risk farm, low risk cows (mid to late-stage animals (> 150 DIM))

LH: Low risk farm, high risk cows (early to mid-lactation stage animals (≤ 150 DIM))

LL: Low risk farm, low risk cows (mid to late lactation stage animals (> 150 DIM))

SCC = Somatic cells counts

PAR = Parity

SE= Standard error

4.5 Discussion

The main objectives of this study were to determine if the concentrations of MAA and the proportions of fatty acids (FAs) in milk can be used as biomarkers to diagnose SARA. This study was conducted as the current technique for this diagnosis, which is based on measuring the rumen pH, is invasive, inaccurate, and, therefore, not commonly used on farms (Li *et al.,* 2012; Mitchell *et al.*, 2016). As a result, we were not able to use the rumen pH to confirm that SARA was present in the cows included in our study. Hence, other measures had to be used to determine which cows were at risk of SARA. As symptoms of SARA include a reduction in the milk fat content, a reduction of *de novo* milk fat synthesis, and an increase in the proportions of long-chain unsaturated FAs in milk (Coppa *et al.,* 2012; Li *et al.,* 2012), we assumed that farms were at Risk of SARA when the milk fat content in the bulk tank was below 4.01 %, the content of *de novo* FAs in this milk was below 0.88 g/100 g of milk fat, and the proportion of polyunsaturated FAs in this milk was higher than 3.58 g/100 g of total FAs. For controls, i.e., farms that were not at risk of SARA, we selected farms in the same geographical regained and with similar management, but with a *de novo* FA content in the bulk tank greater than 1.12 g/100 g of milk.

As cows in early and peak lactation are at a higher risk of SARA compared to cows in mid to late lactation (Enemark, 2008; Li *et al.,* 2013), we selected 7 cows with 150 or fewer days in milk (DIM) on each farm and considered these cows to be at high risk of SARA. We also selected 7 cows with more than 150 DIM on each farm and considered these cows to be a low risk of SARA. Hence, our study included four groups of cows, i.e., High SARA Risk cows on high SARA Risk Farms (HH), low SARA Risk cows on High SARA Risk farms (HL), high SARA Risk cows on low SARA risk farms (LH), and low SARA Risk cows on low SARA Risk farms (LL).

Low dietary content of NDF and a high content of NFC increases the risk of cows experiencing SARA (Abdela, 2016; Villot *et al.,* 2018). In a herd context, these types of rations would often be fed to high-producing cows in earlier stages of lactation, as their requirements of nutrients are high due to the high demand for milk production. On the other hand, cows in later stages or with lower production levels would get rations containing more fiber and less fermentable carbohydrates, as was observed in the current study where lows SAA Risk cows on low SARA Risk farms (group LL) had the highest average dietary NDF content of 39.1 %DM, and the lower average dietary NFC content of 36.9% DM. Despite this, the dietary NDF and NFC content of the other experimental groups also met NRC recommendations (NRC 2001; Krause and Oetzel 2006; Plaizier *et al.,* 2018). Although the chemical compositions of the diets met the nutritional recommendations, this does not infer that SARA did not occur, as factors other than this composition, such as dietary particle size, feeding behavior, sorting, and bunk space affect the SARA risk of cows also (Kleen and Cannizo, 2012; Kitkas *et al.,* 2013; Plaizier *et al.,* 2018). Nevertheless, the differences between the SARA risk groups in our study were lower than those in studies during which SARA was experimentally induced. Hence, these differences may not have been large enough to affect the milk fatty acid profile as much as experimentally grain-induced SARA.

Milk fat depression is a recognized symptom of excessive grain feeding and SARA (Bauman and Grinarii, 2003; Krause and Oetzel, 2006; Plaizier *et al.,* 2018). This depression has been associated with a change in rumen biohydrogenation of dietary FAs, which produces fat-inhibitory FAs (*trans*10 C18:1) that could cause MFD (Bauman and Grinarii, 2003; Prasanth and Ajithkumar, 2016). In our study, the Farm Risk of SARA did not significantly affect the milk fat content, although this content was numerically higher in not-at-risk farms. The lack of a difference in the

milk fat content of high and low SARA Risk farms is likely due to rations of these two groups of farms being more similar between control and SARA cows in experimental trials. Low SARA Risk with over 150 DIM had higher milk fat content compared to earlier lactation (high SARA Risk) cows. Nevertheless, the milk fat content of high SARA Risk cows on high SARA Risk farms does not suggest that substantial milk fat depression occurred (Bauman and Grinarii, 2003; Krause and Oetzel, 2006; Plaizier *et al.*, 2018). Mid and late-lactation cows are less likely to suffer from SARA compared to early and peak lactation dairy cows, as they are commonly fed less grain than early and peak lactation dairy cows (Krause and Oetzel, 2006).

The milk fat content of the HH group did not suggest that milk fat depression occurred (Shingfield and Grinarii, 2007; Enemark, 2008). This is additional evidence that if SARA occurred in cows with high SARA Risk cows with a DIM below 150 days on high-risk farms, it would not be severe. Previous studies have shown that SARA is caused by feeding diets with excessive starch content, insufficient dietary fiber, and coarse/physically effective fiber, and *de novo* milk fat synthesis is reduced (Baumgard *et al.*, 2000; Bauman and Griinari, 2001). It is assumed that this reduction is due to alterations in the biohydrogenation of dietary unsaturated FAs in the rumen that increase the passage of intermediates of this biohydrogenation, such as *trans*10 C18:1 and *trans*10 *cis*12 C18:2 to the mammary gland where they reduce the synthesis of *de novo* short-chain FAs (Bauman and Grinarii, 2003; Bernard *et al.*, 2008; Dewanckele *et al.*, 2020). The farms that were at risk of SARA indeed had higher milk fat proportions of *trans*10 *cis*12 C18:2 compared to not-at-risk farms, but this was not associated with a lower milk fat content, and changes in the proportions of *de novo* milk FAs. The lack of significant differences in the milk FA profile between high and low SARA Risk farms in our trial may be linked to the fact that rations compared on our farms

were not as extreme in terms of starch and fiber content, as previously reported in experimental trials.

In both groups of farms, the milk fat proportion on *trans*10 *cis*12 C:18:2 was higher in low SARA Risk cows than in high SARA Risk cows. Colman *et al.* (2013) observed that an experimental grain-induced SARA increased the proportions of C6:0, C10:0, C12:0, C14:0, *cis*9 C14:1, C16:0 and *cis*9 C16:1 in milk fat, while not affecting this proportion of C8:0 and reducing that of C4:0. Next to increasing the dietary starch content and lowering the dietary fiber content, reducing dietary particle size, and the addition of saturated rumen inert fat affects the FA composition of milk by increasing the proportions of these saturated FAs and reducing the proportions of short and medium-chain FAs in milk fat (Grant *et al.,* 1990; Mosely *et al.,* 2007; Colman *et al.,* 2010). A "naturally-occurring" SARA may not have the same effects on *de novo* milk FAs as experimentally grain-induced and ground forage-induced SARA (Kleen and Cannizzo, 2012; Plaizier *et al.,* 2018). Diagnosing SARA based on the proportions of *de novo* milk FAs is further complicated by dietary factors other than fiber and starch contents that affect these proportions, such as the addition of saturated rumen inert fats to the diet (Mosely *et al.,* 2007; Kargar *et al.,* 2012).

According to Coppa *et al.* (2012), the concentration of milk C4:0 can be affected by the acetyl coenzyme-A carboxylase pathways and the malonyl coenzyme-A carboxylase pathways, with *de novo* synthesis in the mammary gland accounting for half of the total concentration of these FAs. Acetate and β-hydroxybutyrate are the precursors of C4 and are obtained from the fermentation of carbohydrates in dairy cows (Coppa *et al.,* 2012). When a diet containing rapidly degradable carbohydrates is fed to dairy cows, the population of rumen bacteria that act upon such diet increases rapidly, which leads to increased acetate and butyrate production in the rumen, and an

increase in the supply of these precursors for *de novo* milk fat production in the mammary gland. Coppa *et al.* (2012) suggested that this was the mechanism behind the increase in the C4 content of milk fat. Colman *et al.* (2013) showed that experimentally induced SARA by feeding pellets of ground forage without increasing the dietary starch content, reduced the milk fat percentage and proportions of C4:0, C6:0, C8:0, C10:0, and *cis*-9 C14:1 while increasing the proportions of C16:0 and *cis*9 C16:1. The study by Colman et al. (2013), therefore, showed that similar rumen pH depressions can have different effects on the milk fat content and milk FA profile. Enjalbert *et al.* (2008) induced SARA by feeding diets containing up to 34% of wheat to dairy cows and reported that this reduced the milk fat content from 4.41 to 2.24%, reduced milk fat proportions of C6:0, increased these proportions of C10:0, C12:0, C14:1, and C16:1, while not affecting these proportion of C8:0, C14:0, and C16:0. Hence, the studies of Enjalbert *et al.* (2008) and Colman *et al.* (2013) agree that grain-induced SARA increases the milk fat proportions of C8:0, C10:0, C14:1, and C16:1. The effects of increased grain feeding on the proportions of short and medium-chain FAs in milk, therefore, depends on the severity of the rumen pH depression.

In our study, low SARA Risk cows had higher milk fat proportions of *de novo* FAs, except for C8:0 and C10:0 that were not affected. Palmquist *et al.* (1993) reported that the proportion of *de novo* FAs increased until 8 to 10 weeks of lactation. Also, Bilal *et al.* (2014) showed that the milk fat proportion of C6:0 to C14:0 increased during the first 100 days of lactation, whereas these proportions of long-chain saturated, and unsaturated FAs decreased during that period. In part, these effects may be due to a negative energy balance of early lactation cows that causes mobilization of fat from adipose tissues and the resulting incorporation of the mobilized long-chain saturated FAs, such as C16:0 and C18:0, in the milk fat (Palmquist *et al.*, 1993; Stoop *et al.*, 2009). In agreement, the milk fat proportion of C18:0 was higher in high SARA Risk cows

compared to low SARA Risk cows. However, the opposite was observed for cows on low SARA Risk farms. The relatively low *de novo* milk fat synthesis in early lactation may be explained by relatively low dietary NDF content during this stage, and the relatively high inclusion of long-chain FAs from tissue mobilization.

Odd and branch-chain FAs have been proposed as biomarkers for SARA (Vlaeminck *et al.*, 2006; Enjalbert *et al.*, 2008; Fievez *et al.*, 2012). These FAs include *iso* C13:0, *iso* C14:0, C15:0, *iso* C15:0, *anteiso* C15:0, *iso* C16:0, C17:0, *iso* C17:0 and *anteiso* C17:0 (Fievez *et al.*, 2012). These FAs are synthesized by rumen bacteria, but these bacteria differ in the odd and branch chain FAs that they synthesize (Vlaeminck *et al.*, 2006; Fievez *et al.*, 2012). Branched-chain FAs are predominantly synthesized from branched-chain amino acids (Vlaeminck *et al.*, 2006; Fievez *et al.*, 2012). In contrast, propionate is used for the synthesis of C15:0 and C17:0 (Cabrita *et al.*, 2007; Plaizier *et al*., 2018). Both the size of the flow of bacteria out of the stomach and the composition of that flow affect the proportions of the intestines and in the milk fat (Vlaeminck *et al.*, 2006; Fievez *et al.*, 2012). It appears that forage-based diets result in higher proportions of *iso* FAs, and that concentrate-based diets result in higher proportions of *Anteiso* FAs (Vlaeminck *et al.*, 2006; Fievez *et al.*, 2012).

As a higher proportion of grains in diets increases the production of propionate in the rumen (Khafipour *et al.*, 2009a; Pourazad *et al.*, 2016), it may be assumed that increasing the dietary grain content increases the production of C15:0 and C17:0, and the proportions of these FAs in milk fat (Vlaeminck *et al.*, 2006). In agreement, Enjalbert *et al.* (2008) found that a grain-based SARA challenge increased the milk fat proportion of C15. However, the proportion of C17 was not affected by SARA in this study. Also, in agreement, Guo *et al.* (2013) also observed that odd chain FAs had the highest milk fat proportions when the rumen concentration of propionate was

elevated, and the rumen pH was reduced. Colman *et al.* (2013) reported that both grain-induced SARA and a SARA induced by feeding pellets of ground forage increased the milk fat proportion of C15. However, in their study, the milk fat proportion on C17 was only increased by the ground-forage-induced SARA. According to Guo *et al.* (2013), the molar proportions of odd chain FAs increase when the molar proportion of propionate and the concentration of total volatile FAs in the rumen increase, and the rumen pH decreases. In their study, while the effects of the risk of SARA were not significant on C15:0 and C17:0, the effects of stage of lactation were significant for both FAs. An increase in the concentration of C17:0 has been reported as a potential SARA indicator (Fievez *et al.*, 2012). Next to this, C15:0 is assumed to be one of the FAs related to the synthesis of *de novo* odd chain FAs from propionate in the rumen along with C11:0, C13:0, and total odd chain FAs (Guo *et al.*, 2013). The concentration of C15:0 was shown to increase when the dietary inclusion of fine ground wheat increased (Guo *et al.*, 2013). The increase in the total odd chain FAs as found in other studies could not be confirmed, since C11:0 and C13:0 were not analyzed in this study. Fievez *et al.* (2012) reported that while increases in C15 and C17 were associated with SARA, cows were already exhibiting this change in the FA profile in the week before symptoms of rumen acidosis became distinct, suggesting their potential for the early diagnosis of SARA.

In our study, the Farm Risk of SARA did not affect the milk fat proportions of C15:0 and C17:0, but earlier lactation high SARA Risk cows had a higher milk fat proportion of C17:0. This agrees with previous research, as higher rumen propionate concentrations are expected in these cows (Krause and Oetzel, 2006; Plaizier *et al.*, 2018). However, in our study, the opposite was observed for C15:0. Rumen propionate contents could not be determined in our study, but as low SARA Risk later lactation cows received diets with higher NDF and lower NFC contents compared to

earlier lactation high SARA Risk cows, it can be assumed that the production of propionate in the low SARA Risk later lactation cows was lower and that the proportions on C15:0 and C17:0 reacted differently in response to the change in rumen propionate production.

Several studies have reported the effects of excessive grain feeding and grain-induced SARA on the milk fat proportions of branch chain FAs. A reduction in the rumen pH appears to reduce the milk fat proportion of iso FA (Fievez *et al.*, 2012). According to Colman *et al.* (2010), the proportion of anteiso C13 increased after a change from a high grain diet to a low grain diet. The same authors reported that the milk fat proportion of *iso* C15 decreased after feeding dairy cows with concentrates, while that of *anteiso* C15 decreased, and returned to normal after the resumption of feeding lower concentrate diets. In this study, the effects of risk on the percentage abundance of *iso* C15 plus *anteiso* C15 were not significant. It has been reported that the milk fat proportion of *Iso* C14 was reduced when the dietary content of physically effective fiber and rumen pH was reduced (Colman *et al.*, 2013). Hence, a decrease in the milk fat proportion of Iso C14:0 may be a suitable biomarker for SARA (Fievez *et al.*, 2012). In our study, the Farm Risk of SARA did not affect the proportions of branch chain FAs. However, later lactation low SARA Risk cows had higher proportions of total branch chain FAs, *iso* C13, *anteiso* C13, *iso* C14, *anteiso* C15/*iso* C15, and *cis*10 C15:1/*iso* C16 compared to earlier lactation high SARA Risk cows. In contrast, the milk fat proportions of *iso* C17 and *anteiso* C17 were not affected by the Cow Risk of SARA. The lower proportion of *iso* C14 in high SARA Risk cows may be explained by higher grain feeding to these cows, compared to low SARA Risk cows (Krause and Oetzel, 2006; Plaizier *et al.*, 2018). However, the lower milk fat proportions of *Iso* C13 and *cis*10 C15:1/*iso* C16 in the high SARA Risk cows suggest the opposite. Also, in our study, the opposite effects of the stage of lactation and Cow Risk of SARA

on *iso* and *anteiso* branched-chain FAs were not detected. Hence, differences in the proportions of branch chain FAs between low and high SARA Risk cows may not solely be explained by the difference in the prevalence of SARA between these groups of cows.

Guo *et al.* (2013) also reported that the milk fat proportions of monounsaturated FAs- C14:1, C16:1, C17:1, and C20:1 and the Δ9-desaturase index were highest in the milk of dairy cows fed the highest proportion of finely grounded wheat in their diet. The same authors reported that the milk fat proportions of polyunsaturated FAs increased when the dietary inclusion rate of finely grounded wheat increased. One of the FAs reported by these authors was C20:3(*n-6*), which was significantly different between the high SARA Risk cows on high SARA Risk farms and the low SARA Risk cows on low SARA Risk farms in our study. The milk fat proportions of Total saturated FAs and C18:0 were also reported by Guo *et al.* (2013) to be reduced when SARA was induced. Guo *et al.* (2013) ascribed this to modifications in the various steps of biohydrogenation of unsaturated FAs in the rumen. According to Jing *et al.* (2018), monitoring changes in the milk fat proportions of C18:1 *trans*-11, and the C18:1 *trans*-10 and the ratio between *trans*10, C18:1, and *trans*11, C18:1 are useful for the diagnosis of SARA.

In the current study, the milk fat proportion of C18:0 was higher in high SARA Risk cows on high SARA Risk farms than in low SARA Risk cows on low SARA Risk farms. Also, low SARA Risk cows on low SARA Risk farms had the lowest proportion of total saturated FAs. Jing *et al.* (2018) reported that milk fat depression was associated with increases in the milk fat proportions of the total *trans* 18:1 and total CLA, including *cis*9, *trans*11 CLA. In addition, Colman *et al.* (2013) observed that the milk fat proportion of *cis*9 *trans*11 C18:2 decreased when the rumen pH decreased following an increase in the dietary starch content (Colman *et al.*, 2013). In our study, the agreement was that the milk fat proportion of *cis*9, *trans*11 CLA in our study was indeed higher

for low SARA Risk cows on low SARA Risk farms than for high SARA Risk cows on high SARA Risk farms.

*Trans*10, *cis*12 CLA is an inhibitor of the *de novo* synthesis of milk fat and will, therefore, cause milk fat depression (Baumgard *et al.*, 2003; Lanier and Corl, 2015). This FA is a precursor of *trans*12 C18:1 and *trans*10 C18:1. Baumgard *et al.* (2002) described that an increase in *trans*10, *cis*12 C18:2, causes a reduction in the abundance of mammary messenger RNA for genes that control the regulatory points of *de novo* milk fat synthesis, including circulation, desaturation, fat secretion, and intracellular transport of FA, and triglyceride synthesis. However, the change in the proportion of *trans*10, *cis*12 CLA is not always sufficient to explain the MFD experienced in dairy cows (Perfield *et al.*, 2006). Rumenic acid (*cis*9, *trans*11 C18:2) and Vaccenic acid (*trans*11 C18:1) decrease when the grain content of dairy cow diets increases, which, in turn, increases the synthesis of *trans*10, C18:1 and *trans*10 *cis*12 C18:2 (Coppa *et al.*, 2012; Bauman and Grinarii 2003; Dewanckele *et al.*, 2020). Other FAs whose proportions increased during high grain feeding include *cis*10, *trans*12 C18:0 (Sæbø *et al.*, 2005), and *trans*9, *cis*11 C18:2 (Perfield *et al.*, 2007). In our study cows on at-risk farms had a lower milk proportion of *trans*10 *cis*12 C: 18:2, therefore the relationship between NDF and NFC contents of the diets given to the cows could not be established. However, in disagreement with earlier research, the milk fat proportions of *cis*9, *trans*11 C18:2, and *cis*9 C18:1/*trans*11 C18:1 were not affected by the risk level of SARA. The latter suggests that the difference in dietary starch and fiber content between at-risk and non-at-risk farms may not have been large enough to affect the milk proportions of poly-unsaturated FAs. The lack of a risk level of SARA effect on the milk fat proportion of *de novo* FAs suggests the same and shows that increases in the synthesis

of *trans*10 *cis*12 C18:2 are not always accompanied by milk fat depression and a reduction in *de novo* milk fat synthesis.

In our study, later lactation lows SARA Risk cows had higher milk proportions of *trans*10 *cis*12 C18:2, *cis*9 *trans*11 C18:2 in milk fat and C20:3 *(n-6)*, and lower proportions of *cis*9 *cis*12 C18:2 and C:20:5 *(n-3)*, but also a higher milk fat content than earlier lactation high SARA Risk cows. Also, the milk fat proportion of total poly-unsaturated FAs tended to be lower in later lactation lows SARA Risk cows. This confirms that increases in the synthesis of *trans*10 *cis*12 C18:2 are associated with milk fat depression and that that early lactation low SARA Risk cows did not experience changes in the biohydrogenation pathway of dietary FAs in the rumen. This may in part be explained by the absence of severe milk fat depression in any of the experimental groups, including the high SARA Risk cows on high SARA Risk farms. The SCC and parity of cows affected milk fat proportions of *cis*11 C20:1, *cis*9 *cis*12 *cis*15 C18:3, C20:0 *(n-6)*, C20:5 *(n-3)*, and *cis*9 *trans*11 C18:2, showing the complexity and multifactorial aspects of the regulation of milk fat synthesis.

Colman *et al.* (2013) also reported that the milk fat proportion of trans 11 C18:1 was affected by SARA. In our study, this FA was measured combined with *cis*9 C18:1. The milk fat proportion of *Trans*11 C18:1 reduced when lower amounts of physically effective fiber are fed, but not only due to a reduction in the rumen pH (Colman *et al.*, 2013). This suggests that the rumen pH and the milk fat proportion of FA do not have direct and linear relationships (Colman *et al.*, 2013). The authors also reported an inverse relationship between the milk fat proportions of *trans*11 C18:1 and *trans*10, *cis*12 C18:2. In this study, c*is* 9 C18:1 and *Trans* 11 C18:1 were measured together. Hence conclusions on *trans*11 C18:1 as a single compound could not be drawn. Colman *et al.* (2010) also reported that there was a gradual decrease of *cis*11 C18:1 during the first 4 weeks

of lactation. In agreement, our results show that the milk fat proportion of thus FA was higher in High SARA Risk cows than in low SARA Risk cows. Coppa *et al.* (2012) showed that when the dietary forage to grain ratio in dairy cow diets increased, there was an increase in the milk fat proportions of *n-3* FAs and a decrease in those of the saturated FAs, and that this dietary change increased the milk fat proportions of *cis*9, *trans*11 CLA and C18:3(*n-3*) (Coppa *et al.,* 2012). In our study, Farm Risk of SARA did not affect any of these FA, but higher SAA Risk cows tended to have lower milk fat proportions of total saturated and total poly-unsaturated FAs, had higher milk fat proportions of *cis*9, *trans*11 CLA, and *trans* 10 *cis*12 C18:2, but that of C18:3(*n-3*) were not affected.

Cows with a DIM below 150d were considered high SARA Risk cows. This does not imply that they all had SARA, and the random selection of cows from their high SARA Risk group, may have resulted in the selection of cows without SARA. The confirmation of SARA by the measurement of the rumen pH was not possible, as farmers did not agree to it. Hence, the Cow Risk of SARA criteria was used. The challenge with this is that the stage of lactation affects the milk FAs profile through factors other than SARA. Palmquist *et al.* (1993) reported that the proportion of short chain *de novo* FAs increase until 8 to 10 weeks of lactation. In part, this may be due to the negative energy balance of early lactation cows that cause mobilization of fat from adipose tissues, and the resulting incorporation of these mobilized long-chain FAs, such as C16:0 and C18:0 in the milk fat (Palmquist *et al.,* 1993; Stoop *et al.,* 2009). However, the inhibition of *de novo* synthesis of short-chain FAs that increases with increased chain length may also play a role (Palmquist *et al.,* 1993).

Experimentally induced SARA by high grain feeding has been associated with an acute phase response, including a rise in the MAA content (Enemark 2008; Zebeli and Metzler-Zebeli, 2010;

Plaizier *et al.*, 2018). In our study, MAA concentrations were not affected by the Farm Risk level of SARA and Stage of Lactation. This suggests that these factors did not affect the concentration of acute-phase proteins in the blood and that cows on high-risk farms and cows in early lactation were not subject to a diet-related acute phase response. In agreement, Humer *et al.* (2018) reported that the concentration of MAA was not affected by an intermittent SARA challenge. In our study, the concentration of MAA was not affected by Farm Risk of SARA and Cow Risk of SARA. This suggests that the high Farm Risk of SARA and a high Cow Risk of SARA were not associated with the acute phase response. As no cases of SARA were severe, such an acute phase response was not expected. Despite cows with an SCC greater than 200,000ml/L being excluded from the study, MAA was positively correlated with MAA. A large increase in the MAA concentration during mastitis was reported in several studies (Gerardi *et al.*, 2009; Kalmus *et al.*, 2013). Our study shows that subclinical increases in SCC are also associated with increases in the concentrations of MAA, which can increase the understanding of the impact of subclinical mastitis. As the concentration of MAA is a direct indication of inflammation in the mammary gland, the analysis of this acute-phase protein can contribute to the diagnosis of subclinical mastitis. This diagnosis, based on MAA, can, however, not stand on its own, as other inflammations can affect the concentrations of acute-phase proteins in blood and milk also (Petersen *et al.*, 2004; Nazifi *et al.*, 2008).

On commercial dairy farms, SARA is naturally occurring, unlike the experimentally induced SARA (Kleen and Cannizzo, 2012; Plaizier *et al.*, 2018). Kleen and Cannizzo (2012) concluded that many of the symptoms of experimentally induced SARA, including laminitis, milk fat depression, and poor body condition were not present in cows on commercial dairy farms that were diagnosed with a depressed rumen pH using rumenocentesis. As a result, these authors

suggested that experimental grain-induced SARA may not be representative of "naturally-occurring" SARA. The experimental grain-induced SARA involves increasing the dietary starch content to 30% DM or more (Dohme *et al.,* 2008; Khafipour *et al.,* 2009a; Pourazad *et al.,* 2016; Plaizier *et al.,* 2018). It may be unlikely that starch levels above 30% DM commonly occur on dairy farms. However, other risk factors for SARA, such as improper dietary particle size, feed sorting, and insufficient bunk space, are present on farms and may contribute to SARA (Coppa *et al.,* 2012; Jing *et al.,* 2018; Plaizier *et al.,* 2018). Hence, "naturally-occurring" SARA may not be solely due to excessive grain feeding but also to undesirable feeding behavior. Khafipour *et al.* (2009b) showed that rumen pH depression achieved by feeding pellets of ground forage did not result in the acute phase protein response, whereas this response did occur in cows with a similar rumen pH depression resulting from high grain feeding. Hence, a rumen pH depression during "naturally-occurring" SARA may not cause an acute phase response and an increase in MAA of the same magnitude as experimentally grain-induced SARA.

Our results show that the milk FA profile of dairy cows is affected by many animal and farm factors, including SARA and stage of lactation effects related to and not related to SARA and grain feeding. Abnormal milk FA profiles are undesirable as they indicate health problems in cows, the healthiness of milk, and the quality of dairy products. Hence, monitoring the milk FA profile of cows is a good management tool, if it can be incorporated in routine herd management. Currently, milk analysis is gaining a lot of attention as a tool for the diagnosis of infectious and metabolic diseases (Oetzel, 2007).

However, as these diagnoses need to be performed for individual cows, milk samples from individual cows need to be taken (Oetzel, 2007). The current practice involves testing milk fat in the milk tank, which makes it difficult to spot outliers. Also, if lactating cows have non-

synchronized MFD, milk fat testing may not be efficient unless done daily (Oetzel, 2007). The use of milk biomarkers for diagnostic purposes is, therefore, not without challenges. If these challenges can be mitigated, then milk testing may become comprehensive enough to develop a trend that can assist the early diagnosis of SARA.

In summary, several and confounding factors affect the FA profile and MAA content of milk. Hence the consideration and integration of these factors when diagnosing SARA is important. As discussed previously, these factors include the repetition and severity of SARA, feed sorting, feed delivery, presence of other metabolic diseases that alter milk FA profile (mastitis), animal variability, breed and genetics, parity, feeding patterns, and quite importantly, the history of the animal. In this study, several of these factors were considered, including parity and SCC. Incomplete cow history on feeding, rumen conditions, and disease occurrences. The limited inadequate history available to us, therefore, complicated the diagnosis of SARA. Despite that, it was distinctly noted from this study that we can diagnose SARA on farms irrespective of the farm risk level of SARA. This was because we identified that the stage of lactation, which is a major cow risk factor for SARA, affected the milk FA profile greatly. As a result, we can focus the diagnosis of SARA on high-risk early and mid-lactation cows and monitor them throughout their transition and peak yield periods. This will enable farmers to adjust feed patterns and aid in the process of preventing and reversing gut health disorders, such as SARA, and enhancing the quality of the milk.

4.6 Conclusion

Our results show that there were considerable variations in milk fat content, milk FA profile, and MAA among experimental groups and cows within these groups. As the study consisted of a survey, several and confounding factors, including non-dietary factors, may have been responsible

for the differences between high SARA Risk farms and low SARA Risk farms and between low SARA Risk cows and high SARA risk cows. Results obtained in our study are representative of on-farm conditions, whereas studies during which SARA was induced may not be representative of these conditions. Dietary NDF and NFC contents also differed among groups, with diets for low SARA Risk cows on low SARA Risk farms having the highest dietary NDF content and the lowest dietary NFC content. However, these contents for the other experimental groups did not suggest that, due to these contents, cows were at risk of a severe SARA. This does not preclude cows from undergoing SARA, as many other dietary, animal, and management factors put cows at risk of this disorder.

The MAA concentrations showed that cows did not suffer from an acute phase response. As cows with SCC content above 200,000 were excluded from the trial, high MAA values due to mastitis were also not expected. As the dietary NDF and NFC contents were not excessively low, respectively high, an acute phase response due to severe SARA could also not be expected. Hence, our study was not able to assess if a severe SARA occurring on-farm can be diagnosed using the milk MAA content. The correlation between SCC and MAA that was observed in our study does, however, suggest that MAA measurement may be a useful tool to detect subclinical mastitis. Although the milk fat content varied among experimental groups, and the high SARA Risk cows had the lowest milk fat content of 3.94%, cows in this group did not experience milk fat depression. This confirms that cows in the group did not experience severe SARA. Studies that induced SARA experimentally mainly fed diets with excessively high starch contents to reduce the rumen pH to a level that was considered to be representative of SARA. It is questionable if these models are reprehensive for “naturally occurring” SARA on fairy farms, like many other dietary, animal, and management factors other than the NDF and NFC content of the diet may cause “naturally

occurring" SARA. It is, therefore, not unexpected that the effects and symptoms of "naturally occurring" SARA and experimentally induced SARA differ. Hence, diagnosing "naturally occurring" solely based on the fat content and FA profile of milk of individual cows may not be sufficiently accurate.

Our research was able to confirm that the milk FA profile is a useful tool in the diagnosis of abnormal proportions of milk FAs and the underlying causes of these abnormal proportions. As normal milk fat proportions vary among cows, the baseline milk FA profile of individual animals must be known. So far, the milk FA profile is being explored as it is related to dietary composition and stage of lactation. In the future, the milk FA profile can be further explored by exploring the effects of diet, lactation stage, breed, parity, feed delivery (frequency and TMR vs. component feeding) on this profile, and integration of these factors to define thresholds that define abnormal milk FA profiles. Challenges to this include variability in the normal FA profile and sensitivities to these factors among cows. This can be addressed by considering individual animal variation, the use of ranges, and the correction of these ranges based on regions and management factors. Until now, SARA has been considered a herd-based disorder. However, to allow treatment of individual cows, diagnosis of individual cows, such as by assessing their current and past milk FA profile history, is needed.

5.0 GENERAL DISCUSSIONS, CONCLUSIONS, AND FUTURE DIRECTIONS

5.1 General Discussion

This study sought to determine if MAA and the FA profile in milk are accurate tools for the diagnosis of SARA in individual dairy cows. This disorder is currently diagnosed by the evaluation of the pH of rumen fluid. The interest in biomarkers for the diagnosis of SARA in dairy cows that are not based on rumen pH measurements has arisen, due to the variation in the sensitivity of individual cows to rumen pH depression, poor representation of collected rumen fluid, and inaccuracies of the measurement of the pH of rumen fluid (Li *et al.*, 2012; Villot *et al.*, 2018). SARA is common in intensive production systems because in these systems high-producing dairy cows are fed large quantities of starch and low quantities of fiber to meet their high energy requirements for milk production (Villot *et al.*, 2018). In Canada, this intensive system of management is the most common. Hence, cows on most Canadian dairy farms are at risk of SARA. Normally, ruminal fermentation in dairy cows is characterized by stability in fermentation pattern and ruminal pH ranges between 5.6 and 6.5 with an occasional pH reduction (<5.6) for a short time, usually after feeding (Plaizier *et al.*, 2018). The diurnal rumen pH variation is related to the accumulation of VFAs (Kooman *et al.*, 2018, Plaizier *et al.*, 2018). If SARA is mild, there will hardly be any impact of VFAs on the barrier the rumen epithelium provides, especially when it is for a short period of time (Kooman *et al.*, 2018).

The fat content, fatty acid profile, and the concentration of MAA in milk are affected by experimentally induced SARA (Prasanth and Ajithkumar, 2016; Plaizier *et al.*, 2018). In experiments in which SARA was induced, the milk protein percentage increased while the milk fat percentage decreased (Prasanth and Ajithkumar, 2016; Plaizier *et al.*, 2018). Some authors have found a relationship between milk fat content and SARA, but in some other studies no significant

relationship was found (Kooman *et al.*, 2018; Plaizier *et al.*, 2018). In our study, however, SARA was not induced, as diagnosing SARA in a commercial farm using the milk FA and MAA varies from was expected to differ from that in a setting in which SARA was induced first. The change in milk fatty acid profile is assumed to be brought about by the change in the pattern of rumen biohydrogenation of fatty acids influenced by a SARA inducing diet, which results in the production of *de novo* fat synthesis-inhibitory fatty acids (*trans*10 C18:1) that cause MFD in cattle (Bauman and Grinarii, 2003; Prasanth and Ajithkumar, 2016). The change in MAA is expected to be due to the acute phase response triggered by SARA (Plaizier *et al.*, 2012). Cows naturally reduced their feed intake temporarily to restore homeostasis (for lack of a better word for balance) in the rumen (Pourazad *et al.*, 2016). This might result in SARA lasting only for a short period (Pourazad *et al.*, 2016). However, upon resumption of the feed intake, SARA can occur again, possibly with greater severity (Pourazad *et al.*, 2016).

Coppa *et al.* (2012) reported that feeding high grain and high-fat diets increase the polyunsaturated fat content in the milk of dairy cows. This supports the assumption that the bulk tank polyunsaturated fatty acids might be useful in identifying the farm risk of SARA. Secondly, it emphasizes the significance of dietary factors in determining the farm risk of SARA. However, Finally, non-dietary cow and farm characteristics, including feeding behavior, lactation stage, and parity, also have a major impact on the milk fatty acid profile (Coppa *et al.*, 2012). The risk of SARA is especially high in early lactation cows, due to their limited feed intake, they need to receive high grain diets (Coon *et al.*, 2019).

The risk and severity of SARA are affected by several farm and cow factors. Firstly, the regulation of the pH of the rumen as well as the severity of SARA by several factors including time of exposure to SARA, substrates supplied for microbes in the rumen, the absorption and passage of

VFAs, and buffering by saliva (dependent on feed intake level, animal activity and supply of dietary fiber), feedstuffs and rumen epithelium (Pourazad *et al.*, 2016). Also, the severity of SARA increases with repeated bouts of SARA (Dohme *et al.*, 2008). Secondly, some animals have a higher risk of SARA, because they differ in their sensitivity to errors in feed delivery, feedstuff quality, and processing, abrupt feed change associated with the transition from dry to lactating cows, feed intake, and feed formulation (Kooman *et al.*, 2018). Thirdly, the effects of feed sorting and differences in that sorting among cows affect the risk of SARA (Coon *et al.*, 2019).

Pourazad *et al.* (2016) recommended that more studies on prolonged bouts of SARA and recovery from SARA need to be carried out. Pourazad *et al.* (2016) described the short-term occurrence of SARA as a transient, and the long-lasting SARA as persistent, with the former triggering more severe conditions when it reoccurs. When the nutrients supplied to cows are inconsistent, ruminal microbes may be impacted negatively by a bout of SARA, the establishment or re-establishment of gut microbiota may take time (Pourazad *et al.*, 2016). This might also help explain why primiparous cows are more prone to SARA than multiparous cows (Enemark, 2008). In our study, we included both primiparous and multiparous cows. The study carried out by Dohme *et al.* (2008) discovered that when early lactation cows were fed a high-concentrate diet, they had lower pH profiles than mid-lactation cows that were fed a high forage diet, although both groups of cows experienced SARA (Dohme *et al.*, 2008). The study by Pourazad *et al.* (2018) may explain why repeated SARA bouts in the study from Dohme *et al.* (2008), increased the severity of ruminal acidosis. It was concluded by Dohme *et al.* (2008) that when risk factors of acidosis are reduced, this may give some protection to cows against SARA. However, Dohme *et al.* (2008) postulated that there could be a recovery time for about of SARA which might reduce the severity of subsequent bouts.

Kooman *et al.,* 2018 reported that the adaptation of the rumen wall to high grain diets requires several weeks. This adaptation involves increasing the length and density of the rumen papillae to increase the absorption of VFA (Kooman *et al.,* 2018). Hence, increases in the dietary grain content need to be made gradually. Ruminal microbiota also requires ample time to adapt to the increases in the grain content of the post-partum diet (Kooman *et al.,* 2018). Primiparous cows have a higher risk of SARA than older cows, and this can be attributed to their first-time exposure to high grain diets (Kooman *et al.,* 2018). The consumption of the diet, rather than the composition of the diet provided to the dairy cows, and, therefore, sorting behavior can lead to SARA (Coon *et al.,* 2019). Research has shown that animals tend to change their sorting behavior when lactating. Cows tend to sort their feed, selecting in favor of small feed particles, thereby increasing the risk of and the consumption of an unbalanced diet (Coon *et al.,* 2019).

In our study, the severity of SARA is relevant because the animals on at-risk farms were expected to experience SARA. Confirming this proved to be difficult, as the main qualifying factor for SARA, i.e., the rumen pH, was not available. Upon comparison of the bulk tank parameters, we differentiated between high and low risk of SARA farms. Our findings, however, showed that the farm risk level of SARA did affect the fatty acid profile and MAA concentration of milk of individual cows. FA. This might be due to, in part, the further selection process that caused animals with high SCC to be excluded from our study-seeing how MAA and SCC are strongly correlated, and the random selection of cows to be included on each farm. Individual variation among cows can in their susceptibility to SARA is affected by variation in microbial populations in the rumen, age, genetics, and previous bouts of acidosis (Dohme *et al.,* 2008). Hence, the rumen conditions of cows fed the same diet may react differently to increased grain feeding. This may also explain

why some cows experience SARA, despite dietary adaptation efforts during the transition from the dry to the lactation stage (Dohme *et al.*, 2008).

In diagnosing animals with SARA on commercial farms, knowing that the sensitivity to SARA will help producers monitor their animals for SARA more accurately, and may also encourage a shift in the outlook of SARA from being a herd problem to being a cow problem (Shabani and Ceroni, 2013). Kooman *et al.* (2018) concluded that to have a diagnostic tool that is next to the rumen pH, measures like feed intake, strength and number of rumen contractions, rumination, fecal pH and composition, and rumen fill need to be evaluated. This agrees with our findings that several factors need to be considered when diagnosed with SARA based on the fatty acid profile and MAA in milk. The concept of individual animal variation in the susceptibility to SARA and the effects of rumen depression, as described by Dohme *et al.* (2008) and Villot *et al.* (2018), also confirms why the integration of factors into the diagnosis of SARA is important.

Our results showed that the farm risk level of SARA was not as strong as the factor affecting the milk fatty acid profile and MAA as the stage of lactation. This stage of lactation effects can be both herd and animal specific. Some farms in our study had the same diets for all animals, irrespective of lactation stage, whereas other farms had diets that differed among states of lactation. All diets in our survey, however, met the NRC recommendations (NRC, 2001). While we are trying to move from a herd-based SARA diagnosis, Kooman *et al.* (2018) suggested that this diagnosis should remain herd-based, because the stage of lactation was a major factor affecting the risk for SARA, due to stage differences in feed intake and diet composition (Kooman *et al.*, 2018). However, the inability to quantify SARA in farms based on the fat content and fatty acid profile of bulk tank milk makes the monitoring of individual cows necessary. However, to confirm that changes in these measures are caused by SARA, other measurements are needed, including MUN

(Gao and Oba, 2014) and milk lactose (Stefańska *et al.*, 2020). Hence, conducting these measurements on the milk of the cows included in our study to confirm or exclude the cows that were experiencing SARA would have been useful.

5.2 General conclusion

Since the diagnosis of SARA based on the measurement of the rumen pH has several issues with accuracy and cow health, there is an increased interest in alternative methods for this diagnosis, particularly a method that can be adopted in commercial dairy farms. SARA affects rumen health, but also the FA profile in milk. SARA may also increase MAA concentrations because it has been shown to trigger the immune response in cows when SARA is experimentally induced. Relationships exist between parity, SCC, MAA, and inflammations including mastitis. Hence, the accuracy of the diagnosis of SARA by MAA analysis is dependent on additional confirmatory tests, particularly when the SARA is mild. There is a potential to diagnose SARA by evaluating the milk FA profile, especially by considering the relationship between the milk FA profile and the stage of lactation of cows. However, as the milk FA profile of cows that are not experiencing SARA varies among these cows, analyzing milk samples from a single test day might not suffice for this diagnosis. A more comprehensive approach based on the history and baseline milk fat and milk FA profile values may be needed to accurately diagnose SARA.

In our study, the Farm Risk of SARA did not affect the milk FA profile. This may be due to the absence of an effect of this risk factor on rumen fermentation, as previous reports have shown that changes in ruminal fermentation cause changes in the milk FA profile, but that these changes differ among cows (Enemark, 2008; Colman *et al.* (2010 and 2013); Coppa *et al.* (2012). Hence, the diagnosis of SARA will require regular testing to assess if changes from the base levels of individual cows occur. The use of a quick laboratory procedure (FAME) is useful in the diagnosis

of SARA through observation of the milk FA profile of milk in dairy cows. Early diagnosis of SARA will reduce losses that could occur due to this disease. The random selection of cows without the certainty that SARA is present in these cows, as done in our study, may have affected the outcome of our research. Future research will confirm if it would have been better if animals that experienced SARA as suggested by milk fat depression had been chosen. Previous authors have also used more than one test to diagnose SARA. For example, Gao and Oba (2014) reported that the MUN may provide information on the prevalence of SARA.

The accuracy of milk fat analysis for the diagnosis of SARA may be affected by a multitude of factors that affect the milk fat content and its composition. This was also suggested by Stefańska et al. (2020) who suggested that because MFD is not only due to increased ruminal acidity, for milk FA and MAA to be an accurate biomarker for diagnosing SARA, these measurements need to be accompanied by an understanding of factors that put animals at risk of SARA. Besides knowing the suitability of diagnostic tools for SARA on a herd basis, it is important to investigate further to find biomarkers that will be suitable for diagnosing SARA in individual cows.

5.3 Future Directions

There is still limited research about the prevalence and impact of mild SARA on commercial dairy farms in Canada. As discussed extensively in this paper, SARA may not be diagnosed due to several factors, including but not limited to, housing style, lack of distinct symptoms, and individual cow variability. While this study revealed that milk FA profile may contribute to the diagnosis of SARA, follow-up research may include:

1. The investigation of SARA by adding more biomarkers as variables in the model (breed, covariates in the current model (SCC, parity), and other biomarkers (MUN, BHB)). There might also be a need to find these relationships both in animals with experimentally induced SARA and

mild SARA as found in commercial dairy farms with adequate feeding regimes that minimize the susceptibility of cows to SARA.

2. Observation of changes in the FA profile and MAA concentrations over time (cow history) and how it might be useful for naturally occurring SARA. Fatty acids observed using samples from a single test day might not suffice for diagnostic purposes. Farm history before the test day will aid in obtaining reference points that will accurately define SARA in animals.

3. Evaluation of the recovery length of SARA based on its severity and its impact on the milk FA profile. The severity of SARA as discussed in 5.1 has been shown to increase with repeated bouts of SARA.

4. Validation of herd-based and cow-based criteria for selection of herds and animals with SARA, respectively.

5. Validation of milk FA profile thresholds that define SARA for easy application to farms.

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