UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

INSTITUTO SUPERIOR DE AGRONOMIA

CONSEQUENCES OF VARYING DIETARY STARCH CONTENT ON METABOLIC STATUS

AND PRODUCTION LEVELS IN DAIRY COWS

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DISSERTAÇÃO DE MESTRADO EM ENGENHARIA ZOOTECNICA – PRODUÇÃO ANIMAL

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ABSTRACT

Subacute ruminal acidosis (SARA) is one of the most common metabolic diseases in dairy cows. The main cause for SARA is an excessive feeding of fermentable carbohydrates, mainly starch. To test the effect of varying starch levels in the diets on metabolic status and productive performance, six lactating Danish Holstein cows with ruminal cannulas and permanent intercostal artery catheters were used in a replicated 3 x 3 Latin square design. Animals were fed *ad libitum* with a control diet containing 20% starch (DM basis) during the adaptation period (6 days). At day 7 (D1), 9, 11 and 13 (D4) cows received one of the experimental diets containing either 28, 35, 42% starch (DM basis) corresponding to low, medium and high treatments. At day 8, 10, 12 and 14 cows were fed with the control diet. Rumen fluid (ventral and medial), blood, urine and milk samples were collected at day 6, 7 and 13, at -0.5, 1, 2.5, 4, 5.5 and 7 h relative to feeding. Additionally, milk yield, water intake and dry matter intake were recorded. In all samples, pH was measured. In blood samples, partial pressure of O_2 and CO_2 were measured. In milk samples, glucose, glucose-6-phosphate, β-hydroxybutyrate, lactate dehydrogenase, creatinine, malate, isocitrate and urate concentrations were measured using an enzymatic-fluorometric method. Daily milk samples were also measured for fat, protein, somatic cell count (SCC) and lactose using infrared spectrometry. The MIXED procedure of SAS was used with a model including starch level, sampling time and interaction between both as independent variables, and cow as a repeated effect. Production performance parameters (milk yield, fat, protein, SCC and lactose) were not affected by treatment nor were dry matter and water intake ($P>0.05$). Parameters affected by time were due to normal metabolic alterations during rumination. Ventral fluid pH was affected by treatment on D1, increasing from the medium treatment to the high treatment (6.19 ± 0.08 and 6.45 ± 0.08, respectively; $P=0.01$). Blood pH decreased in the medium and high treatments compared to the low treatment (7.50 ± 0.01 and 7.52 ± 0.01, respectively; $P=0.02$) on D4. Milk urate concentration increased in the medium treatment compared to the low treatment on D1 (129.6 ± 3.79 and 155.5 ± 8.48, respectively; $P=0.01$) and D4 (110.9 ± 3.79 and 105.7 ± 8.48, respectively; $P=0.04$). Milk pH decreased in the high treatment compared to the low treatment (6.75 ± 0.01 and 6.80 ± 0.01, respectively; $P=0.02$) on D1. Milk malate and creatinine were affected by the interaction on D4 ($P<0.05$) while isocitrate was affected on D1 ($P=0.03$). Our results demonstrate that all groups had some level of SARA. In general, cows had more metabolic imbalances on D1 than on D4 leading to the conclusion that the rumen microbiome adapted to the high starch content and cows also able to adapt the different metabolic pathways related to excretion of the acid, allowing cows to cope with high levels of dietary starch.

Key-words: acidosis, dairy cow, rumen disorders, metabolism

RESUMO

A acidose ruminal subaguda (ARS) é uma doença metabólica comum em vacas leiteiras. A principal causa é a alimentação com excesso de hidratos de carbono fermentescíveis, nomeadamente o amido. Para estudar o efeito da variação do conteúdo de amido nas dietas no estado metabólico e desempenho produtivo, foi realizado um ensaio com seis vacas *Danish Holstein* lactantes com cânulas ruminais e cateteres na artéria intercostal, num design quadrado latino replicado 3 x 3. A alimentação foi *ad libitum* com dieta controlo com 20% de amido (MS) durante o período de adaptação (6 dias). No dia 7 (D1), 9, 11 e 13 (D4), foi fornecida uma dieta experimental com 28, 35 ou 42% de amido (MS) correspondendo ao tratamento baixo, médio e alto. No dia 8, 10, 12 e 14 foi fornecida dieta controlo. Foram recolhidas amostras de fluído ruminal (ventral e medial), sangue, urina e leite nos dias 6, 7 e 13, às -0.5, 1, 2.5, 4, 5.5 e 7 h relativamente à hora de alimentação. Foram registados nestes dias o leite produzido, consumo de água e ingestão de matéria seca. Foi medido o pH de todas as amostras. A pressão parcial de O_2 e CO_2 nas amostras de sangue foi determinada. Foi medida a concentração de glicose, glicose-6-fosfato, β-hidroxibutirato, lactato desidrogenase, creatinina, malato, isocitrato e urato nas amostras de leite utilizando um método enzimático fluorométrico. Foi também determinada a gordura, proteína, contagem de células somáticas (CCS) e lactose nas amostras de leite utilizando espetrofotometria infravermelha. Foi utilizado o procedimento MIXED do SAS com um modelo que incluía nível de amido, hora de amostragem e interação entre os dois como variáveis independentes e a vaca como efeito repetido. O desempenho produtivo (quantidade, gordura, proteína, CCS e lactose), a ingestão de matéria seca e o consumo de água não foram afetados pelo tratamento (P>0.05). Os parâmetros afetados pelo tempo são resultado das alterações metabólicas durante a ruminação. O tratamento afetou o pH do fluído ventral em D1, aumentando do tratamento médio para o alto (6.19 ± 0.08 e 6.45 ± 0.08, resp.; P=0.01). O pH do sangue diminuiu nos tratamentos médio e alto em comparação com o baixo (7.50 ± 0.01 e 7.52 ± 0.01, resp.; P=0.02) em D4. A concentração de urato aumentou no tratamento médio em comparação com o baixo em D1 (129.6 ± 3.79 e 155.5 ± 8.48, resp.; P=0.01) e D4 (110.9 ± 3.79 e 105.7 ± 8.48, resp.; P=0.04). O pH do leite diminuiu no tratamento alto em comparação com o baixo (6.75 ± 0.01 e 6.80 ± 0.01, resp.; P=0.02) em D1. A concentração de malato e creatinina no leite foram afetados pela interação em D4 (P<0.05) e o isocitrato em D1 (P=0.03). Todos os grupos sofreram ARS. Em geral, as vacas demonstraram mais desequilíbrios metabólicos em D1 do que em D4, concluindo que o microbioma ruminal adapta-se ao tratamento e que os animais ajustam as vias metabólicas de excreção do ácido, desvalorizando o elevado conteúdo de amido na dieta.

Palavras-chave: acidose, vaca leiteira, doenças ruminais, metabolismo

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and the corresponding abstract

The effect of a sudden dietary starch inclusion increase on metabolic status and milk production in dairy cows

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ABREVIATIONS

AHTC – haematocrit

AMS – automatic milking systems

ARS – acidose ruminal subaguda

ATP – adenosine triphosphate

AU – Aarhus university

BHB – B-hydroxybutyrate

BW – body weight

CCS – contagem de células somáticas

CTRL – control

CP – crude protein

DIM – days in milk

DIP – days in period

DKK – Danish krone

DMI – dry matter intake

EU – European Union

G6P – glucose-6-phosphate

LDH – lactate dehydrogenase

LW – live weight

NADPH – reduced nicotinamide adenine dinucleotide phosphate

NADP+ – nicotinamide adenine dinucleotide phosphate

NDF – neutral detergent fiber

NEB – negative energy balance

NUTS – *nomenclature des unités territoriales statistiques* (nomenclature of territorial units for statistics)

OM – original matter

pCO_2 – partial pressure of carbon dioxide

pO_2 – partial pressure of oxygen

SARA – subacute ruminal acidosis

SCC – somatic cell count

SCFA – short chain fatty acids

SFU – Scandinavian feed unit

VFA – volatile fatty acids

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1. INTRODUCTION

Controlling rumen disorders in dairy cows is critical to ensure the successful herd management, health and productivity. Dairy cows have high nutritional demands during lactation (Humer et al. 2018) and are prone to different metabolic diseases such as ketosis, retained placenta, milk fever, displaced abomasum and particularly ruminal acidosis (Radostits et al. 2007; Liang et al. 2017). Subacute ruminal acidosis (SARA) is a prevalent metabolic disorder in high-producing dairy herds (Gao and Oba 2014). This disorder is associated with feeding diets with high-energy contents, particularly starch. Indeed, increased starch content on lactation diets can modify rumen bacteria populations. The term SARA is often used as a synonym for poor rumen health. Being subclinical, SARA lacks clear symptoms and is therefore difficult to diagnose and to control on farm (Humer et al. 2018).

Cows in physiological imbalance are defined as cows with altered function of the digestive tract, metabolic state and immune state. Consequently, these animals have increased risk of developing production diseases (clinical or subclinical) and reduced production and/or reproduction performance (Ingvartsen 2006).Biomarkers in milk may be used to reflect the physiological status of the animal (Larsen and Moyes 2014). Biomarkers in blood reflect the rate and extent of tissue mobilization and they have been previously used to predict the energy status of the animal (Bjerre-Harpøth et al. 2012).

Based on these facts, this thesis aims to evaluate the effect of the sudden inclusion of different starch levels in the diet on milk composition and milk production performance. The experimental trial and laboratory analysis of this study where held in AU Foulum, Department of Animal Science, Denmark. AU Foulum is a part of Aarhus University were research in food and agriculture is conducted.

1.1. Denmark – an overview on agricultural status

Denmark is the southernmost and the smallest of the European Nordic or Scandinavian countries (Danish Agriculture & Food Council 2016) with an estimated area of 4.3 million hectares (Eurostat 2016). Statistics Denmark sum more than 5.8 million people residing in the country in the first quarter of 2019.

Denmark is part of the Kingdom of Denmark, which also includes Greenland and the Faroe Islands (Statistics Denmark 2017). This accounts to a total of 222 million hectares. The Constitution (Parliment 1953) that applies to the kingdom states that the government form is of a parliamentary constitutional monarchy. Denmark is divided in five regions (EU standard NUTS 2) and eleven provinces (EU standard NUTS 3). The five regions which are Capital, Zealand, Southern Denmark, Central Jutland and Northern Jutland (Danish Regions 2012), are mapped in Figure 1.

Figure 1 – Map of the Danish regions and main cities

(Adapted from: Danish Regions, 2012)

The continental part of Denmark, known as the Jutland peninsula (Danish Agriculture & Food Council 2016), shares its southern border with Germany and accounts for 69 % of Denmark's total area (Statistics Denmark 2017). The capital region, although the smallest with 2.541 km^2, has more than 30 % of Denmark´s inhabitants (Danish Regions 2012). Copenhagen is the capital. Zealand and Funen (part of Southern Denmark) are the largest islands, but the Danish archipelago is formed by more than 400 islands (Central Intelligence Agency 2019). Denmark has been part of the European Union since 1973, or European Economic Union at the time, but not the Eurozone (Central Intelligence Agency 2019) and uses the Danish krone (DKK) as currency.

Apart from sharing its southern border with Germany, Denmark is surrounded by the Baltic Sea and the North Sea (Central Intelligence Agency 2019). Being the seventh smallest country of the European Union, Denmark has an extraordinarily long coastline that stretches for more than 7,300 km (Statistics Denmark 2017).

The mean elevation is 34 m, being *Lammefjord* the lowest point with - 7 m and the highest *Mollehoj/Ejer Bavnehoj* peaking at 171 m (Central Intelligence Agency 2019). Between 2006 and 2015, The Danish Meteorological Institute reported an average temperature of 8.9ºC, with January being the coldest month (average 1.4ºC) and July the

hottest (average 17.4ºC) (Cappelen 2019), but Danish weather is known for being very variable (Statistics Denmark 2017). Thermometers have reached -31ºC, on January 1982, and 36ºC in July 1975. Within the same time frame mentioned above, the average precipitation a year was 791.5 mm (Cappelen 2019). Denmark's Statistical Yearbook stated that it rains or snows every other day (171 days of precipitation in a year) and in 2017 there was a total of 37.8 frost days for the country as a whole. Due to its latitude, Denmark has a considerate variation of sunshine hours during a year. In July, there has been in average more than 8 hours of sunshine a day, that eventually turn out to be less than 2 hours a day in December (Cappelen 2019). Figure 2 summarizes all this information with county-wise temperature, precipitation and sunshine average from 2006 to 2017 graphic.
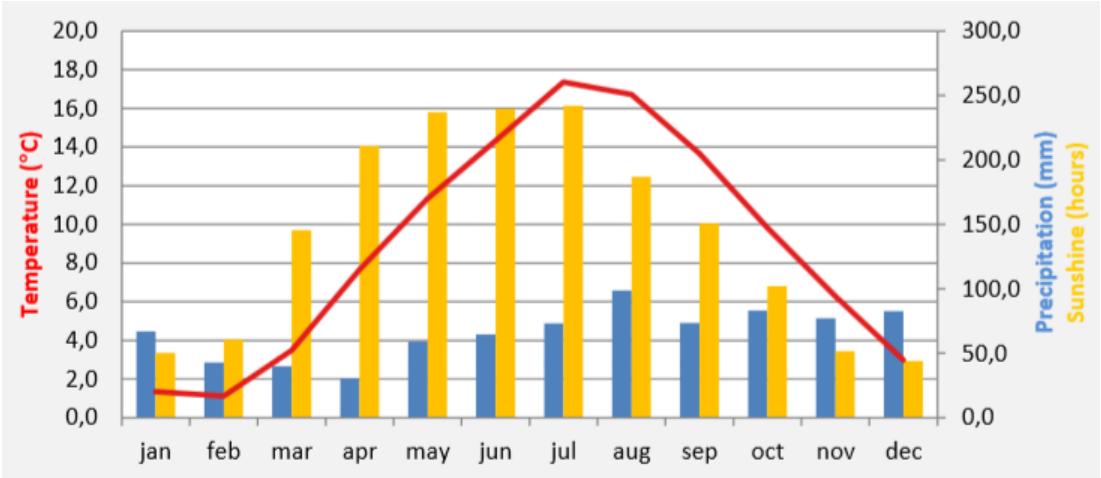
Figure 2 – Denmark country-wide thermopluviometric graphic
(source: Danish Meteorological Institute, 2019)

1.1.1. Agriculture in Denmark

Because of the above-mentioned climate characteristics, Denmark has the perfect conditions for agriculture. In 2016, agricultural crops occupied more than 60 % of all Danish land (Statistics Denmark 2018). Cereals are the dominating type of crop and 75 % is used in animal feeding (Danish Agriculture & Food Council 2016). Danish agriculture is amongst the most efficient in the World. The Danish Agriculture and Food Council (2016), attributes Denmark's high productivity to well-educated farmers, both public and private intensive research and innovation, well-organized farmer owned cooperatives, efficient knowledge transfer within the value chain, colleague like relationship between farmers rather than competitive, returned profits from the value chain to incentive and improve effectiveness. Denmark has an immense environmental awareness, being the European country with the highest consumption of organic products, focus on animal welfare and reduced environmental footprint. It is important to remark that recently Denmark has been able to

increase production, while reducing the environmental footprint (Danish Agriculture & Food Council 2016).

1.1.2. Dairy production in Denmark

With 5.4 million tons of cow milk produced in 2016 (Eurostat 2017), Denmark's temperate climate conditions and abundant rainfall, seems favourable for an environmental balanced dairy production. Denmark has Europe's highest productivity, with an apparent yield of 9,621 kg/head (Eurostat 2017) and the average milk yield per cow has almost doubled in the past 30 years (Danish Agriculture & Food Council 2016). Exports of dairy products account for more than 20 % of all Danish agricultural exports, with a value of 1.8 billion euros annually (Statistics Denmark 2017).

The Danish dairy industry consists of the international dairy group Arla Foods and 30 smaller dairy companies, together processing 4.7 billion kg of milk from a total of 61 production plants in Denmark. Arla Foods is cooperatively owned by Danish and Swedish milk producers and it is Europe's largest dairy group (Statistics Denmark 2017).

Organic agriculture and foods are popular in Denmark, with Danish consumers buying more organic food items than any other European country. Seven percent of this country's farmland is cultivated organically, and 33 % of dairy consumption is organic (Danish Agriculture & Food Council 2016).

1.1.3. Central Jutland

Central Jutland, or *Midtjylland*, is the heart of Danish milk production. This region alone is responsible for 30 % of the national production and has one of the highest apparent milk yields in Europe with 9,533 kg/head (Eurostat 2017). Jutland even has a history of having its own cattle breed. Jutland cattle history begins in the middle age, when thousands of black and white cows were imported annually from Germany and the Netherlands. These cows were breed in Jutland until the 1950's. During the 1960's, bulls were commonly imported from the Netherlands to improve the Danish heard. North American semen was introduced to make the Danish breed competitive in the 1970's. Today it is denominated Danish Holstein and is considered a mutual partner in exchange of genetics between countries (Christensen 2019).

Automatic milking systems (AMS) are also an important part of Danish milk production. More than 90 % of the world's AMS farms are in north-western Europe, with the greatest adoption of this technology being in Denmark, in terms of percentage of dairy farms (Barkema et al. 2015). The option of AMS has taken place in areas where there is high density of dairy production (the case of Jutland), since it requires a high amount of technical

support (Koning 2010). This system allows less milking labour, which puts farmers under better social conditions and allows more time for management and control activities. It has also proven to increase milk productivity (Koning 2010) and animal health and well-being, after adequate adaptation to AMS.

In most European countries, especially Scandinavia, grazing during summer is a common routine. North western Europe consumers believe grazing is essential for cows. Arla (2019) states that their grazing season in usually between April and November. Besides grazing or roughages, cows get concentrates (cereal, soybean meals, rapeseed) to ensure their milk production genetic potential is met.

1.2. The dairy cow production cycle

The production life of any dairy cow starts with the 15-18 month old heifer being artificially inseminated so that it calves, ideally at the age of two years and two months old (Gillespie and Flanders 2010). At this time, the cow begins its milk production. The cow will then be milked for approximately 10 months (305 days), followed by a dry period of 60 days. In the meanwhile, the cow will be again inseminated 85 days after parturition, so that calving matches with the end of the dry period (Gillespie and Flanders 2010). Production cycle of a dairy cow is summarized in Figure 3. Efficient milk production requires the dairy cow to experience gestation and parturition each year (Goff and Horst 1997).
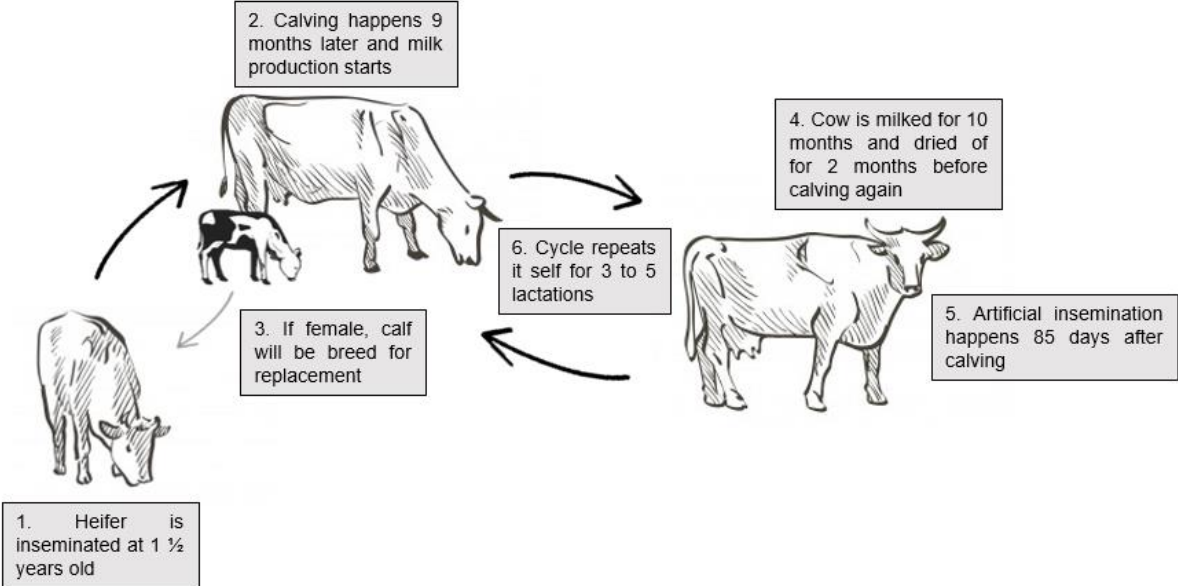
Figure 3 – Dairy cow production cycle

(adapted from: https://www.freepik.com/premium-vector/vector-set-cows_3893708.htm, 2019)

Calves are kept on artificial milk and weaned at 35-45 days. Female heifers are needed for replacement since average culling rate in Denmark's dairy farms was 25.4 %

between 2007 and 2010 (Nor et al. 2013). Culling rate is defined as "the percentage of cows removed from a herd because of sale, slaughter, salvage or death" (Fetrow et al. 2006). The most common reason for culling (32.5 %) is low milk production (Gillespie and Flanders 2010), that commonly happens after 3-5 lactations. Nevertheless, other reasons such as infertility, lameness, metabolic diseases or mastitis also contribute significantly to culling rates.

1.2.1. Most common diseases in dairy cows

Disease related culling only happens when cows show repeated health problems. Still, about 20 % of culling is due to diseases. The incidence of most common diseases is described in Table 1.

Table 1 – Incidence rate of most common diseases in dairy cows.

Disease		Incidence	Reference
Mastitis		20 %[b]	(Liang et al. 2017)
Lameness		30 %[a]	(Liang et al. 2017)
Metabolic diseases:	Ketosis	12 %[a]	(Liang et al. 2017)
	Retained placenta	12 %[b]	(Liang et al. 2017)
	Milk fever (hypocalcemia)	8 %[b]	(Fikadu et al. 2006)
	Displaced abomasum	3 %[b]	(Liang et al. 2017)
	Acidosis	19[c] –26[d] %	(Gao and Oba 2014)

a) primiparous and multiparous cows; b) multiparous cows; c) early-lactation; d) mid-lactation

Mastitis and lameness, although common, are relatively uncomplicated since they are generally easily detected and cured. Mastitis is an inflammation of the parenchyma of the mammary gland due to pathogenic bacteria invading and multiplying. Even its subclinical form is easily and cheaply detected by a somatic cell count (SCC) test. Mastitis is most common where hygiene is poor (Radostits et al. 2007), therefore appropriate prevention, management and sanitation is sufficient to lower the incidence of mastitis. Laminitis or lameness is a foot or leg condition caused by acute degeneration of the sensitive primary and secondary laminae of the hoof. The cause of this degeneration is unknown, but its predisposition appears to be inherited and it affects mostly heifers after calving (Radostits et al. 2007). High incidence in heifers can be explained by imposing too much weight in the hoofs with pregnancy and the udder on a relatively young animal. Hence, proper genetic management and appropriate surface of the yards can reduce the incidence of lameness. Metabolic diseases, on the other hand, are more complex.

1.2.2. An overview on major metabolic diseases in dairy cattle

Metabolic disorders are defined as disturbances of one or multiple metabolic processes, related to the regulation of certain metabolites in the body fluids and are a manifestation of the cow's inability to cope with metabolic demands (Sundrum 2015). Amongst domestic farm animals, metabolic diseases have more importance in dairy cows, since in other species these diseases occur only sporadically (Radostits et al. 2007). The transition phase, defined as the period between three weeks before to three weeks after parturition, is the most challenging and critical period in relation to the dairy cow´s health status during the lactation cycle (Sundrum 2015). Gestation alone, is already very demanding on the metabolism. By the end of gestation, daily foetus development requires, among other things, 0.82 Mcal of energy, 117 g of protein and 10.3 g of calcium. Beyond that, the production of just 10 kg of colostrum in the day of calving requires 11 Mcal of energy, 140 g of protein and 23 g of calcium (Goff and Horst 1997). These high demands on nutrients cannot be met just by feed intake, the cow will need to mobilize body tissues namely adipose tissue. When the amount of energy that a cow obtains from dietary source is not enough to cover the animal's energetic needs, the cow is in a state of negative energy balance (NEB) (Sundrum 2015). In addition to lactation increasing daily, the shift from pregnant non-lactating to non-pregnant lactating, imposes tremendous physiological challenges to the homeostatic mechanisms of the cow (Goff and Horst 1997), resulting in metabolic "malfunctions", or diseases.

Increased milk production in dairy cows increases the incidence of metabolic diseases. Increased incidence seems to be related to the extremely high turnover of fluids, salts and soluble organic materials during the early part of lactation (Radostits et al. 2007).

Ketosis is a consequence of constant negative energy imbalance. It is more common at early lactation, when cows mobilize high amount of fat reserves as a source of energy. However, there is a limit to the amount of fatty acids that can be oxidized by the liver. When this limit is reached, and the tricarboxylic acid cycle is blocked, the acetyl-CoA that is not incorporated in the cycle is converted to acetoacetate and β-hydroxybutyrate. Consequently, the cow will have low blood sugar resulting in reduced feed intake, drop in milk production, loss in body weight and overall dullness (Gillespie and Flanders 2010).

Retained placenta, as the name indicates, is a condition in which the placenta is not discharged within 12 to 24 hours after calving. Infection in the reproductive tract during pregnancy, deficits in vitamin A or E, iodine and selenium, out of balance calcium to phosphorus ratio in the diet, cow being fat or fed to much carbohydrates, stress at calving and inseminating too soon after calving are some causes of this illness (Gillespie and Flanders 2010). Retained placenta is widely considered to be a predisposition factor for metritis, which is an inflammation of the uterus due to bacterial invasion (Liang et al. 2017).

Milk fever, or hypocalcemia, is caused by low levels of calcium in the blood (Radostits et al. 2007). It usually occurs within a few days after calving and symptoms include loss of appetite, excitation in an early state followed by depression, bloating, cold skin, paralysis and, if not treated, death (Gillespie and Flanders 2010).

In a nonpregnant cow, the abomasum occupies the ventral position of the abdomen. As a cow becomes pregnant and the foetus grows, the uterus occupies an increasing amount of the ventral cavity. This forces the abomasum forward and slightly to the left side of the cow. After calving, the uterus retracts back toward the pelvic area (Goff and Horst 1997). The necessary metabolic processes, that allow that abomasum to return to its original place, may not occur causing displaced abomasum.

Ruminal acidosis is a fermentation disorder in the rumen characterized by a decreased ruminal pH (Hernández et al. 2014). This condition is thoroughly described in the following section.

1.3. Ruminal Acidosis

A common practice to meet high nutritional requirements in early and mid-lactation dairy cows is the inclusion of large quantities of concentrate in the diet at the expenses of a decreased fibre content. Inclusion of rapidly fermentable carbohydrates generates large amounts of short chain fatty acids (SCFA). When the production of acids surpasses the absorptive, buffering and outflow capacity of the rumen, ruminal pH decreases (Aschenbach et al. 2011).

1.3.1. Definition

Decreased ruminal pH and changes in the ruminal microbiota population are responsible for acidosis (Owens et al. 1998). The most recent definition of subacute ruminal acidosis (SARA) states that it occurs when intermittent drops of ruminal pH have a sever, long and frequent effect on the rumen function (Owens et al. 1998). Current definitions of SARA are based on ruminal fluid pH (Plaizier et al. 2009). However, the precise definition of SARA is still controversial and there is also no agreement on which rumen pH depressions have negative consequences on health and performance of dairy cows. The current guideline is that the risk of SARA increases when ruminal pH drops below 5.6 for more than 3 hours a day (Plaizier et al. 2009) or below 5.8 for more than 5 to 6 hours a day (Zebeli et al. 2012). Figure 4 summarizes the causes, consequences and symptoms of acidosis in cattle.
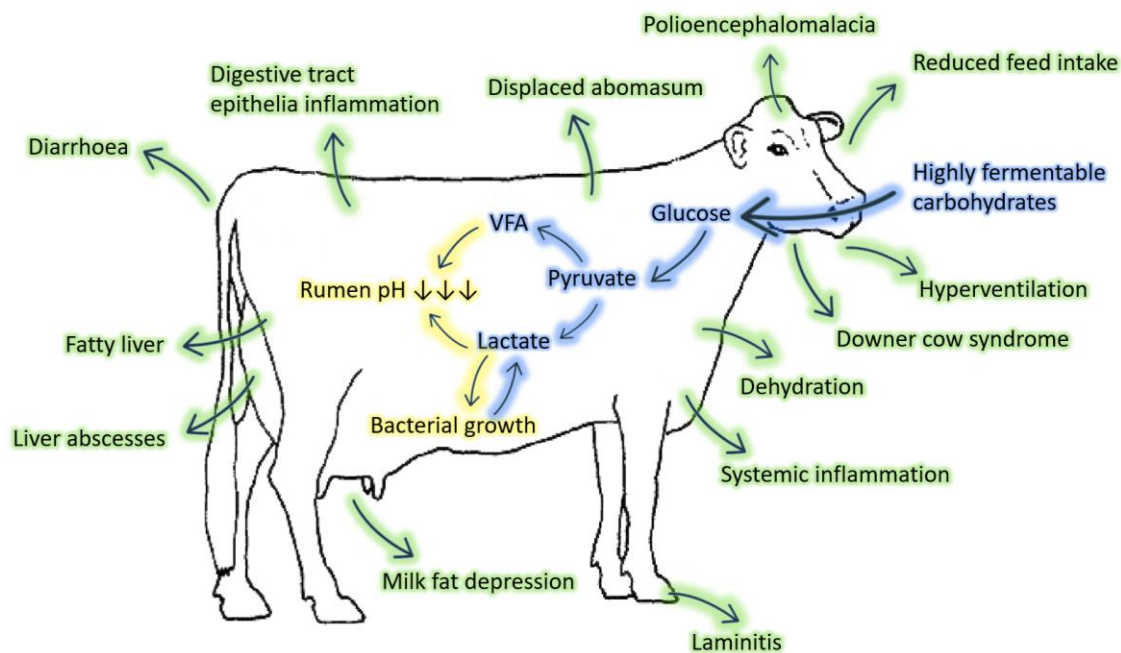
Figure 4 – Clinical picture of acidosis

Causes are highlighted in blue, consequences in yellow and symptoms in green.

(adapted from: http://www.cattle-empire.net/sites/default/files/stomachs.jpg, 2019)

1.3.2. Causes

Ruminal pH is determined by the balance between acid production in the rumen and acid buffering or removal from the rumen (Allen 1997). Hernandez et al. (2014) described that a) bad bunk management, b) high non-structural carbohydrates intake and c) inadequate ruminal buffers capacity are the main causes for SARA.

a) The susceptibility for acidosis varies among animals. Several factors such as feed intake, eating rate, feed selection, salivation rate, inherent ruminal microbial population, previous exposure to acidosis, rate of passage of feed from the rumen and other aspects of physiology and behaviour (Hernández et al. 2014), for example hierarchy and dominance patterns. This social behaviour, if not properly managed, leads to interruption in normal feed patterns and stress. Drastic diet changes also contribute to higher acidosis risk.

b) Acid production begins with the consumption of a diet rich in grains (Hernández et al. 2014). Grain have a large amount of starch and other rapidly fermentable carbohydrates. The amylolytic bacteria of the rumen will then ferment such carbohydrates, firstly into glucose, then pyruvate and finally into volatile fatty acids (VFA) (Hernández et al. 2014) or lactate (Owens et al. 1998). With more substrate and less competition, the growth rate of all bacteria increases, thereby increasing total VFA production, mainly acetate, propionate and butyrate (Bramley et al. 2008). Lactate is also produced as a consequence of bacteria fermentation processes and it is normally present in the digestive tract in small amounts. However, abruptly carbohydrate supply, causes lactate accumulation in the rumen (Owens et

al. 1998). When the absorptive capacity of the rumen papillae is maximal and the ruminal VFA concentration increases, pH decreases sharply. Bacteria in the rumen is often classified as "lactate producers" or "lactate users". Balance between these two groups determines whether lactate accumulates or not (Owens et al. 1998). Within the pH range between 6 and 5.6, the fermentative capacity and growth rates of major lactate-producing bacteria and lactate-consuming bacteria exist in balance. Therefore, at this point lactate produced is immediately consumed (Goad and Nagaraja 1998). Most lactate-using bacteria are sensitive to low pH, whereas most lactate producers are not (Owens et al. 1998). As pH keeps dropping, lactate-consuming gram (-) bacteria disappear. The extra space left by the gram (-) bacteria are taken over by gram (+) bacteria, that produce lactate (Hernández et al. 2014). Ruminal microbes produce two lactate isomers D (+) and L (-), respectively. This furthermore decreases pH, leading to a second bacteria population change due to the increasing L-lactate production. Only pH resistant bacteria can grow in this condition, producing D-lactate. At this point, a drop of ruminal pH of up to 3.8 can occur, and as the acid crosses the ruminal wall and reaches the blood stream and a metabolic acidosis occurs (Hernández et al. 2014).

c) The absorption of the ruminal acid through the rumen epithelial cells, neutralization by buffers and passage to the lower digestive tract (Allen 1997) causes removal of acids from the rumen. There are several buffers acting in the rumen. Saliva is the first buffer neutralizing ruminal acids. However, grain-based diets used to replace high-fiber forages, compromise the physically effective fibre, or NDF, content (Humer et al. 2018). Therefore, chewing activity is not stimulated, and saliva secretion is reduced compromising the buffering capacity. Urea crossing the ruminal wall allows for ammonia to bond with the acid, neutralizing it. Also, a reduced proportion of the ruminal acid passes into the lower gastrointestinal tract (González et al. 2012). These three buffers neutralize about 30-50 % of the ruminal acid and the rest is absorbed by the ruminal papillae (Hernández et al. 2014).

1.3.3. Symptoms

There are two clinical forms of acidosis: acute, or metabolic, and subacute, or ruminal (Hernández et al. 2014). While in the acute form, clinical signs will appear in the animal, the subacute form does not show obvious symptoms. Reduced and erratic feed intake, diarrhoea, milk fat depression, liver abscesses, systemic inflammation, local inflammation of the epithelia of the digestive tract (Plaizier et al. 2017), displaced abomasum, fatty liver, downer cow syndrome (Humer et al. 2018) polioencephalomalacia, dehydration, laminitis, lameness, hyperventilation, cardiac arrest and death (Hernández et al. 2014) are some of the symptoms during acidosis. While in the acute form, clinical signs will appear in the animal, the subacute form (i.e. SARA) does not show obvious symptoms.

As SARA diagnosis is difficult under farm conditions, this metabolic disease may remain undetected. In this sense, it is more of a problem than acute acidosis that will immediately be detected and treated. The consequences for cattle health and productivity may then be quite extensive (Humer et al. 2017).

1.3.4. Consequences

Subacute ruminal acidosis has negative consequences on production cost and animal welfare (Plaizier et al. 2017). One field survey in the United States indicated that SARA incidence is 19 % in early-lactation dairy cows and 26 % in mid-lactation cows (Gao and Oba 2014). Lean et al. (2000), described SARA as the most significant disorder in lactating dairy cows.

Subacute ruminal acidosis reduces the richness, diversity, stability and functionality of rumen and large intestine microbiota (Plaizier et al. 2017). Cows rely on microbiota in the digestive tract to use nutrients (Plaizier et al. 2017), including the components needed for milk production. SARA has been associated with alterations in the biohydrogenation of unsaturated fat in the rumen (Danscher et al. 2015).

1.4. The use of biomarkers in dairy production

At the onset of lactation, the nutrient demand increases drastically. A cow with a maximum milk yield of 50 kg produces approximately 2 kg of milk fat daily, 1.6 kg of milk protein, 2.5 kg of lactose, 65 g of Ca, 50 g of P and 8 g of Mg, which of course, increases the demand for energy, protein and minerals. The nutrient demand that happens, particularly for lactation, calls for a coordination of the biological processes in different tissues, resulting in metabolic changes that try to ensure that the cow´s genetic potential for milk yield is exploited concurrently with maintenance of the homeostasis physiological parameters (Ingvartsen 2006).

The term "metabolism", literally meaning "change", is used to refer to all the chemical and energy transformations that occur in the body (Ganong 2005). Metabolic status is often evaluated by the analysis of diverse variables in rumen fluid, urine and blood. However, these fluids are difficult to collect and therefore are not useful at the farm level. Rumen pH, together with data obtained from potential biomarkers in milk may provide tools for early detection of physiological imbalance (Bruckmaier and Gross 2017).

Biomarkers or biological markers are defined as indicators of processes, events or changes in a biological system (DeCaprio 2006). This definition implies that biomarkers can reflect, predict or take the place of biological endpoints that cannot be easily or directly measured or that take extended periods of time to become apparent. In theory, anything that one can measure in an organism can represent a marker for some biological event or

process (DeCaprio 2006). Although clinical symptoms of a disease are endpoints for diagnosis themselves, biomarkers allow, in many cases, for early detection and, therefore, prevention of diseases (Griffiths et al. 2002).

1.4.1. Rumen, blood and urine (bio)markers for SARA

As previously described, acidosis begins in the rumen when pH drops. Rumen epithelial walls then absorb the acids to the blood stream. Blood is filtered by the kidneys and some of the acids could potentially be excreted by urine. Analysing pH in these three fluids allows to study the net flux of the acids formed.

1.4.1.1. Rumen pH

Rumen pH is used to assess rumen health status and detect disorders such as SARA because it is a direct homeostatic result of the acid-base balance regulation efforts (Allen 1997). This balance can be measured after the collection of rumen fluid. Diagnosis of SARA requires standardization of the timing of rumen fluid collection and the threshold for SARA needs to reflect the sampling time (Plaizier et al. 2009). Therefore, the sampling method and timing used to measure rumen pH may affect the pH value itself.

Additionally, comparing ruminal pH may be challenging because ruminal pH is not homogeneously distributed throughout the rumen (Aschenbach et al. 2011). The standardized sampling site for ruminal pH is the ventral sac, because it is where most mixing of ruminal content occurs. Therefore, it provides the most integrated information on the pH status of the whole rumen (Duffield et al. 2004).

The pH values in the medial portion of the rumen have been reported to be 0.16 to 0.75 units lower compared to those from the ventral part. Therefore, the pH conditions under which the major part of fermentation occurs, should be slightly more acidic than predicted from ventral ruminal pH. Thus, additional measurements in the ruminal ventral portion have a potential to improve the precision when predicting fiber digestibility, fermentation patterns, and health consequences of feeding regimens based on easily fermentable carbohydrates (Aschenbach et al. 2011).

1.4.1.2. Blood pH and gas analysis

Blood pH depends on the relative concentrations of bases, acids and buffers in solution. Blood pH below 7.35 is indicative of metabolic acidosis (Owens et al. 1998). There is a close relationship between rumen pH and blood pH (Gianesella et al. 2010) and blood pH has been reported to decline when lactate concentration increases in the rumen (Brown et al. 2000). However, decreased blood pH mainly occurs during acute ruminal acidosis

(Humer et al. 2017), not subacute. Although slightly lower and occurring following nadir ruminal pH (Brown et al. 2000), studies show no significant differences in blood pH of SARA induced cattle (Li, Gozho, et al. 2012; Danscher et al. 2015).

Most fermentation products are transported by the bloodstream. Therefore, blood gas analysis can be used to detect the early onset of SARA (Gianesella et al. 2010), as it provides a good assessment of acidosis while being less invasive than rumen pH analysis. Partial pressure of carbon dioxide has been investigated for its potential to serve as an indirect diagnostic marker for SARA (Humer et al. 2017). Although some studies show no significant changes in SARA induced animals (Li, Gozho, et al. 2012; Danscher et al. 2015), acidotic cows have decreased partial pressure of oxygen (pO_2) and increased partial pressure of carbon dioxide (pCO_2) (Morgante et al. 2009; Gianesella et al. 2010; Brscic et al. 2015), especially when ruminal pH is below 5.5. Decreased pCO_2 has been attributed to an increase in anaerobic metabolism and O_2 consumption.

1.4.1.3. Urine pH

Urine pH reflects changes in the metabolic acid-base load in the systematic circulation and not the actual changes of acid-base homeostasis in the rumen. It is important to note that the main organ for base excretion in lactating dairy cows is not the kidney, but the salivary gland (Humer et al. 2017). The normal range of urine pH is considered between 8.2 and 8.4 (Cozzi et al. 2011). Although some studies have shown decreased urine pH in induced SARA cows (Gianesella et al. 2010; Danscher et al. 2015), most of them show no significant correlation between rumen pH and urinary pH (Duffield et al. 2004; Gianesella et al. 2010; Li, Gozho, et al. 2012; Humer et al. 2017) recommend that urinary variables of acid-base status be interpreted very cautiously, due to different factors affecting this parameter.

1.4.2. Commonly used (bio)markers for SARA

Water intake and dry matter intake are some of the parameters regularly supervised on farm level, that can indicate metabolic irregularities.

1.4.2.1. Water intake

Water is the most significant nutrient for dairy cows. Enough supply of clean water is generally accepted as essential to prevent negative effects on animal health, performance and welfare (Meyer et al. 2004). Ulrich et al. (2004) suggested that the main factors determining water consumption are average ambient temperature, milk production, dry matter intake, dry matter content of ration, body weight, lactation rank, lactation day, Na intake, K intake and relative humidity (Meyer et al. 2004).

Appuhamy et al. (2016) gathered 55 published studies and concluded, with significant heterogeneity, that lactating cows consume on average 78.4 ± 2.6 kg of water each day.

1.4.2.2. Dry matter intake

Regarding feed intake, often expressed in dry matter intake (DMI), there are numerous factors, which can affect it. Generally, sudden changes in the diet should be avoided and instead animals should be fed with gradual changes in diet (Ingvartsen 2006). Decreased DMI has been used as a clinical sign to diagnose SARA (Keunen et al. 2002) and has been reported by Krause and Oetzel (2005), Danscher et al. (2015), Brown et al. (2000), Kleen et al. (2003) and Plaizier et al. (2009). But other researches find no incidence of reduced feed intake to cows with ruminal pH depression (Krause and Oetzel 2005; Gozho et al. 2007; Khafipour et al. 2009; Li, Gozho, et al. 2012). Reduced feed intake can be caused by reduced fiber digestibility, increased VFA concentrations, especially propionate, and altered rumen osmolarity (Plaizier et al. 2009). This discrepancy among studies suggest that rumen pH depression during SARA alone does not necessarily reduce feed intake (Li, Gozho, et al. 2012).

While reduced DMI is often associated with SARA, it should be noted that low DMI followed by a rapid increase in DMI is also a risk factor for SARA (Humer et al. 2017). Fluctuating feed patterns are explained by (Humer et al. 2017), stating that animals typically refuse to eat after their first meal, due to a dramatic decline in ruminal pH. As soon as ruminal pH returns to its phycological value, appetite is restored. Because experimental studies usually record DMI once a day, these fluctuations are therefore not noticed.

1.4.3. Milk quality biomarkers for SARA

Milk samples are more accessible at the farm level. Therefore, special interest exists on biomarkers that can be measured in milk rather than, for example, blood or rumen fluid (Koster et al. 2019). Also, milk payment systems are constructed as a method of approximating the true value of milk based on its components and are based on volume (yield), fat and protein contents (Costa et al. 2019) as well as somatic cell count (SCC). Therefore, evaluating these parameters is common in dairy farms. Although in the past, lactose has been considered a low-value milk component, the importance of this parameter has changed in recent years and has gained economic interest at international level (Costa et al. 2019).

1.4.3.1. Milk yield

A field study found that SARA reduced milk yield by 2.7 kg/day, milk fat production by 0.3 % and milk protein production by 0.12 % (Stone 1999). However, a fair amount of more recent studies inducing SARA, showed no significant difference on milk yield (Lean et al. 2000; Gozho et al. 2007; Li, Gozho, et al. 2012; Danscher et al. 2015), although some do (Kleen et al. 2003). Koster et al. (2019) clarifies that milk yield is not a cause of metabolic imbalance or disorders, but rather the cow´s individual ability to cope with the metabolic challenges of early lactation.

1.4.3.2. Milk fat

Studies have shown that SARA can negatively impact various milk parameters, especially milk fat content (Nocek 1997; Kleen et al. 2003; Li, Gozho, et al. 2012). This is a major concern, as the decrease in milk fat content lowers the milk energy efficiency despite the enhanced milk yield obtained by feeding high-grain diets. As described by Bauman and Griinari (2003), rumen pH depression reduces milk fat production, which promotes the synthesis of trans fatty acids in the rumen. However, the negative effects of SARA on milk are still controversial, as it is not the only factor affecting milk fat synthesis. Some experiments inducing SARA, show no significant signs of milk fat depression (Lean et al. 2000; Gozho et al. 2007; Gao and Oba 2015) and even an increase in milk fat content (Danscher et al. 2015).

It has been suggested that the inconsistent response in milk fat in experimentally induced SARA may be related to the duration of the bouts of SARA, with short ones being unlikely to influence milk fat content (Plaizier et al. 2009).

1.4.3.3. Milk protein

Most studies determine no significant differences in milk protein yield in SARA induced cows (Lean et al. 2000; Gozho et al. 2007; Danscher et al. 2015). However, two other studies have showed increased milk protein percentage in cows with SARA (Khafipour et al. 2009; Li, Gozho, et al. 2012).

1.4.3.4. Milk lactose

Lactose is a simple method for following mammary gland metabolism *in vivo*. According to recent studies, lactose concentration is a better indicator than SCC in reflecting reduction in milk yield and milk clotting parameters (Silanikove et al. 2014). Based on this fact, lactose is currently considered a potential additional trait for inclusion in udder health indexes, along with SCC (Costa et al. 2019).

Lactose is synthesised in the Golgi apparatus of mammary gland cells, by enzymatic condensation of glucose with UDP-galactose, which is derived from glucose (Silanikove et al. 2014). Glucose also serves as the main source for production of ATP and NADPH, which are essential for sustaining the mammary gland metabolism and synthetic capacity (Silanikove et al. 2014). Lactose percentage is strictly dependent on blood circulating glucose (Costa et al. 2019). Hence, it is important to highlight the dependence of milk yield on lactose yield and that the uptake of glucose from the blood to synthetize lactose is a metabolic priority in specialized dairy animals.

1.4.4. Milk parameters and metabolites

Further analysing milk samples can help asses a cow's metabolic status. Determining milk pH, glucose, glucose-6-phosphate (G6P), B-hydroxybutyrate (BHB), lactate dehydrogenase (LDH), creatinine and organic acids, seem to provide an overall picture of cow's rumen and metabolic health.

1.4.4.1. Milk pH

The normal cow's milk pH is approximately 6.6 (Faulkner and Peaker 1982). Even though no studies were found concerning SARA affecting milk pH, it seems rational to measure milk pH to see if significant decreases occur.

1.4.4.2. Glucose and glucose-6-phosphate

Glucose is essential for lactose synthesis, because it is a limiting factor for milk synthesis. More than 80 % of the glucose turnover is used by the mammary gland for the synthesis of lactose during peak lactation (Bruckmaier and Gross 2017). The mammary gland cannot synthesize glucose, and therefore, relies on circulating glucose for its requirements (Bjerre-Harpøth et al. 2012). Due to ruminal fermentation, glucose is scarcely absorbed directly from most feeds. Because it is metabolized in the gastrointestinal tract, glucose is predominantly derived from hepatic gluconeogenesis in ruminants (Bruckmaier and Gross 2017).

Glucose-6-phosphate (G6P), is both an intermediate compound during lactose synthesis and the first step in both glycolysis and the pentose phosphate pathway. Therefore, G6P can also be considered a limiting factor for milk synthesis. Also, glucose in milk is dependent on the quantity of glucose absorbed from blood to the mammary gland. Although the concentration of free glucose in cow's milk is negligible compared to oligosaccharides, glucose and galactose are the main free monosaccharides in milk. Free glucose and G6P concentrations in milk are usually less than 1 mmol/L and 30-60 µmol/L,

respectively (Larsen 2015). The concentrations of G6P in milk are always lower than glucose concentrations. Milk glucose concentration have been correlated to milk yield (Silanikove et al. 2014). Changes in concentration of mammary epithelial cells metabolites in milk reflect a reduction in glucose availability for lactose synthesis and shift to glycolytic metabolism at the expense of mitochondrial metabolism.

1.4.4.3. β-hydroxybutyrate

β-hydroxybutyrate (BHB) is the predominant circulating ketone body in ruminants, therefore it is often used to assess energy status (Santschi et al. 2016). It has been proven that milk composition is unaltered even in case of breakdown of body reserves. Increase in energy demand in high-yield cows could result in a negative energy balance and mobilization of fat reserves from tissue to blood, passing through the liver. Supporting this view, milk BHB is one of the most common indicators of ketosis in dairy cows (Larsen and Moyes 2014). Ketone bodies are known to increase during negative energy balance (Zarrin et al. 2017). BHB concentrations, both in plasma and milk, can be used as a biomarker relating to body tissue mobilization (Bjerre-Harpøth et al. 2012).

1.4.4.4. Lactate dehydrogenase

Lactate dehydrogenase (LDH) is an enzyme found in the cytoplasm of all cells and tissues in the animal body, yeasts and bacteria (Larsen 2005). This glycolytic enzyme is involved in the reversible conversion of pyruvic acid to lactate (Doornenbal et al. 1988). A positive correlation between LDH activity and cell counts in milk contaminated with pathogens has been proven. Larsen (2005) concluded that the activity of LDH in individual milk samples was an important indicator of bovine mastitis.

1.4.4.5. Creatinine

Creatinine is the excretion form of creatine compounds. Creatine is phosphorylated in the muscle, forming a backup of readily available high energy (Doornenbal et al. 1988). Higher values of blood creatinine concentrations have been recorded when cows are subjected to metabolic stress, due to tissue mobilization (Cozzi et al. 2011). Creatine is synthesized in the liver from methionine, glycine and arginine.

1.4.4.6. Malate and isocitrate

The citric acid cycle, or Krebs cycle, is a series of chemical reactions used by aerobic organisms to release stored energy. Malate, or malic acid, and citrate, or citric acid, are part of the metabolism and intervenient in this cycle (Shapiro and Silanikove 2011). Both organic

acids take part in the enzymatic reactions that regulate the metabolism using $NAD^+/NADP$ as cofactors (Shapiro and Silanikove 2011), therefore report to the animal's energy status. Oxidation of isocitrate is believed to deliver large fractions of energy (Larsen 2014).

1.4.4.7. Urate

Urate, or uric acid, is a naturally occurring component in milk and a well-known antioxidant in many biological systems. Østdal, Andersen, and Nielsen (2000) consider urate the main antioxidant in milk. It is formed from ruminal breakdown of microbial nucleotides and thereby influenced by rumen metabolism and feed composition. A negative correlation between daily milk production and urate concentrations in milk has been reported. This indicates that the urate concentration can be controlled by the intensity of the milk production and by feeding regimes promoting high ruminal activity in order to achieve optimal antioxidative activity of this compound in milk (Østdal et al. 2000).

1.4.4.8. Glutamate

Glutamic acid is an amino acid found in proteins with acidic an acidic side chain (Ganong 2005). Glutamine is incorporated into proteins and exists as a free amino acid in the liver, muscles, blood and milk. Although it is considered a non-essential amino acid (since the organism can synthesize the amount needed), glutamine participates in several unique physiological processes. Glutamate is formed when the terminal amide group of glutamine is cleaved. This process can happen enzymatically or spontaneously through heat or, more important for this case, low pH.

1.4.5. The importance of biomarkers

Figure 5 summarizes biomarkers used in this study to determine cow's metabolic status. Analysis are separated into "on lab" and "on farm". This separation is made so that we can study the effects of SARA on both biomarkers that farmers can use and analyse the metabolic related load. Research analysis will allow us to go further on determining the consequences of SARA in cows.

On lab

Rumen fluid
- Medial fluid pH
- Ventral fluid pH

Blood
- pH
- pO_2
- pCO_2

Urine
- pH

Milk metabolites
- pH
- Glucose
- Glucose 6P
- BHB
- LDH
- Creatinine
- Malate
- Isocitrate
- Urate

On farm

Commonly used
- Body weight
- Water intake
- Dry matter intake

Milk quality
- Yield
- Fat
- Protein
- Somatic cell count
- Lactose
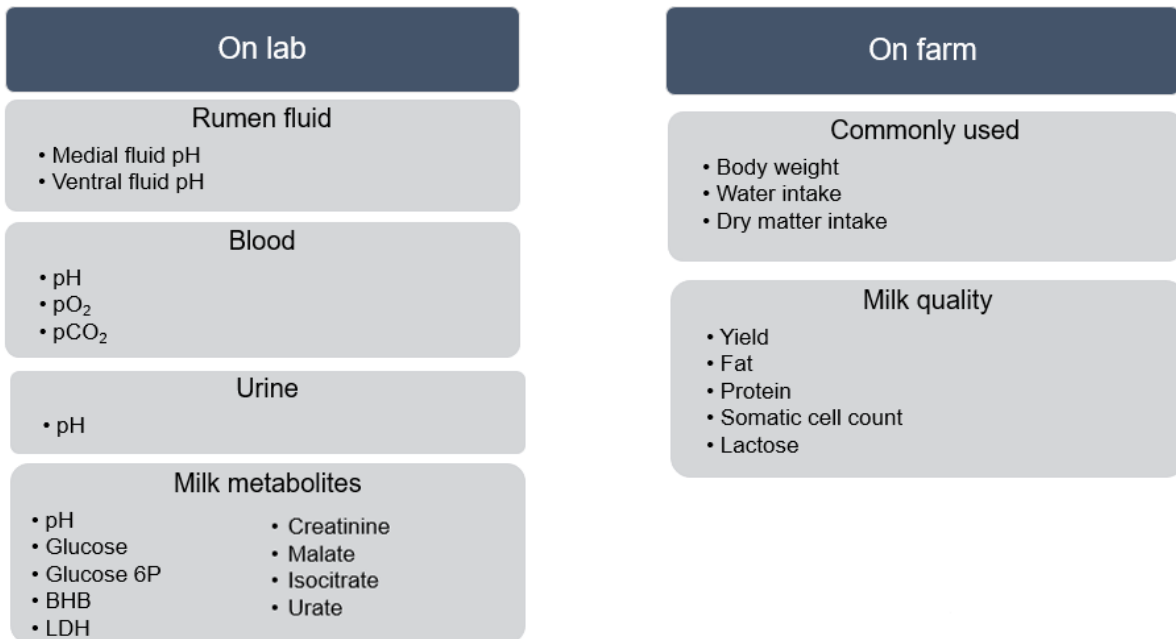
Figure 5 – Biomarkers used in this study to determine the cow´s metabolic status

Data collected from the rumen will allow us to study the epicentre of SARA. From here blood analyses will reveal which consequences it can have on the overall organism of the animal. Milk quality analysis will determine the consequences the disorder can have on farms income. Milk metabolite analysis will describe the cow's metabolic status, since they take part in the most common metabolic processes occurring in the organism

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2. MATERIALS AND METHODS

The materials and methods section thoroughly describes every procedure conducted in the experimental trial. It is organized in three sections: experimental design, sampling procedures and statistical analysis in a way that the experiment can be replicated.

2.1. Animals and experimental design

Experimental design explains the overall conditions the animals were kept in and how the experiment was conducted.

2.1.1. Animal welfare disclaimer

The Animal Experiments Inspectorate under the Danish Veterinary and Food Administration and following pertinent Danish and EU Legislation approved the experiment with the following references: Project number: 15-12-02189; Permit number: 2013-15-2934-00854.

2.1.2. Experimental groups

In this study, six multiparous Holstein-Friesian dairy cows (LW = 702 ± 61; DIM = 127 ± 48) were used. Animals were production cows and had the following four last numbers on their earing identification: 7907, 6492, 6663, 6448, 6343, 6488. In all six cows, ruminal cannulas (Figure 6) were placed one month before the beginning of the experiment by specialized veterinary personnel. Additionally, a permanent indwelling catheter in the aorta artery was surgically implanted three weeks before the beginning of the experiment. Cows were randomly allocated to the three experimental diets in a crossover design (balanced for six cows) with 14 days periods.

2.1.3. Experimental conditions

The animal trial was conducted in the Danish Cattle Research Centre, in Foulum (56º29'17.9''N; 9º35'06.0''E) and animals were placed in barn K33-2. Cows were allocated in tie stalls (Figure 6). Tie stalls have the cow fastened by the neck chain to the front of the stall. There was a pipe across the front of the stall to prevent the cow from walking forward through the stall (Gillespie and Flanders 2010). Individual housing is necessary to control what that specific cow drinks and eats, especially in experiments where the feed is the challenge. They also allow individual attention to each cow and makes it easier to observe and collect samples. Tie stalls provide greater comfort to the cow than stanchions, that are the only other form of individual housing (Gillespie and Flanders 2010). For this experiment, tie stalls were 300 x 120 cm, this allowed them to be long enough for cows to lie down with

the udders on the platform, but short enough that faeces fall in the gutter placed immediately at the end. Width allowed the necessary room for the cow and the person milking it Figure 6). Stalls were arranged in two rows with cows facing in. Bedding on the stalls was sawdust wood over rubber mats. Water was provided *ad libitum* with automatic water cups.

Figure 6 – Tie stalls and cannula
Cow were placed in a barn with individual housing with a tie stall method and cannulas were on the left side.

2.1.4. Experimental design: Experimental calendar

The experiment started on the 12th of September 2018 and ended on the 25th of October 2018. The experiment was divided in tree periods (14 days each) in order to test all experimental diets in all six cows. For further details, please refer to Figure 7.
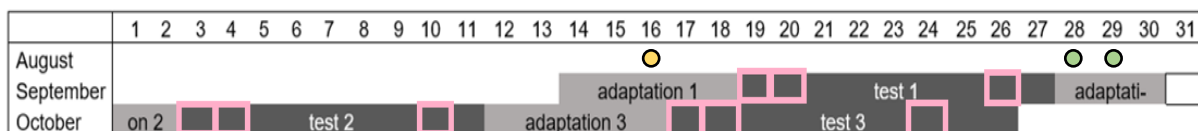
Figure 7 – Experimental calendar
The experiment was divided into three period, each one 14 days long. Periods had and adaptation (light grey), where cows were fed a control diet, and a test period (dark grey), where the challenge feed was provided. The calendar also presents sampling days (borderline in pink), canula surgery (yellow circle) and catheter surgery (green circles).

Ruminal cannulas (#1C; Bar Diamond, Parma, ID, USA) were fitted four weeks before trial according to Duffield, 1999. Catheterization occurred two weeks before the beginning of the trial, in two days, as it is a complex and time consuming surgery. Therefore, to ensure the veterinarian and the animal's well-being, three cows were catheterized each day. Surgery was done according to Kristensen et al. (2007), except that intercostal catheterization was performed under infiltration analgesia. The artery was exposed in the last intercostal space and a Tygon catheter (1.02 mm i.d. x 1.78 mm o.d.; S-54-HL, Buch & Holm A/S, Herlev, Denmark) was inserted 35 cm into the artery, placing the tip of the catheter in the aorta. This

was done by specialized veterinarians with the master student´s assistance. Because cows could potentially lick the catheters (Figure 8) and remove them, which could be harmful and unsafe for them, common horse blankets were adapted to fit them and cover the catheter (Figure 9).

Figure 8 – Catheter
A permanent indwelling catheter was surgically implanted in the aorta artery for blood sampling.

Figure 9 – Adapted horse blanket
To prevent that the cows lick the catheters, horse blankets were adapted to cover them.

2.1.5. Experimental design: Nutritional treatments

Each period started with five days of adaptation, where cows were fed a control diet (CTRL). The test days of each period consisted in switching control and experimental diets. This way, it is done in opposition to what Ingvartsen (2006) suggests in terms of avoiding sudden changes in the diet, so that SARA is induced and animals are not able to adapt. Therefore, in days 6, 8, 10, 12 and 14 of every period was provided control diet and on days 7, 9, 11 and 13 was given a challenge diet. Figure 10 summarizes the feeding schedules for every period.

			adaptation								test				
	date	14/set	15/sep	16/sep	17/sep	18/sep	19/sep	20/sep	21/sep	22/sep	23/sep	24/sep	25/sep	26/sep	27/sep
	DIP	1	2	3	4	5	6	7	8	9	10	11	12	13	14
COW	7907	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	MED	CTRL	MED	CTRL	MED	CTRL	MED	CTRL
	6492	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL
	6663	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL
	6448	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL
	6343	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	MED	CTRL	MED	CTRL	MED	CTRL	MED	CTRL
	6488	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL

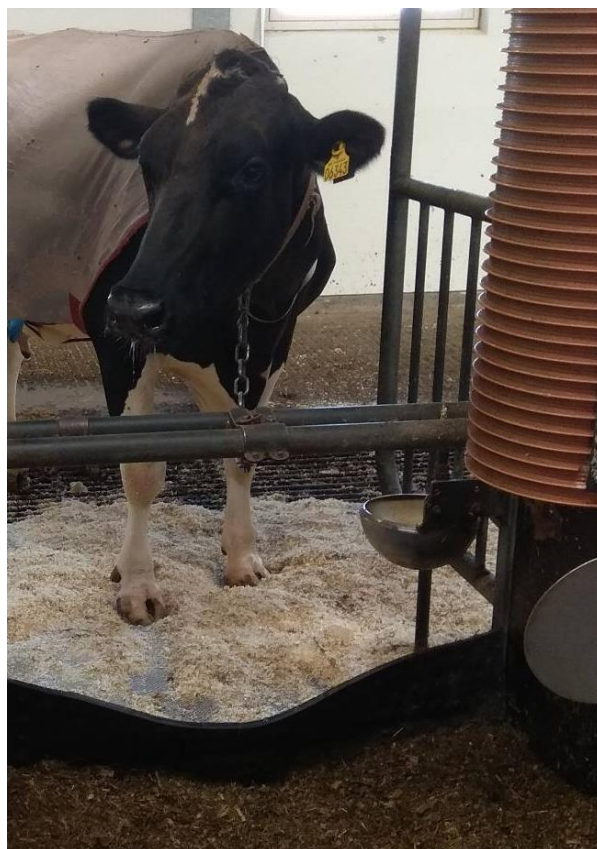|			adaptation								test				
	date	28/set	29/sep	30/sep	01/oct	02/oct	03/oct	04/oct	05/oct	06/oct	07/oct	08/oct	09/oct	10/oct	11/oct
	DIP	1	2	3	4	5	6	7	8	9	10	11	12	13	14
COW	7907	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL
	6492	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	MED	CTRL	MED	CTRL	MED	CTRL	MED	CTRL
	6663	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL
	6448	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL
	6343	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL
	6488	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	MED	CTRL	MED	CTRL	MED	CTRL	MED	CTRL

			adaptation								test				
	date	12/oct	13/oct	14/oct	15/oct	16/oct	17/oct	18/oct	19/oct	20/oct	21/oct	22/oct	23/oct	24/oct	25/oct
	DIP	1	2	3	4	5	6	7	8	9	10	11	12	13	14
COW	7907	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL
	6492	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL
	6663	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	MED	CTRL	MED	CTRL	MED	CTRL	MED	CTRL
	6448	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	MED	CTRL	MED	CTRL	MED	CTRL	MED	CTRL
	6343	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL	LOW	CTRL
	6488	CTRL	CTRL	CTRL	CTRL	CTRL	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL	HIGH	CTRL

Figure 10 – Diet schedule calendar
Specific daily feed in each period. Days in period (DIP) are mentioned on the second line of each period. During adaptation, cows were fed control diet (blue) and in the adaptation, control and challenge feed was alternated. Challenge feed could either be of low (green), medium (orange) or high (red) starch content. On the date line of the scheme, pink days indicate sampling days.

Every feed was provided *ad libitum*, calculated as an additional 10% than the expected intake and corrected if needed during the trial. Experimental diets were formulated based on starch content resulting in control diet having 21% starch, diet 1 having with 28% starch (low content), diet 2 having 35% starch (medium content) and diet 3 having 42% starch (high content). Further details on the experimental diets can be found in Table 2. Recent studies analysing feeding schedules show a growing tendency in Scandinavia towards increased

content of starch per SFU (Scandinavian feeding unit for cattle) to the detriment of sugar content and digestible cell walls, especially at the expense of barley (Enemark et al. 2002). Therefore, the cereal base chosen was barley. Cows were also offered a salt mineral block (KNZ Tradition, KNZ Salt Licks, Hengelo, the Netherlands).

Table 2 – Diet composition

Ingredients	CTRL 21% starch	LOW 28% starch	MED 35% starch	HIGH 42 % starch
Spring barley (Kg DM)	6.70	9.80	12.9	16.0
Rapeseed expeller	1.50	1.20	0.90	0.60
Soybean, decorticated (Kg DM)	2.50	2.70	2.90	3.10
Beet pulp, dried (Kg DM)	9.0	6.0	3.0	0.0
Clover grass silage (OM, 40 Kg DM)	2.0	2.0	2.0	2.0
Maize silage (Kg DM)	4.0	4.0	4.0	4.0
Limestone (kg DM)	0.0	0.07	0.14	0.21
Type 1, granuleret (Kg DM[a])	0.2	0.2	0.2	0.2
STARCH (expected) %	20.9	28.1	35.2	42.3
CONCENTRATE (Kg)	19.9	19.9	20.0	20.1
SILAGE (Kg)	6.0	6.0	6.0	6.0
TOTAL (Kg)	25.9	25.97	26.0	26.1

[a]Premix of minerals and vitamins formulated with calcium, phosphorus, magnesium, potassium, sodium, chloride, sulphur, cation anion balance, iron, manganese, zinc, copper, cobalt, selenium, molybdenum, iodine, vitamin A, vitamin D, β-carotene and vitamin E.

Table 3 covers the analytical composition of the diets determined by chemical analysis. Feed samples were dried (60ºC, 48h) to determine DM concentration. Combustion (525ºC, 6h) was used to determine ash concentration. Crude protein (CP) was calculated as N x 6.25, when N was determined according to the Dumas method (Hansen 1989). Neutral detergent fiber (NDF) was determined as described by Mertens (2002). Starch was determined as described by Kristensen et al. (2007) modifying determination by Salomonsson et al. (1984).

Table 3 – Analytical composition of the experimental diets

	CTRL	LOW	MED	HIGH
Humidity (%)	37.5	38.3	38.6	39.4
DM (%)	62.5	61.7	61.4	60.6
NDF (% DM)	28.1	25.3	22.2	19.5
Ash (% DM)	4.64	4.78	4.65	4.65
CP (% DM)	16.4	16.5	16.4	16.9
Starch (% DM)	20.5	27.1	34.5	41.3

2.1.6. Experimental design: Daily and weekly tasks

Daily tasks were mostly conducted by the barn staff and the MSc student. This included, in the morning, measuring water consumption of the day before, morning milking at 06.30h, removing feed residue, collecting a feed sample and providing daily feed at 08.00h, and afternoon milking at 17.00h. Milk yield was recorded in both daily milking. On sampling days, a milk sample (50 mL) from the two consecutive milking was collected. Animals were weighed every Tuesday, corresponding to eight weight recordings in total. To ensure proper animal welfare, cows were brushed, cannulas washed, and blankets replaced every two days.

2.2. Sampling procedures

Sampling days occurred every period on day 6, 7 and 13. This way, day 6 would represent the cow under control conditions, day 7 would represent the cow after one day of challenge and day 13, a cow after 8 days of alternated challenge, corresponding to the 4th day of challenge feed. Blood, rumen, urine and milk samples were collected at 07.00h, 09.00h, 10.30h, 12.00h, 13.30h and 15.00h. The sampling team consisted in four people, a pair for every three cows. Each pair would always collect from the same three cows in the same order to avoid random errors and make the process as similar as possible every time. Ideally, urine would be collected by two people first, as the cow naturally urinates after standing up, which would be a consequence of people entering the barn. Secondly milk collection should take place, also by two people. Finally, one member of the pair would collect blood samples while the other one collected rumen samples. Besides this, daily milk samples from the two milkings were also analysed. Figure 11 schematizes the samples that were collected and what components were analysed.
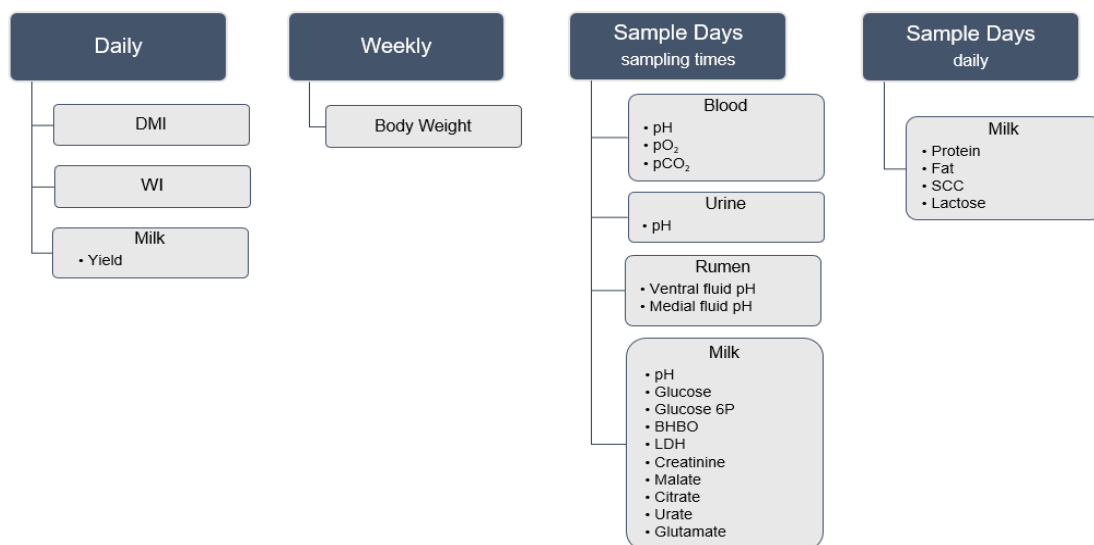
Figure 11 – Samples collected and components analysed

2.2.1. Sample collection

Urine was sampled by stimulating the cow to urinate by hand sweeping the supra-mammary region and collecting into a cup as described in Figure 12. Milk was always collected from the right back quarter, after the first two jets were discarded, into a 50 mL tube (Figure 13). Ruminal fluid was collected at the medial and ventral rumen compartment. Medial rumen fluid was sampled by grabbing the ruminal mat thought the cannula. One large handful was obtained, 10 to 15 cm beneath the mat surface in the midsection of the rumen. The fluid associated to ruminal mat sample was squeezed from the sample through one piece of cheese cloth into 50 mL tubes (Figure 14). Rumen fluid from the ventral sac was sampled using an extended suction strainer (#RT, Bar Diamond, Parma, ID, USA) using a 60 mL syringe. The first 10 to 20 mL collected were discarded and after the syringe was filled, fluid was transferred to a 50 mL tube (Figure 15). Immediately after collection, urine, milk and rumen fluid (both medial and ventral) samples were measured for pH using a pH meter calibrated at pH 4.000 and 7.000 (PHM 240, Hach Lange APS, Brønshøj, Denmark).

Figure 12 – Urine sampling
Urination was induced by stimulation and collected into a cup.

Figure 13 – Milk sampling
Milk was hand collected from the right back quarter into a tube.

Figure 14 – Medial rumen fluid sampling
Rumen sampling was done through the cannula. Ruminal content was grabbed from the midsection, the corresponding handful was squeezed through a cloth and the obtained medial fluid was collected into a tube.

Figure 15 – Ventral rumen fluid sampling
Rumen sampling was done through the cannula. Ventral fluid was collected using an extended suction strainer using a syringe that was later transferred to a tube.

Blood sampling began with catheters being flushed by drawing a minimum of two times the catheter volume into a new syringe and discarding the blood. Blood samples were collected for blood gas and oximetry measurements using heparinized 2 mL gas syringes (PICO50, Radiometer A/S, Copenhagen, Denmark). Samples were immediately placed at 4ºC (crushed ice). Blood samples were also drawn using 20 mL disposable syringes and the blood was immediately transferred to heparin vacuettes (#455051, Greiner BioOne GmbH, Kremsmuenster, Austria) and placed in crushed ice at 4ºC. Arterial blood pH, gases and oximetry variables where measured using an ABL700 Blood Gas Analyzer (Radiometer A/S, Brønshøj, Denmark). Haematocrit was determined right after collection of arterial samples by centrifugation in capillary tubes at 13,000 x g for six minutes at ambient temperature.

Since this trial was a part of a larger study, all the biological fluids were further preserved for future analysis. Figure 16 summarizes all the process that the samples were subjected to, being that only milk and milking samples were relevant for this dissertation are highlighted.
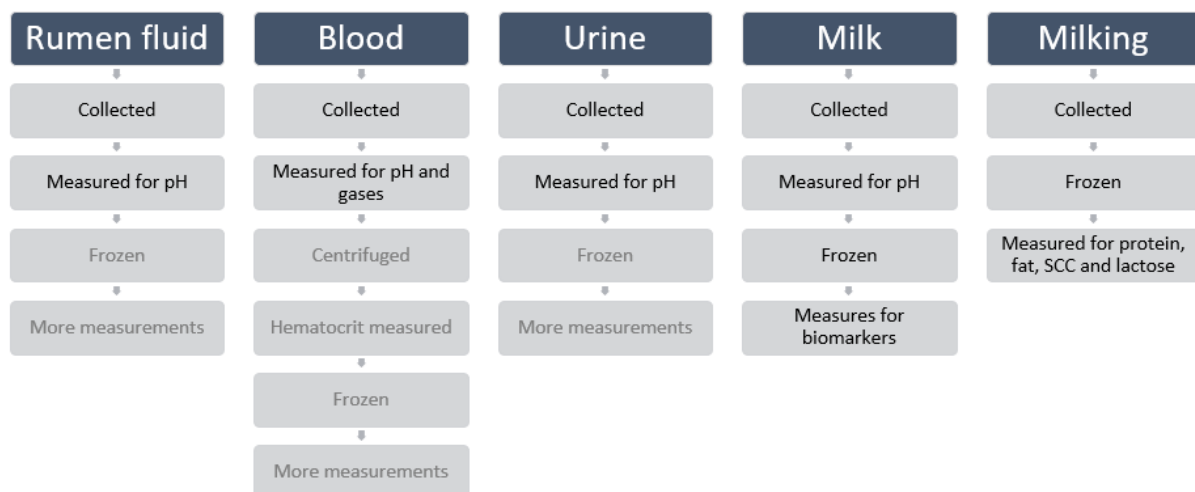
Figure 16 – Sample processing
Several fluids were collected, processed, stored and analysed, but only milk and milking are relevant for this paper. Analysis that were not needed are portrayed in light grey.

2.2.2. Sample Preservation

Milk samples collected from the barn staff at milking were aliquoted into one 10 mL tube to be preserved at -20ºC and one 5 mL tube to be preserved at -80ºC. Milk samples from sampling time were aliquoted in to three 10 mL tubes to be preserved at -20ºC.

2.2.3. Milk Laboratory Analysis

Glucose, glucose 6P, isocitrate, BHB, LDH, glutamate, malate, and urate were determined using an enzymatic-fluorometric method. In the enzymatic procedures, the objective was to obtain equimolar levels of a fluorescent product equivalent to the biomarker

which was being measured. The non-fluorescent resazurin was used as an intermediate and reduced quantitatively by NADPH+H$^+$ or NADH+H$^+$ or ADHP, as shown in tables 4, 5 and 6, respectively. It produces the highly fluorescent substance resorufin. Subsequently, resorufin is read using fluorometry (excitation 544 nm, emission 590 nm) in a fluorometer (FLUOstar/Galaxy, BMG, Germany).

Table 4 – Chemical reactions with NADPH

	Chemical reaction	Reference
Glucose & G6P	D-glucose + ATP → glucose-6-phosphate + ADP	Larsen 2015
	D-glucose-6-phosphate→ D-gluconate-6-phosphate+NADPH+H$^+$	
Isocitrate	Isocitrate + NADP+ → 2-oxoglutarate + NADPH + H$^+$	Larsen 2014
	NADPH + H$^+$ + resazurin→ resorufin + NADP$^+$	

Table 5 – Chemical reactions with NADH

	Chemical reaction	Reference
BHB	BHB + NAD$^+$ → acetoacetate + NADH + H$^+$	Larsen and Nielsen 2005
LDH	L-Lactate + NAD$^+$ → piruvate + NADH + H$^+$	Larsen 2005
Glutamate	L-glutamine + H$_2$O → L-glutamate + NH$^+_4$	Larsen and Fernández
	L-glutamate + NAD$^+$ + H$_2$O → 2 oxo-glutarate + NH$^+_4$ + NADH	2017
Malate	Malate + NAD$^+$ → oxaloacetate + NADH + H$^+$	Shapiro and Silanikove
		2011
	NADH + H$^+$ + resazurin → resorufin + NAD$^+$	

Table 6 – Chemical reaction with ADHP

	Chemical reaction	Reference
Urate	Uric acid + H$_2$O + O$_2$ → 5-hydroxyisourate + H$_2$O$_2$ + ADHP	Larsen and Moyes 2010
	ADHP + resazurin → resorufin + H$_2$O	

Creatinine was determined according to standard procedures (ADVIA 1800, Siemens Diagnostics, Erlangen, Germany) after precipitation of protein and fat with EtOH, as indicated by manufacturer. Milk samples from milking were analysed for fat, protein, lactose and somatic cell count (SCC) by infrared spectrometry using a MilkoScan 4000 (Eurofins Steins A/S, Holstebro, Denmark) as indicated by manufacturer.

2.3. Statistical analysis

All statistical analyses were performed using SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA) based on a linear mixed model with repeated measures (PROC MIXED procedure). The model included treatment (diet 1 vs. diet 2 vs. diet 3), time relative to

morning feeding (from -1h to +7h), and their interaction as independent variables. Cow was set as the repeated subject. Model assumptions were verified based on residual plots and tests for normality. A Tukey-Kramer test was used to evaluate differences between groups. Values were considered significant when $P < 0.05$. Results are presented as means ± SEM. The glucose-6-phosphate (D1 and D4), LDH (D1), glutamate (D1), milk pH (D4), SCC (D1 and D4), pO_2 (D1 and D4) and pCO_2 (D4) were log transformed to obtain the normal distribution of data

.

3. RESULTS

In the first section of the results – Metabolic response to treatment –, data in the tables is presented in the form of a differential to the control (CTRL) value. Therefore, real results are obtained by adding the control value to the challenge (Chlg). In the second section – Production performance –, data is presented with the real average values obtained.

3.1. Metabolic response to treatment

Ruminal pH for medial and ventral fluids are presented in Table 7. No differences were observed in medial fluid pH for any of the treatment experimental groups on challenge D1 and D4 (P>0.05). Control medial fluid pH mean was 5.65 and control ventral fluid pH was 6.17. However, ventral fluid pH was increased in the high treatment, compared to the medium treatment on challenge D1 (P<0.05), but not on challenge D4 (P>0.05), although a tendency is visible (P=0.07), as shown in Figure 17. Ventral fluid pH was the lowest after feeding and subsequently increased time on challenge D1 (P<0.05), but not on challenge D4 (P>0.05), as shown in Figure 18. Medial fluid pH increased significantly after feed and then decreased with time on challenge D4 (P<0.05), but not on challenge D1 (P>0.05), as shown in Figure 19.

Table 7 – Ruminal fluid pH

pH	CTRL	Chlg	LOW	MED	HIGH	SEM	P-value Treatment	Time	Interaction
Medial Fluid	5.65 ± 0.03	D1	0.20	0.09	0.16	0.05	0.46	0.12	0.26
		D4	0.18	0.02	0.02	0.06	0.10	0.002	0.19
Ventral Fluid	6.17 ± 0.03	D1	0.20^{ab}	0.02^{b}	0.28^{a}	0.08	0.01	0.02	0.21
		D4	0.22	-0.14	-0.01	0.14	0.07	0.13	0.26

Average pH values on challenge days (Chlg) from different starch content diets: low starch content (LOW), medium starch content (MED) and high starch content (HIGH); data from these three diets are in the form of differential from the control diet (CTRL). Standard error means is presented (SEM). Values with different superscripts indicate significant differences (P<0.05) for the treatment effect. Time and interaction affects are also presented.
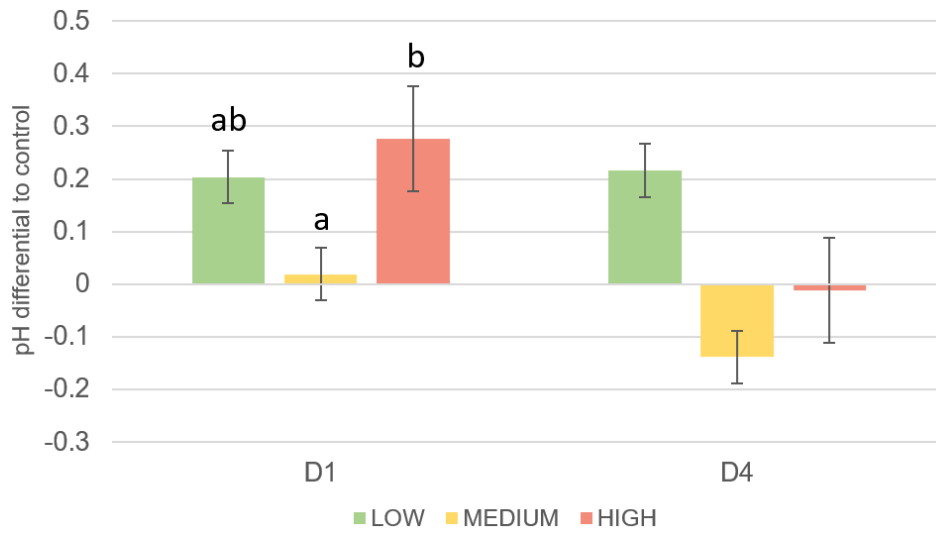
Figure 17– Ventral fluid pH (treatment effect)
Differential ventral fluid pH for the treatment effect in comparison to control values, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).
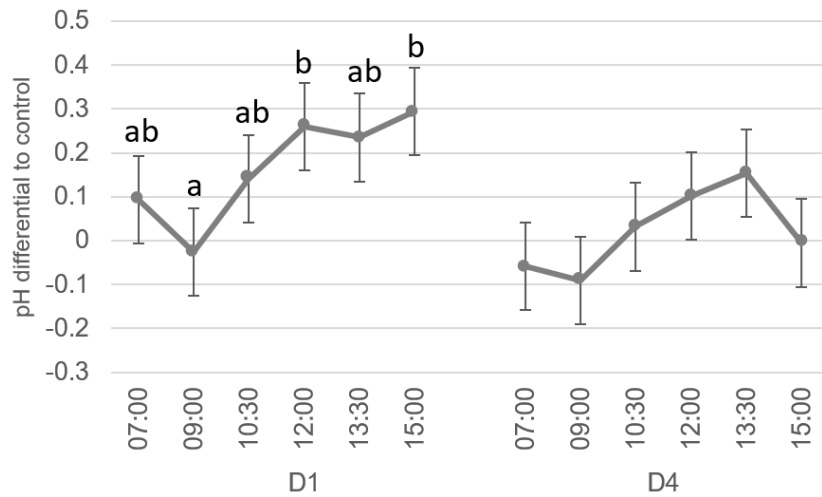
Figure 18 – Ventral fluid pH (time effect)
Differential ventral fluid pH for the time effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).
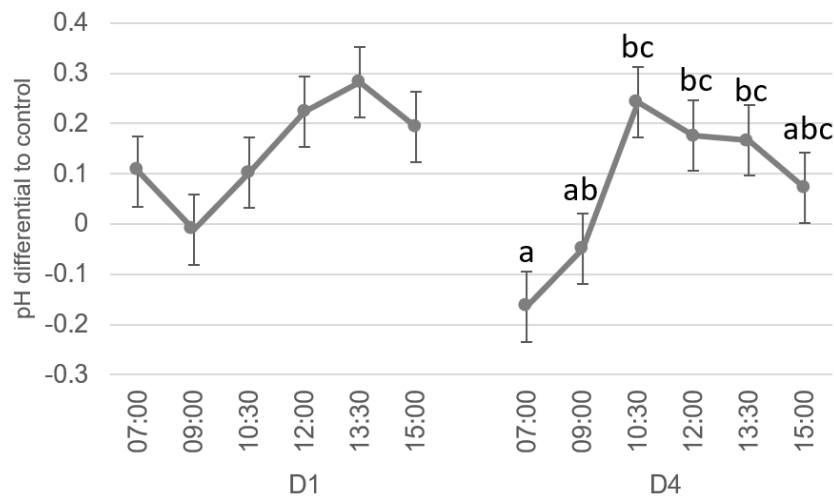
Figure 19 – Medial fluid pH (time effect)
Differential medial fluid pH for the time effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).

Blood parameters are presented in Table 8, as well as urine pH. No differences were observed in partial pressure of oxygen, partial pressure of carbon dioxide nor urine pH for any of the experimental treatments or with time on challenge D1 and D4 (P>0.05). However, blood pH was decreased in the medium and high treatments compared to the low treatment on challenge D4 (P<0.05) but not on challenge D1 (P>0.05). As shown in Figure 20. Time affected the haematocrit (AHTC) on D1, increasing with time, as shown in Figure 21.

Table 8 – Blood pH, gas analysis and urine pH

	CTRL	Chlg	LOW	MED	HIGH	SEM	P-value Treatment	Time	Interaction
Blood pH	7.49 ± 0.003	D1	0.02	0.01	-0.003	0.01	0.22	0.27	0.32
		D4	0.03[a]	0.01[b]	0.01[b]	0.01	0.02	0.17	0.69
pCO_2 (mmHg)	41.4 ± 0.28	D1	-1.29	-0.37	-1.3	0.82	0.52	0.96	0.98
		D4	-1.61	0.23	-0.96	0.80	0.36	0.90	0.47
pO_2 (mmHg)	127 ± 3.05	D1	6.59	6.42	-4.29	5.78	0.29	0.99	0.63
		D4	0.93	-1.39	-5.82	7.50	0.18	0.64	0.71
AHTC (%)	29.1 ± 0.24	D1	-0.37	-0.86	-0.35	0.30	0.43	0.001	0.86
		D4	0.85	0.30	0.03	0.51	0.36	0.45	0.32
Urine pH	8.11 ± 0.02	D1	0.08	0.02	0.04	0.03	0.29	0.99	0.99
		D4	0.02	0.03	0.06	0.03	0.51	0.70	0.90

Average blood parameters and urine pH values on challenge days (Chlg) from different starch content diets: low starch content (LOW), medium starch content (MED) and high starch content (HIGH); data from these three diets are in the form of differential from the control diet (CTRL). Standard error means is presented (SEM). Values with different superscripts indicate significant differences (P<0.05) for the treatment effect. Time and interaction affects are also presented. pO_2 – partial pressure of oxygen; pCO_2 – partial pressure of carbon dioxide; AHTC – haematocrit.

Figure 20 – Blood pH (treatment effect)
Differential blood pH for treatment effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).

Figure 21 – AHTC (time effect)
Differential haematocrit (%) for the time effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).

Milk metabolites and biomarkers are presented on Table 9. No differences were observed in glucose, glucose 6P, BHB, LDH nor glutamate for any of the experimental treatment groups or with time on challenge D1 and D4 (P>0.05). Although isocitrate showed no significant differences affected by treatment, there is a tendency in D4 (P=0.07). Urate concentration in milk, and milk pH were affected by treatment, urate in both on D1 and D4, and milk pH only in D1 (P<0.05). Urate concentration was increased in the medium treatment compared with the low treatment (P<0.05), as shown in Figure 22. Milk pH is decreased in the high treatment compared with the low treatment (P<0.05), as shown in Figure 23. Isocitrate concentration in milk and milk pH were affected by time, on D1 and D4, respectively, although milk pH shows a tendency in D1 (P=0.05). Isocitrate concentration

was its highest at 12.00h and decreased to its lowest at 15.00 (P<0.05), as shown in Figure 24. Milk pH was its lowest before feed, increasing to its highest immediately after feed (P<0.05), as shown in Figure 25. Milk malate, creatinine and isocitrate concentrations differences were observed from the effect of the interaction between treatment and time for (P<0.05) D4, D4 and D1, respectively. Malate concentration was affected by the interaction at 15.00h, being the highest with the high starch treatment and the lowest with the medium starch treatment, as shown in Figure 26. Creatinine concentration was affected at 12.00h, being higher with the high starch treatment and lowest with the low starch treatment, as shown in Figure 27. Isocitrate concentration at 12.00h, being the highest in with the medium starch treatment and the lowest with the low starch treatment, as shown in Figure 28.

Table 9 – Milk metabolites and pH

	CTRL	Chlg	LOW	MED	HIGH	SEM	P-value Treatment	Time	Interaction
Glucose (µM)	51.7 ± 3.16	D1	-0.31	1.81	-2.19	1.24	0.10	0.63	0.52
		D4	5.03	12.4	2.71	5.39	0.41	0.55	0.86
G6P (µM)	187 ± 9.11	D1	-0.95	7.47	31.3	10.8	0.27	0.93	0.80
		D4	-2.05	6.04	15.90	15.9	0.38	0.19	0.72
BHB (µM)	87.5 ± 2.53	D1	-7.13	-5.81	2.56	6.92	0.57	0.48	0.81
		D4	6.94	-0.43	10.30	7.36	0.60	0.43	0.48
Malate (µM)	83.4 ± 2.32	D1	-5.85	2.60	0.48	4.51	0.38	0.44	0.12
		D4	8.81	-4.03	13.2	7.31	0.26	0.86	0.03
LDH (U/l)	5.66 ± 0.30	D1	0.54	-0.54	-1.94	1.33	0.90	0.21	0.58
		D4	-0.13	-1.70	-1.91	1.48	0.65	0.79	0.14
Glutamate (µM)	65.8 ± 5.99	D1	-5.53	7.92	5.34	6.12	0.80	0.35	0.81
		D4	-2.31	10.9	5.83	11.4	0.72	0.94	0.46
Urate (µM)	113 ± 4.42	D1	-2.14[a]	16.6[b]	7.65[ab]	3.79	0.01	0.11	0.33
		D4	-7.28[a]	22.5[b]	3.34[ab]	8.48	0.04	0.47	0.50
Creatinine (µM)	134 ± 1.46	D1	0.30	2.68	5.82	2.01	0.14	0.22	0.12
		D4	0.05	2.37	7.80	5.40	0.44	0.80	0.01
Isocitrate (µM)	128 ± 2.61	D1	-2.6	-2.16	2.72	4.68	0.65	0.03	0.03
		D4	5.28	-20.0	14.9	9.91	0.07	0.20	0.32
pH	6.77 ± 0.01	D1	0.03[a]	0.00[ab]	-0.02[b]	0.01	0.02	0.05	0.93
		D4	0.03	0.01	0.003	0.02	0.41	0.04	0.75

Average metabolite concentrations on milk and milk pH values on challenge days (Chlg) from different starch content diets: low starch content (LOW), medium starch content (MED) and high starch content (HIGH); data from these three diets are in the form of differential from the control diet (CTRL). Standard error means is presented (SEM). Values with different superscripts indicate significant differences (P<0.05) for the treatment effect. Time and interaction affects are also presented. G6P – glucose-6-phosphate; BHB – β-hydroxybutyrate; LDH – lactate dehydrogenase.
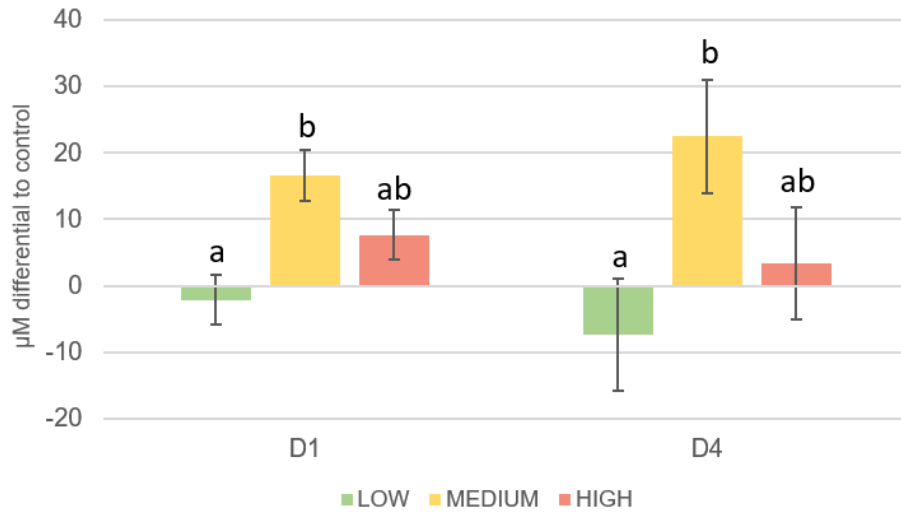
Figure 22 – Milk urate concentration (treatment effect)

Differential milk urate concentration (µM) for the treatment effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).
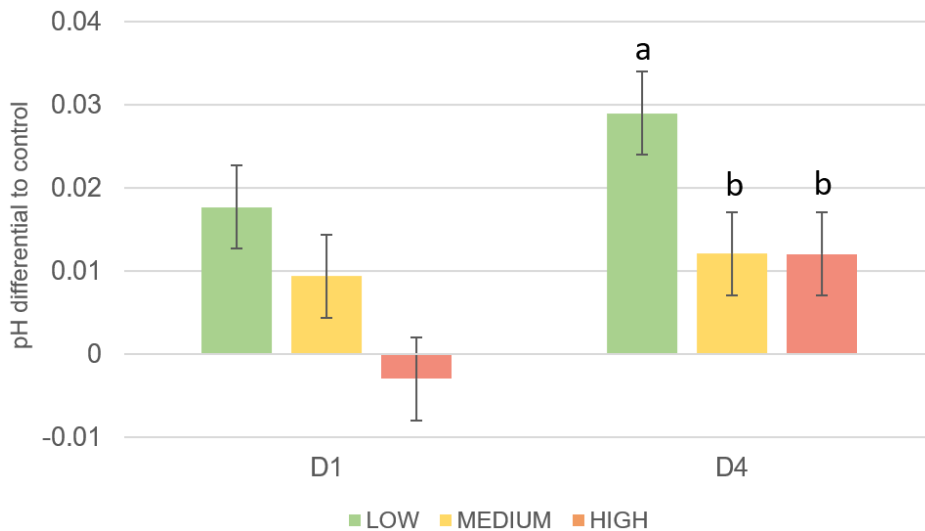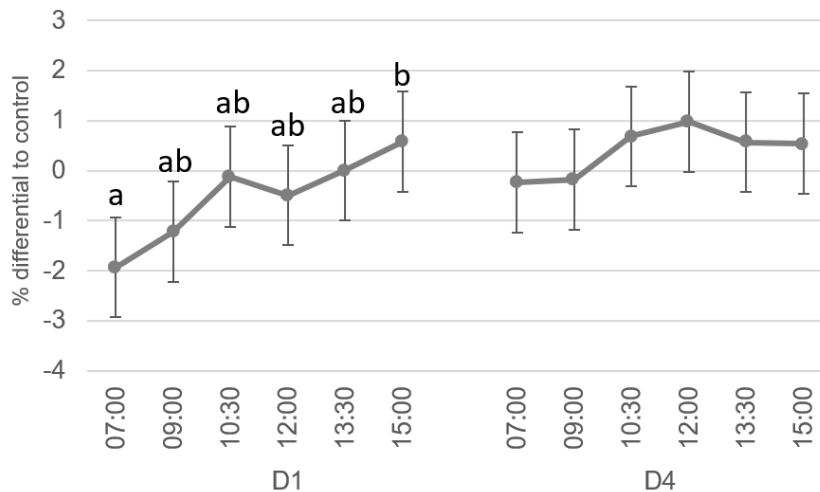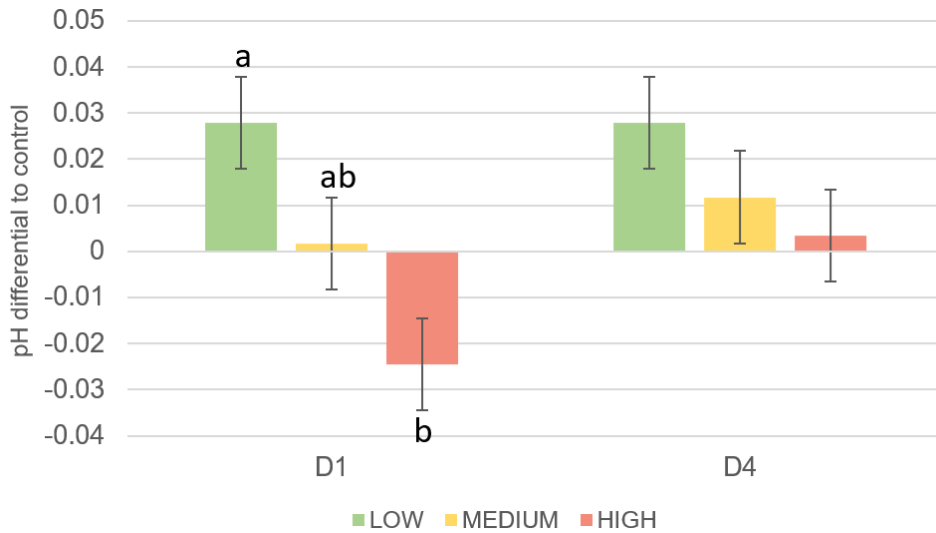

Figure 23 – Milk pH (treatment effect)

Differential milk pH for the treatment effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).
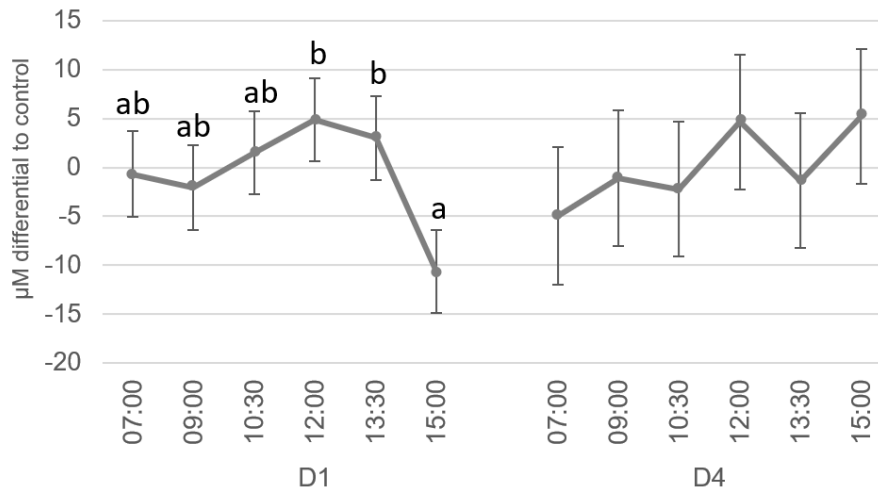
Figure 24 – Milk isocitrate concentration (time effect)

Differential milk isocitrate concentration (µM) for the time effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).
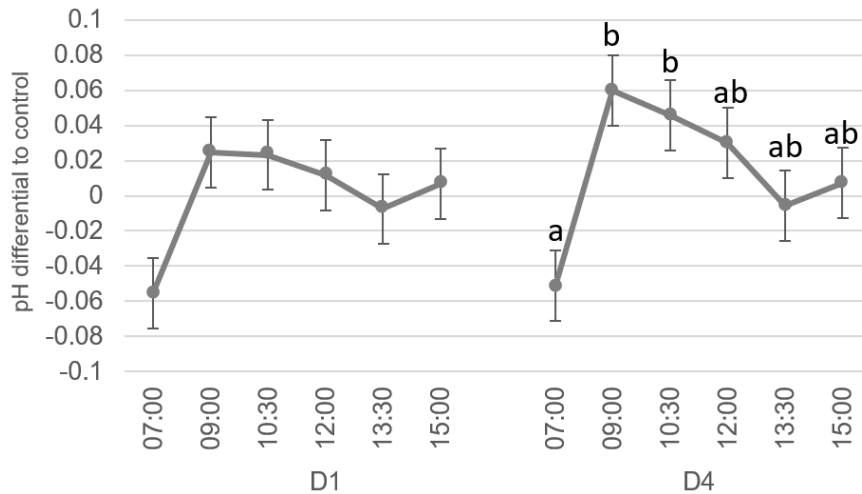
Figure 25 – Milk pH (time effect)

Differential milk pH for the time effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).
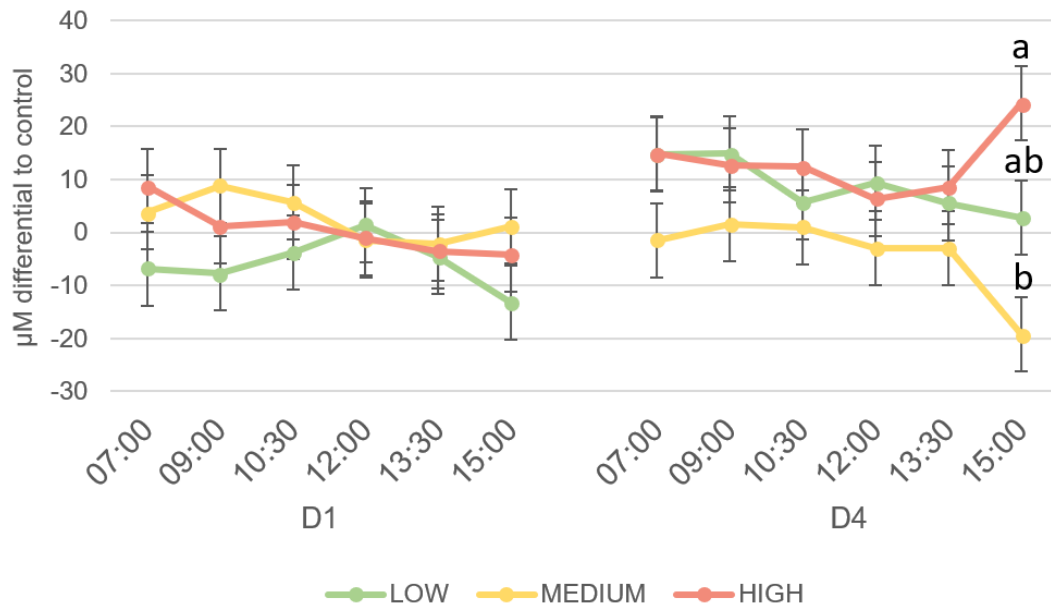
Figure 26 – Milk malate concentration (interaction effect)
Differential milk malate concentration (µM) for the interaction between treatment and time effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).
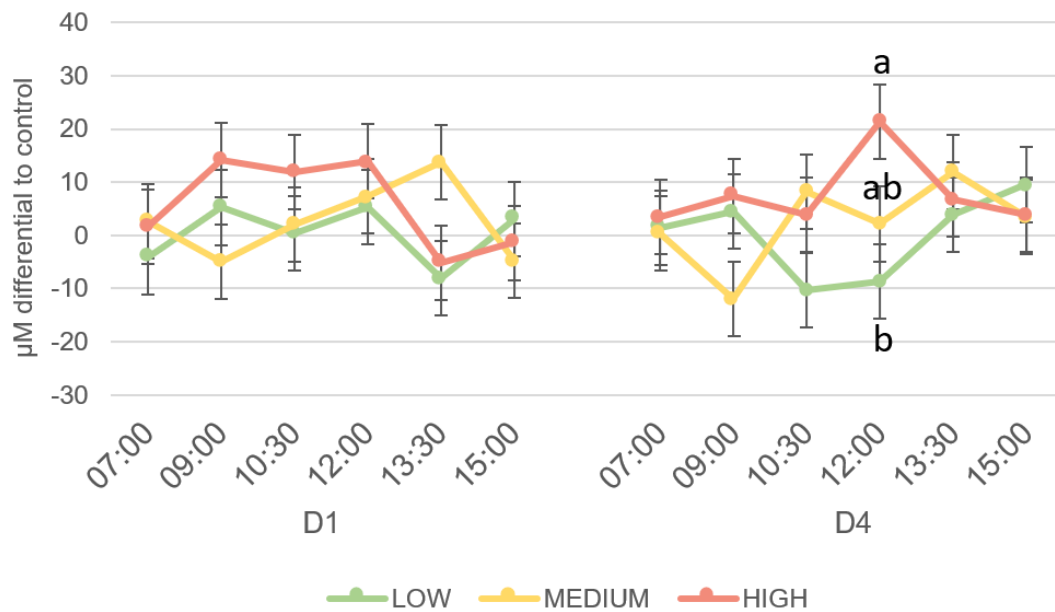
Figure 27 – Milk creatinine concentration (interaction effect)
Differential milk creatinine concentration (µM) for the interaction between treatment and time effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).
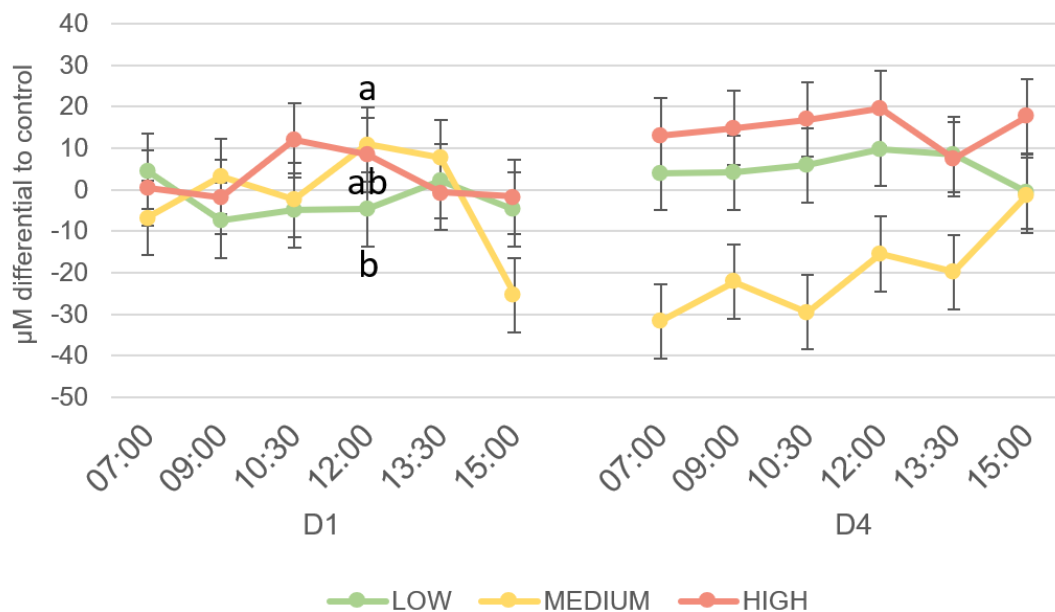
Figure 28 – Milk isocitrate concentration (interaction effect)

Differential milk isocitrate concentration (µM) for the interaction between treatment and time effect in comparison to control value, with standard deviations. Bars with different superscripts indicate significant differences (P<0.05).

3.2. Production performance

Dry matter and water intake records are presented in Table 10. Milk quality analysis results, explicitly yield, protein, fat, SCC and lactose, are described in Table 11. No differences were observed in any of the parameter for any of the experimental groups on challenge D1 and D4 (P>0.05).

Table 10 – Dry matter and water intake

	CTRL	Chlg	LOW	MED	HIGH	SEM	P-value Treatment
DMI (kg)	20.7 ± 0.51	D1	21.1	20.7	19.4	1.36	0.51
		D4	21.8	20.6	21.1	1.11	0.74
WI (L)	95.8 ± 3.17	D1	88.8	85.2	87.3	7.58	0.77
		D4	95.2	87.7	97.7	8.19	0.35

Average values on challenge days (Chlg) from different starch content diets: low starch content (LOW), medium starch content (MED) and high starch content (HIGH). Standard error means is presented (SEM). DMI – dry matter intake; WI – water intake.

Table 11 – Milk quality analysis

	CTRL	Chlg	LOW	MED	HIGH	SEM	P-value Treatment
Yield (kg)	30.2 ± 1.14	D1	31.8	32.5	32	3.45	0.97
		D4	31.8	27.7	32.7	4.27	0.55
Protein (%)	3.62 ± 0.08	D1	3.51	3.52	3.56	0.10	0.94
		D4	3.73	3.55	3.55	0.19	0.63
Fat (%)	3.87 ± 0.14	D1	4.00	3.59	3.44	0.24	0.27
		D4	3.37	4.34	2.82	0.54	0.19
SCC (cells/ml x 10^3)	8690 ± 3163	D1	1712	1607	3522	543	0.06
		D4	855	2350	1021	1379	0.67
Lactose (%)	4.72 ± 0.05	D1	4.83	4.83	4.81	0.12	0.98
		D4	4.98	4.77	5.05	0.19	0.44

Average values on challenge days (Chlg) from different starch content diets: low starch content (LOW), medium starch content (MED) and high starch content (HIGH). Standard error means is presented (SEM). SCC – somatic cell count.

4. DISCUSSION

In order to keep the same rationale of the results section, this discussion is organized in two parts. The first – Metabolic response to different starch inclusion in the diet – concerns acidosis, where the acidification occurs in the rumen and how it affects other parts of the animal's organism. The second part – Production performance – concerns the consequences of acidosis in the cow's organism, particularly from a production perspective.

4.1. Metabolic response to different starch inclusion in the diet

This section describes altered function of the digestive tract, discussing rumen pH, and metabolic state, discussing blood parameter and milk metabolites. These are two of the three parameters needed to evaluate cows potential physiological imbalance (Ingvartsen 2006).

4.1.1. Rumen pH

It is fundamental to start discussing the results by rumen pH, as it is the basis of SARA diagnosis. Indeed, SARA depends on the occurrence of low rumen pH levels, lower than those of a healthy individual, as well as the persistence of various clinical signs and an increased risk of culling (Duffield et al. 2004). As acknowledged before, pH from ventral fluid is considered to be the most accurate parameter (Aschenbach et al. 2011 and Duffield et al. 2004). Contrary to our expectations, different starch inclusion in the treatments did not affect ruminal pH. Indeed, it would be expectable that starch would be cleaved by bacteria into glucose, then pyruvate and finally VFAs and lactate. This would decrease ruminal pH (Hernández et al. 2014) in a direct proportion of starch inclusion in the diet. Other authors that induced SARA concluded an overall rumen pH decline, namely Gozho et al. 2007, Li et al. 2012a, Li et al. 2012. Indeed, in our study, ventral fluid pH values were higher in all challenged cows than in control cows. Additionally, cows from the medium starch treatment group decreased ventral fluid pH at a greater extent than those from the high starch treatment group. According to these results, it seems that the higher amount of starch provided in the high treatment group is not completely digested by the rumen bacteria in the following six hours after morning feeding. Therefore, they are not able to decrease ventral fluid pH the same way as in the cows fed with medium starch treatment. In order to test this hypothesis, longer experimental periods (i.e. 12 hours) could be tested in the future.

As previously discussed, the ability of a cow to cope with a metabolic overload is very dependable on the individual. This could possibly also have influenced the results, explaining why the high starch treatment did not present the lowest ruminal pH.

Typically, in cows fed once a day, ruminal pH is highest just before feeding and declines for approximately 5 to 7 hours, before gradually increasing back again. This pattern

is consistent with our findings. Although mean pH values vary across individuals, such pattern was observed. Diurnal pH ranges can differ in cows fed the same diet due to several factors, for example rate of passage of digesta and VFA from the rumen, efficiency of VFA absorption from the rumen and the effectiveness of buffering from salivation (Duffield et al. 2004; Palmonari et al. 2010). Decreased pH values returning to normal pH values after rumination, can be explained by the gradual decreased acid production and increased VFA absorption, combined with increased saliva production (Palmonari et al. 2010).

As described above, ventral fluid pH was only affected in D1 and medial fluid pH was only affected in D4. Although treatment was not the cause of these significant differences, with time starch levels probably contributed to a more unstable ruminal pH. It seems important to remember that ventral pH is measured the closest to the ruminal wall – where acid absorption occurs. We can therefore assume that the rumen papillae adapted over time to an overall more acid environment, improving the absorption effectiveness. On the other hand, medial ruminal fluid was more stable during D1. This means that the increase starch inclusion in the treatment promote growth of specific bacteria populations in the rumen. As reviewed above, amylolytic bacteria, produces VFA and lactate from starch, which classifies them as "lactate-producers". On the other hand, "lactate-consumers" cannot endure the low ruminal pH caused by these acids and cease activity or even die, leaving more substrate and less competition for the amylolytic bacteria. This consequently leads to the growth of this population and more lactate production (Goad and Nagaraja 1998).

4.1.2. Blood parameters

Blood pH indicates whether the acid state was contained and buffered by the rumen or absorbed into the bloodstream. Metabolic or acute acidosis occurs when blood pH is below 7.35 (Owens et al. 1998). Control blood pH was 7.49 and blood pH was not affected by treatment, therefore, we successfully maintained the cows away from acute metabolic acidosis. Of all the blood parameters measured, only blood pH was affected by the increased starch inclusion in the diet. Additionally, haematocrit was affected by time.

Blood pH was the lowest in cows from the high starch treatment group compared to those from the low starch treatment group in D4, although a tendency was already observed in D1. However, ventral fluid pH was not affected either on D1 or on D4. It seems that the absorptive capacity of acids by the rumen increased throughout the experiment, indicating an adaptation of the rumen microbiome after several challenges.

The reference interval for haematocrit, or packed blood cell volume, is 21-30 % (Douglas and Wardrop 2010), therefore all values obtained in this study were within normal range. This variable was affected in all groups by time on D1, but not on D4. Because this

decrease in not seen in D4, there could have been an adaptation during the course of the challenge.

4.1.3. Milk metabolites

Milk metabolites allow drawing some conclusions on the cow's metabolic state, providing information on any type of physiological imbalance.

Milk urate was affected by treatment, but once again, cows consuming the medium starch treatment were more affected than cows on the high starch diet. Urate is considered a general antioxidant in milk, therefore, increased milk urate concentrations indicates higher oxidative stability (Østdal et al. 2000). This parameter may also be a potential indicator of rumen microbial protein yield (Larsen and Moyes 2010). Based on this fact, it seems that rumen bacteria are able to produce higher amounts of protein in cows from the medium starch treatment group than those from the high starch treatment group. Although starch fermentation products, such as VFA, are not the ones needed for protein production, it has long been proved that the rate of carbohydrate fermentation has influence on ammonia capture in the rumen (Lewis and McDonald 1958). This happens because fermentation products are considered energy-yielding nutrients and contribute to the overall pattern of nutrient use (Oldham 1984). This could explain the high level of urate in the medium starch treatment animals, since it would have potentially been an optimal level.

Milk pH was also affected by treatment. On both D1 and D4 challenges, cows from the high starch treatment group showed the lowest milk pH. However, these differences were more evident on D1 than D4, which supports our previous discussion about the cow´s adaptation through the time of the experiment. Because not many parameters were affected by treatment, it could be speculated that part of the metabolic acid load could have been compensated through milk. Volatile fatty acids are common components in milk. Although long chain fatty acids are synthesised in the mammary gland, most short chain fatty acids are filtered directly from the blood stream (Brown et al. 1962). Therefore, VFA produced in the rumen, have a direct pathway to milk and most likely have been released this way. In future studies this can be confirmed by also analysing VFA and lactate presence in milk during SARA challenge. No other experiments about the inclusion of different starch levels in the diet and its effect on milk pH were found in the literature.

As stated above, time effects are a direct consequence of normal ruminal digestion. Milk isocitrate concentration was mostly affected by time in D1. Because isocitrate is an indicator of the animals energy status (Shapiro and Silanikove 2011), we can assume that D1 digestion required more energy than D4. This could mean that, after successive starch challenges, the rumen might adapt and reduced energy required for digestion.

Malate milk concentration was increased in cows from the high starch treatment group compared to those from the medium starch treatment group only on D4. On the other hand, milk isocitrate concentration was affected only on D1 four hours after the morning feeding, where the medium starch treatment concentration was significantly higher than the low starch treatment. Both malate and isocitrate are active components on the Krebs cycle, reporting the animal´s energy status. Therefore, increased concentrations of these components reflect a higher energy status of the animal as a consequence of the starch supply. However, the reason that explains higher concentrations of isocitrate in milk in medium treatment compared to high treatment, remains unclear.

Milk creatinine concentration was affected on D4 four hours after morning feeding, where the high starch treatment concentration was significantly higher than the low starch treatment. For this metabolite, results are proportional to treatment, meaning that starch content influences this parameter. Higher creatinine values in blood have been related to metabolic stress (Cozzi et al. 2011) and, in this experiment, blood pH was also proportionally related to treatment on D4. Therefore, increased creatinine concentrations in milk can be a direct consequence of reduced blood pH. Further studies will be necessary in order to determine the suitability of milk creatinine as a biomarker for metabolic acidosis in dairy cows.

4.1.4. Parameters not affected by the different starch inclusion in the diet

In this study, some parameters were not affected by either treatment, time or interaction. However, unexpected values such as those we obtained should be discussed, as they could support some of the previous findings.

For variables determined in the blood gas analysis (i.e. pO_2, $pCO2$), although it is not significant, treatment mean to induce acidosis did decrease most average pO_2 and increase most average pCO_2, concurring with previous studies on SARA using indirect diagnosis markers (Morgante et al. 2009; Gianesella et al. 2010; Brscic et al. 2015).

Urine pH in control cows was 8.11 and did not differ significantly from cows fed the experimental treatments. Urine pH was not expected to vary since the main organ for base excretion in lactating dairy cows is not the kidney, but the salivary gland (Humer et al. 2017). Therefore, we suggest monitoring salivary excretion in future studies. Morgante et al. (2009) associates the lack of differences in urine pH with the fact that urine pH is related with the dietary cation-anion balance and because diets have the same bases.

4.2. Production performance

Although none of the parameters related to production performance was affected in this study, some of them were considered relevant for discussion.

4.2.1. Dry matter intake

Although reduced DMI is often associated with SARA, this happens mainly after the dramatic decline in ruminal pH, normally after morning feeding (Humer et al. 2017). When ruminal pH returns to normal values, throughout the course of the day, the cows recover their appetite and therefore DMI increases. Therefore, for future studies, we suggest hourly DMI tracking, since daily values do not provide information of this pause of intake and later regain of normal feeding patterns. Also, different results were obtained in several studies, with some resulting in SARA having no influence in DMI (Krause and Oetzel 2005; Gozho et al. 2007; Khafipour et al. 2009; Li, Gozho, et al. 2012) and others stating that SARA did affect DMI (Kleen et al. 2003; Plaizier et al. 2009; Danscher et al. 2015). This suggests that DMI regulation is complex and can be affected by many dietary, environmental and animal factors not taken into consideration during these studies.

On D1 DMI was 20.7 kg/day for cows in the control group and 21.1 kg/day, 20.7 and 19.4 kg/day for cows receiving the low, medium and high starch diets, respectively. Although differences among groups were not significant, DMI numerically decreased with increased starch content in the treatment, which might support the fact that cows suffering acidosis could potentially experience reduced DMI. This linear scenario is not so evident on D4, in which average DMI was 21.8 kg/day, 20.6 kg/day and 21.1 kg/day in cows from the low, medium and high starch treatment groups, respectively, which suggests, once again, that there was some kind of adaptation from the animals to the highest starch diet. Then again, these differences were not significant.

4.2.2. Milk protein and fat

An interesting point of view to discuss is the milk fat and protein ratios. The inversion of milk fat and milk protein percentages is common in cows with ruminal acidosis (Nocek 1997; Stone 1999; Gozho et al. 2007). Indeed, a healthy cow has a higher milk fat percentage than milk protein percentage. Hence, milk composition and rumen pH suggest that during the control diets, cows also experimented a mild ruminal acidosis, which also happened with Gozho et al. 2007.

Milk fat content depression is commonly associated with SARA (Nocek 1997). Similarity to Gozho et al. 2007, milk composition did not differ between challenge diets and the control diet. Gozho et al. 2007, explained this fact by stating that this might have been due to low pH in the cows receiving control diets. Because there are several studies that report no changes in milk fat content when SARA is induced (Lean et al. 2000; Keunen et al. 2002; Enemark et al. 2004; Gozho et al. 2007) but also several studies that report that SARA does result in milk fat depression (Li, Gozho, et al. 2012; Danscher et al. 2015), it has been

suggested that milk fat in experimentally induced SARA may be related to the duration of low pH (Krause and Oetzel 2005).

5. CONCLUSION AND FUTURE PROSPECTS

This study was successful in inducing a subacute ruminal acidosis in dairy cows. We believe it is possible that not only challenged animals were suffering from SARA, but also during the control period animals were undergoing ruminal pH levels below those of healthy animals. We draw this conclusion because ventral fluid pH did not decline during any of the challenges and because of the inversion of milk fat and milk protein yield in milk. We suggest therefore that in further studies, starch levels should be lower.

In general, animals had some metabolic changes that lead to believe that starch treatment resulted in a certain level of physiological imbalance, mostly on the first testing period. The fact that treatments remained the same throughout time and in the last day of challenge, metabolic changes are not so evident, this leads us to believe that animals have the ability to adapt to the inclusion of high starch levels in their diets. This adaptation occurs mostly in the ruminal microbiome.

The purpose of this study was to evaluate the effect of the sudden inclusion of different starch levels in the diet on milk composition and milk production performance. Although there were overall changes in metabolism related parameters, production performance parameters were not affected. This leads to the conclusion that dairy cows are able to cope with sudden increases of starch in the diet, and that they are able to modulate the negative consequences of a sudden increase in starch content in the diet in successive challenges. Further studies with longer experimental periods will be necessary in order to confirm this hypothesis.

Since SARA has a heavy individual component, in the future it would be interesting to have a large number of animals with radio pH indwelling sensors permanently on the rumen and, if the limit of rumen pH for acidosis is reached, then further analysis should be made.

Although many studies are available on subacute ruminal acidosis, the impact of this condition on milk parameters is not fully described, rendering therefore an additional importance to the conducted study. Indeed, this experiment went further in analysing milk components other than yield, fat, protein and lactose concentration and somatic cell count. We consider that the information provided from metabolites in milk is very valuable because milk is easily accessible on farm, being easy to collect, contrary to cannulas or blood withdrawal. Milk analysing is also fairy uncomplicated and quick, providing there is a laboratory available. For this reason, drawing a range of milk metabolites, would exponentially facilitate dairy farmers ability to detect SARA and act accordingly in a short amount of time therefore reducing the mid and long term negative effects the disorder has on animals and productivity. As such, further studies should be conducted regarding this issue to determine exactly the range references of milk metabolites that are indicative of subacute

ruminal acidosis which could possibly lead to their use on farm for instance in the form of a quick diagnosis kit.

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