Characterization of bovine milk from indigenous species from Sri Lanka

Karakterisering av mjölk från inhemska nötkreatursraser från Sri Lanka

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Swedish University of Agricultural Sciences, SLU
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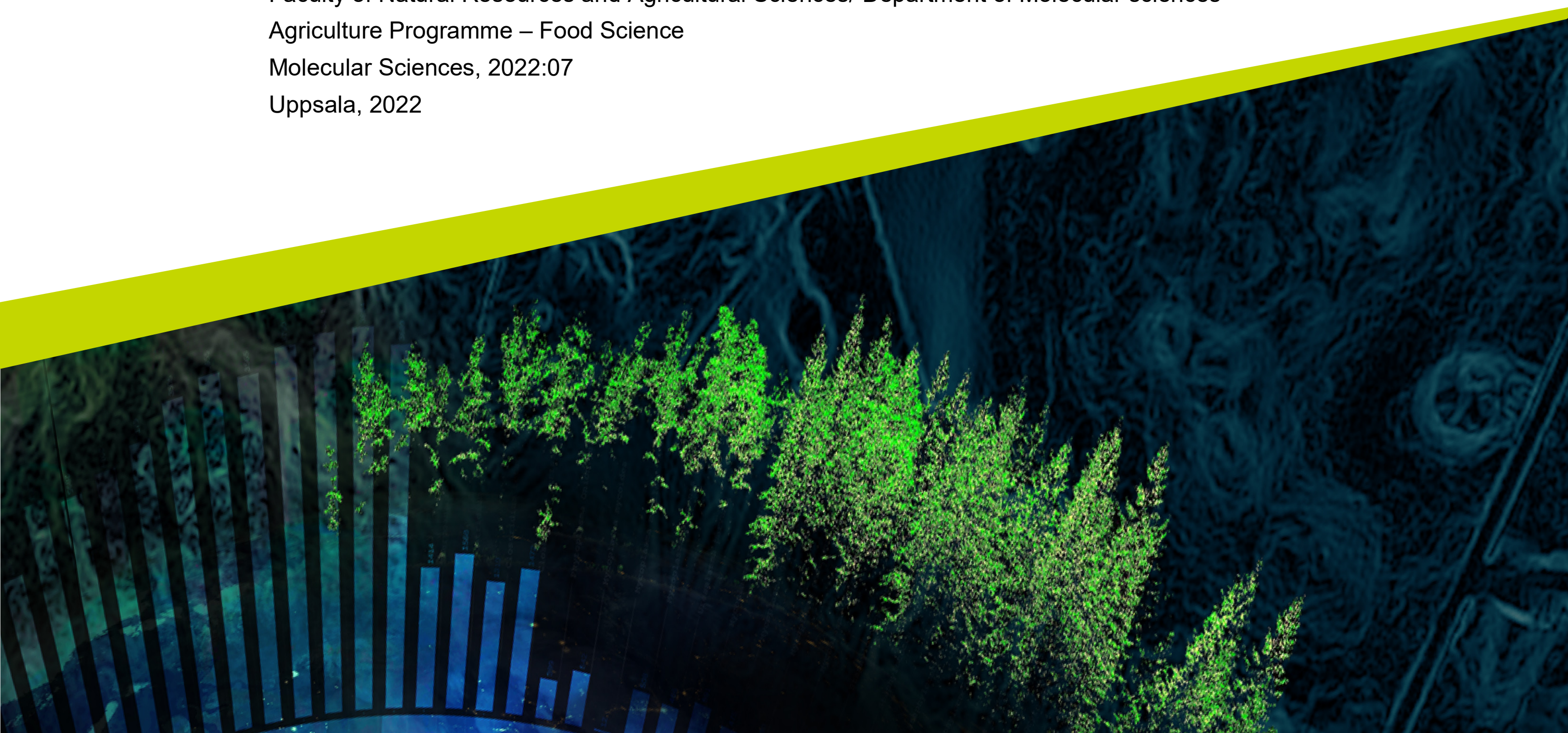

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Jonathan Skager

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Abstract

Sri Lanka is a country in south Asia that from the beginning of the 21st century, until 2016 almost doubled their quantity in milk import, this to meet their national market demand for dairy products. The country is divided into different agroecological zones, which are the up-country, mid-country, and the lowlands. The dry lowland zone is the largest zone of the country, and the environment is severe for cattle holding, especially for European breeds that are used to a more temperate climate. In this zone the indigenous cattle breeds are the most common as they are better adapted to the environment than imported breeds as e.g., Jersey and Friesian. However, the indigenous cattle are known to have a low milk production.

In this study, the objective was to investigate and compare the protein profile and total proteolysis with gross composition, to find potential differences between cattle types. Four different cattle types were investigated: the Thamankaduwa White cattle, Lankan cattle (both indigenous cattle types) and crossbreeds of Jersey cattle and Friesian cattle. A total of 15 milk samples from each cattle type were analyzed by a fluorescence method for total proteolysis and by capillary electrophoresis for identification and quantification of the protein profiles. Each sample represented one individual, and a total of 15 individuals represented each cattle type. The milk samples were collected by a representant from the Rajarata University of Sri Lanka, where the gross composition of the samples was analyzed. Protein profile, total proteolysis and pH-value were measured and analyzed at the Swedish University of Agricultural Sciences, Uppsala.

Total fat, solid non-fat, somatic cell count, pH-value, κ-casein, and total proteolysis differed significantly between the cattle types in this study. Although, for the solid nonfat and somatic cell count the significance was low ($p<0.05$) with a high standard deviation for all cattle types. Thamankaduwa white cattle was the cattle type that differed with the highest significance ($p<0.001$) for the parameters pH-value, κ-casein, and total proteolysis, compared to the other cattle types. However, the significant differences in this study are based on limited number of factors. Also, the low number of individual cows in each group can be considered as a limitation. Therefore, this study can be considered as a contributing part in the work of deeper investigations of the indigenous cattle types in Sri Lanka.

Keywords: Milk, total proteolysis, protein profile, capillary electrophoresis, fluorescence, indigenous cattle types, Jersey cattle, Friesian cattle, Sri Lanka

Sammanfattning

Sri Lanka är ett land i södra Asien som från början av 2000-talet, fram till 2016 nästan fördubblade sin mängd av importerad mjölk, detta för att möta efterfrågan på mejeriprodukter i landet. Landet är uppdelat i olika klimatzoner, dvs. högland, mellanland och lågland. Den torra låglandszonen är den största zonen och miljön är svår för boskapshållning, särskilt för europeiska raser som är vana vid ett mer tempererat klimat. I denna zon är de inhemska boskapstyperna vanligast eftersom de är bättre anpassade till miljön än importerade raser som till exempel Jersey och Fresian. Emellertid är de inhemska boskapstyperna kända för att ha en låg mjölkproduktion.

I denna studie var syftet att undersöka och jämföra proteinprofil och total protolys med bruttosammansättningen i mjölken från de olika boskapstyperna, för att se om det fanns skillnader mellan dem. Fyra olika boskapstyper undersöktes: Thamankaduwa White, Lankan (båda inhemska boskapstyper) och korsningar av Jersey och Fresian, totalt samlades 15 mjölkprover från varje boskapstyp. Mjölkproverna analyserades med en metod baserad på fluorescens för total protolys och med kapillärelektrofores för identifiering och kvantifiering av proteinprofilerna. Varje mjölkprov representerade en individ och totalt 15 individer representerade varje boskapstyp. Mjölkproverna samlades in av en representant från Rajarata universitetet i Sri lanka, där provernas bruttosammansättning analyserades. Proteinprofil, total protolys och pH-värde mättes och analyserades vid Sveriges Lantbruksuniversitet, Uppsala.

Signifikanta skillnader mellan mjölkprover från de olika boskapstyperna observerades för parametrarna total mängd fett, mängd fettfri torrsubstans, antal somatiska celler, pH-värde, κ-kasein och total protolys i denna studie. Signifikansen var låg ($p<0,05$) avseende skillnaderna i fettfri torrsubstans och antal somatiska celler på grund av en hög standardavvikelse i värdena för alla boskapstyper. Thamankaduwa White boskap var den boskapstyp som avvek med högst signifikans ($p<0,001$) för flest parametrar, dvs. pH-värde, κ-kasein och total protolys. De signifikanta skillnaderna i mjölkens sammansättning mellan boskapstyperna baseras dock på ett begränsat antal faktorer. Det låga antalet individuella kor i varje grupp kan också betraktas som en begränsning. Denna studie kan ses som en bidragande del till arbetet med att undersöka och få mer kännedom om de inhemska boskapstyperna i Sri Lanka.

Nyckelord: Mjölk, total protolys, proteinprofil, kapillär elektrofores, fluorescens, inhemska boskaps typer, Jerseyboskap, Fresian boskap, Sri Lanka

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Abbreviations

α_{s2}-CN	alfa$_{s2}$-casein
α_{s1}-CN	alfa$_{s1}$-casein
α-LA	alfa-lactalbumin
β-LG	beta-lactoglobulin
β-CN	beta-casein
κ-CN	kappa-casein
γ-CN	γ-casein
ANOVA	Analysis of variance
AprX	Alkaline metallopeptidase
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
CCP	Colloidal calcium phosphate
CE	Capillary electrophoreses
DAPH	Department of animal production and health (Sri Lanka)
DTT	DL-dithiothreitol
EFSA	European Food Safety Authority
FR	Friesian
JE	Jersey
LAB	Lactic acid bacteria
LC	Lankan Cattle
PCA	Principal component analysis
RB	Run buffer
RT	Room temperature
SB	Sample buffer
SCC	Somatic cell count
SD	Standard deviation
SLU	Swedish University of Agricultural Sciences
SNF	Solids nonfat
TBB	Sodium tetraborate buffer

TCA	Trichloroacetic acid
TP	Total proteolysis
TW	Thamankaduwa White

1. Introduction

Sri Lanka is a country in south Asia and primarily consist of one island which is located in the Indian ocean. The agriculture sector is an important part of Sri Lankas economy, but the livestock sector which mainly consists of dairy cattle, only stand for 8 % of the agricultural sector (Silva et al. 2010).

From the beginning of the 21st century until 2016, the amount of milk imported to Sri Lanka almost doubled to meet the national market demand (Samaraweera et al. 2020). During the years, the government has tried to import European breeds with better milk production efficiency. The selection of the breeds has been based on their milk production properties. However, in Sri Lanka factors e.g. high temperature, humidity, cattle holding systems and feed, can have negative effects on the European breeds production efficiency and health (Silva et al. 2008; Silva et al. 2010; Samaraweera et al. 2020).

Indigenous cattle are a collective name for different endemic types in Sri Lanka. The indigenous cattle are referred to as cattle types instead of cattle breeds, since the knowledge of their genetic differences is limited (Silva et al. 2008). According to the Department of Animal Production and Health in Sri Lanka (DAPH 2021), the total cattle population in Sri Lanka was in 2020 approximately 1.6 million heads. Whereas the total cattle population in 2007 was approximately 1.2 million according to Silva et al. (2008). The knowledge about the proportion of indigenous cattle types in relation to the total cattle population is limited. However, Silva et al. (2008) and Silva et al. (2019) estimated, based on the report from Ibrahim et al. (1999), that about 60 % of the total cattle population consist of diverse indigenous cattle types.

According to Silva et al. (2008), the gap in milk production efficiency between the indigenous cattle and the European breeds is too large to be regulated through genetic selection. In the 1970´s a program of crossbreeding between the indigenous cattle type named Lankan Cattle and the European cattle breeds, Jersey and Friesian was started. The aim of the crossbreeding program was to combine the European cattle’s high milk production and the Lankan cattle´s natural resistance to the severe environmental conditions. Nevertheless, this program was closed as the positive characteristics from the different cattle types disappeared during the crossbreeding (Silva et al. 2008).

According to Silva et al. (2010), the indigenous cattle have an important function for the rural system and livelihood in Sri Lanka. Hence, most of the cattle holding is based on smallholder’s systems in severe environments with high temperatures

and relatively low input of feed. The indigenous cattle have an important function for the smallholders then they are better adapted to these conditions. Even though they have a low efficiency in milk production, they can resist the severe environment and diseases in a better way than the European cattle breeds (ibid).

The conditions for cattle holding in Sri Lanka vary depending on the diverse climate in the different agroecological zones. The country is divided into three different climate zones which are the up-country, mid-country, and the lowlands. The mid-country and lowland zones are also divided into wet, intermediate and dry zones (Kollalpitiya et al. 2012). The up-country zones are at heights between 1200-2000 meters above the mid sea level and get rainfall between 2000-2500 mm per year. Temperatures vary between 10 - 32°C, which makes the up-country zone quite suitable for the European cattle breeds like e.g., Jersey and Friesian (Ibrahim et al. 1999; Kollalpitiya et al. 2012).

The dry lowland zone is the largest zone of the country (Ibrahim et al. 1999), where almost two thirds of the whole cattle population are located. The dry zone gets a rainfall between 1000 -1700 mm per year and have a mid-temperature range between 21-38°C (Silva et al. 2008). Smallholders belong to the most common cattle rearing system in Sri Lanka (ibid.), especially in the dry zones. Here, the indigenous cattle are the most common (Ibrahim et al. 1999)

Since the knowledge related to the indigenous cattle is limited and as they have an important function for the smallholders in a large part of the country (Silva et al. 2008), it is of interest to learn more about these cattle types and the differences between them.

In this study milk samples from Sri Lanka were collected from two different crossbreeds of Jersey and Friesian that originate from Europe. Milk samples were also collected from two indigenous cattle types, Lankan cattle and Thamankaduwa White cattle.

1.1. Objective

The aim of this study was to investigate the protein profile and total proteolysis in milk samples from different cattle types living in smallholder's systems of the dry zone in Sri Lanka. Milk was sampled from two different crossbreeds of Jersey and Friesian and from two indigenous cattle types Thamankaduwa White and Lankan cattle. One of the objectives was also to investigate if there are any significant differences in milk compositional parameters between these cattle types from Sri Lanka. Parameters included in this study were gross composition, somatic cells, pH-value, protein profile and total proteolysis.

2. Background

2.1. Bovine Cattle

Domestication of cattle can be traced back in time to approximately 8 000 years ago in Asia. During all these years, cattle have been domesticated with the purpose of draught power, meat, milk or for religious reasons. The cattle have been divided into cattle breeds, which is a concept that describes different groups of cattle with physical similarities. Cattle breeds have evolved and developed different characteristics depending on the influence of human's demand for different purposes (milk, meat, or draught power). While all cattle primarily originated from the same genus, i.e., *Bos,* cattle breeds later divided into two branches. The genus *Bos* was thus divided in two different species, i.e., *Bos taurus* and *Bos indicus*. From the beginning *Bos indicus* originated from more tropical regions and *Bos taurus* from more temperate regions (Buchanan 2002).

2.1.1. Indigenous cattle types in Sri Lanka

Indigenous cattle contribute to the genetic animal resources in Sri Lanka and are widely discussed in their function in relation to the economy of the agricultural sector. Indigenous cattle are used as draught power or for milk and meat production and are mainly found in the dry zones. The indigenous cattle are small animals, approximately 160 kg in adult age and their milk production efficiency is poor. Hence, the indigenous cattle are often valued as inefficient milk producers in relationship to other dairy cattle breeds. Nevertheless, the indigenous cattle types are better adapted to the Sri Lankan climate and thereby can endure drought and have a better resistance to diseases than the European cattle breeds (Silva et al. 2008).

The milk from the indigenous cattle also has a higher demand in many parts of Sri Lanka, than the milk from the European breeds. The reason for the higher demand of milk from indigenous cattle is due to a higher fat content in the milk, contributing to a firm curd structure and flavor in production of dairy products. Another reason for the high demand of the milk from the indigenous cattle is because the milk is known by tradition to not trigger milk allergy, compared to the milk that produced by European breeds (Silva et al. 2019). There has also been evidence in research that supports this traditional belief. According to Rashidinejad et al. (2017) the genetic milk protein variant β-CN A1 is more common in the milk from European

breeds, whereas in the indigenous cattle, the β-CN A2 variant is more common. Also (Pal et al. 2015) supports the claim of health benefits, further explained in chapter 2.3.2.

One of the indigenous cattle types in Sri Lanka is the *Bos indicus var. ceylonicus,* commonly named Lankan Cattle (LC). It is a breed type that has adapted to the climate during centuries, all the way back to the time when cattle first were introduced in Sri Lanka. Therefore, this cattle type is well adapted to the environment in Sri Lanka (Silva et al. 2019). LC is recognized by its barrel formed body and have a color in a mixture of dark brown and black (DAPH 2010).

Another indigenous breed type is the Thamankaduwa White (TW) cattle. The TW cattle are found in the dry zone of the eastern part of Sri Lanka and differ from the LC (Silva et al. 2008) due to its white color (DAPH 2010). Information about the TW cattle's origin is scarce, but according to Silva et al. (2008), there is a possibility that they are a mixture of ancient LC and Indian white cattle breeds.

2.1.2. European breeds

The Jersey (JE) cattle is an old breed recognized since 1771. These cows originate from the Island of Jersey, which is located at the southern part of the English Channel. The cattle breed is known for its good adaptability to different climates and its good milk production properties. The genetic purity of this breed has been preserved during the years, with the first formation of law regulation established on the island 1776. The law, which prohibited all form of livestock import to the Island, was active until 2008, when the prohibition of livestock import was removed. In contrast, the exportation of Jersey cattle from the island has been ongoing during the century and today the Jersey breed can be found in more than 82 countries. The Jersey cattle is a small breed (average weight 400 kg) and has the highest production of milk in relation to their body weight, of all known breeds (Huson et al. 2020). Nevertheless, the average production of milk in kg/day (19-25), is considered as low compared to other breeds (Buchanan 2002).

The Holstein-Friesian (FR) breed originates from the northern part of the Netherlands from the provinces of north Holland and Friesian. Later, European settlers brought livestock from this place all the way to Holstein in Germany, the place which has also contributed to the name of the breed. Holstein-Friesian cattle are considered as one of the most important dairy cattle breeds in the world, as they have a high average production of milk in kg/day (25-35). Their body weight is between 600-800 kg. Associated to the high milk production, milk from the Holstein-Friesian is characterized by a lower content of fat and protein compared to other breeds, for instance the Jersey breed, which generally has a higher content of fat and protein (Buchanan 2002).

Another difference between the European breeds is the frequency content of β-CN A1 and A2. According to Kamiński et al. (2007) the Friesian breed has a higher frequency of the A1 variant compared to the Jersey breed, that generally has a higher frequency of the A2 variant.

2.2. Principal composition of bovine milk

Bovine milk contains different components which usually appear within the following ranges: water (85.3-88.7%), fat (2.5-5.5%), proteins (2.3-4.4%), carbohydrates (mainly lactose 3.8-5.3%), solids nonfat (7.9-10%) and minerals. Milk is a complex emulsion of these compounds, and their contents vary due to multiple factors. Milk can be divided into different liquid phases which is milk, milk plasma and milk serum. Milk plasma consists of milk without the fat globules and milk serum is milk without fat globules and casein micelles (Walstra et al. 2006).

The carbohydrates in milk mainly consist in the form of lactose, which is a disaccharide consisting of two monosaccharides, galactose and glucose. Lactose cannot be digested without first being hydrolyzed by the enzyme lactase. Lactose has the nutritional function of supplying the offspring with energy, and it contributes to the sweet flavor of milk (Walstra et al. 2006).

2.2.1. Milk proteins

The proteins in milk are usually divided into two large groups: serum (whey) proteins and casein. Approximately 80% of all protein in milk consist of different forms of casein and 20% are whey proteins (Walstra et al. 2006; Nilsson 2017).

Casein

Caseins (CNs) are hydrophobic but have a high net charge due to their phosphate groups. The phosphate groups of caseins interact with the amino acid serine, building up phosphoserine residues, which are ionized at normal milk pH (6.6). Due to this ionization, calcium ions (Ca^{2+}) can bind to the negatively charged residues as well as to the colloidal calcium phosphate clusters, stabilizing the casein micelle. Caseins are heat stable in contrast to the serum proteins, which are easily denatured at elevated temperatures. There are four primary types of caseins, the alpha$_{s1}$-casein (α_{s1}-CN), alpha$_{s2}$-casein (α_{s2}-CN), beta-casein (β-CN) and kappa-casein (κ-CN). In addition, gamma-casein (γ-CN) is sometimes mentioned, although γ-CN is a degradation product of β-CN, containing some of its amino acid residues that are hydrophobic (Walstra et al. 2006).

Serum (whey) proteins

The serum proteins, also referred to as whey proteins, are the proteins in milk that remain soluble in milk serum at the isoelectric point, where caseins precipitate, i.e. pH 4.6 at 20°C (Farrell et al. 2004; Walstra et al. 2006). The serum proteins generally appear as globular proteins and are relatively hydrophobic, and the charge of the molecules are evenly distributed. At high temperatures (70-90°C) and a pH below 6.5 the serum proteins will be denatured. During these conditions, denaturation of the serum proteins leads to irreversible precipitation onto the CN-micelle surface. If the pH instead is 6.7, approximately 30% of the serum proteins aggregate with each other and approximately 70 % precipitates onto the CN-micelle (Walstra et al. 2006).

Bovine milk contains numerous of different serum proteins, for example alpha-lactalbumin (α-LA), beta-lactoglobulin (β-LG), bovine serum albumin (BSA), immunoglobulins, proteose peptones, lactoferrin and enzymes (Walstra et al. 2006). The serum proteins that are of interest in this study include α-LA and β-LG.

2.3. Protein profile in bovine milk

2.3.1. Casein micelles

Since caseins are hydrophobic and not soluble in a water phase, CNs in milk are organized in micelles. Casein micelles have a structure of spherical orbs with a negative net charge. They are rich in calcium phosphate and consist of more water than dry material. Most of the calcium in milk is hidden in the micelle in the form of calcium phosphate nanoclusters, created by interaction between the serine phosphate residues and the colloidal calcium phosphate (CCP). The micellar stability is based on hydrophobic bonds between the proteins, and by cross-links between peptide chains and the calcium phosphate nanoclusters (Walstra et al. 2006). The stabilization by the nanoclusters is possible due to the phosphorylated serine residues. The α_{s1}-CN, α_{s2}-CN, and β-CN are highly phosphorylated, whereas κ-CN is glycosylated (Sadiq et al. 2021). The α_{s1}-CN, α_{s2}-CN, β-CN are known as Ca^{2+} sensitive, since Ca^{2+} may easily bind to the phosphoserine residues. The κ-CN is not sensitive to Ca^{2+} (Walstra et al. 2006; Sadiq et al. 2021).

A final structure of the micelle is not yet determined, but through calculations it is suggested that the α_{s1}-CN, α_{s2}-CN and β-CN are in the core of the micelle. Nevertheless, research have stated that κ-CN is located at the surface of the micelle and creates the "hairy-layer" of the surface. The surface hairs are hydrophilic in contrast to the hydrophobic core, and negatively charged, contributing to its stability to the surroundings, colloidal stability. The negatively charged surface prohibits aggregation of the micelles due to electrostatic repulsion. Enzymatic cleavage of the hairs neutralizes the surface, which can result in aggregation of the micelles. This is an important step in cheese manufacturing (Walstra et al. 2006).

Furthermore, there is a variation in micelle size and the size of the micelle is inversely correlated with the proportion of κ-CN. Hence, when the micelle surface is covered by κ-CN the growth of the micelle ends (Walstra et al. 2006; Lin et al. 2012).

External factors affecting the micellar structure

Storage of milk at low temperatures (at least 4°C in 24 h) affect the micellular structure. During these conditions β-CN partly dissolves and migrate to the milk serum, due to the weakening of the hydrophobic bonds at low temperatures. The other casein variants are also affected, but to a lesser extent. The dissolved form of β-CN is far more susceptible to proteases, such as plasmin at this state. The low

temperature also creates some losses of CCP that partly dissolves. Moreover, the interaction between Ca^{2+} and the α_s-caseins (calcium sensitive caseins) are weakened during these conditions. This results in weaker interactions between the casein molecules in the micelles, with great impact on the technological milk characteristics that are influenced by the caseins in the milk (Walstra et al. 2006).

At freezing conditions (at least -18°C), the casein in milk can aggregate. The aggregation of the caseins can be both reversible and irreversible upon thawing and, depending on the strength of the aggregation. The strength of the aggregation partly depends on how long the milk has been frozen and if the freezing process was fast or slow. Slow freezing increases the risk of irreversible aggregation upon thawing. In addition, freezing and then thawing of whole milk can create partial coalescence of the fat globules, resulting in lumps of fat in the thawed milk (Walstra et al. 2006).

Temperature and pH are two factors that force the structure of micelles to change. Microbial contaminants such as lactic acid bacteria (LAB), can degrade lactose into lactic acid which decreases the pH of the milk (Walstra et al. 2006). During normal conditions in milk the pH-value is approximately 6.6. A decrease in pH results in losses of calcium and phosphate from the micelle. It also affects the micelles electrostatic repulsion since the negative net charge of the κ-CN hairs on the surface will be neutralized. This can result in aggregation of the micelles, which in turn can lead to gelation of the milk (Walstra et al. 2006; Sadiq et al. 2021).

2.3.2. Casein profiles

Different genetic variants of the caseins α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN in milk can be associated with different species (Farrell et al. 2004), e.g. the earlier mentioned *Bos taurus* and *Bos indicus.*

All caseins are considered as phosphoproteins, although the degree of phosphorylation differ widely. α_{s1}-CN and α_{s2}-CN are the most phosphorylated of the caseins and therefore hold more of the phosphoserine clusters (Dalgleish & Corredig 2012; Huppertz et al. 2017; Fang et al. 2018). This compared to β-CN, which is phosphorylated to a degree of 5P and κ-CN that only is phosphorylated to the degree of 1-2P (Goulding et al. 2020). Therefore, it has been suggested that α_{s1}-CN and α_{s2}-CN contribute more to the core stabilization of the micelles (Dalgleish & Corredig 2012; Huppertz et al. 2017; Fang et al. 2018).

Separation of the genetic variants of α_s-casein was first possible after the development of the electrophoresis method, which can separate the α_s-caseins based on their phosphorylation degree (Walstra et al. 2006).

α_{s1}-casein

α_{s1}-CN contributes to approximately 40% of the casein content in bovine milk. There are in total eight genetic variants of α_{s1}-CN (A-H) and the reference variant

for this group is the α_{s1}-CN B 8-P, where B stands for genetic variant and 8-P the degree of phosphorylation, i.e. 8 phosphate groups (Farrell et al. 2004). Nevertheless, the degree of phosphorylation can vary up to nine phosphate groups, depending on the molar amount of α_{s1}-CN and the pH-value of the milk (Walstra et al. 2006; Berry et al. 2020).

According to Walstra et al. (2006) bovine milk generally contains approximately 32 g of α_{s1}-CN per 100 g protein. The α_{s1}-CN B 8-P consists of 199 amino acid residues (Farrell et al. 2004) and the phosphorylated serine groups are located in a cluster between the amino acid residues 43-80 (Berry et al. 2020). This part of the protein is the most hydrophilic, in contrast to the casein's natural hydrophobicity.

α_{s2}-casein

α_{s2}-CN occurs in lower amounts than α_{s1}-CN, constituting approximately 10% of the casein content in bovine milk (Farrell et al. 2004). The α_{s2}-CN can be phosphorylated to different degrees, varying between 10 and 13P (Goulding et al. 2020). The α_{s2}-CN appears in different forms due to their phosphorylation degree and contains intramolecular disulfide bonds. The reference variant for this group is the α_{s2}-CN A-11P, consisting of 207 amino acid residues (Farrell et al. 2004). According to Walstra et al. (2006) a typical bovine milk protein profile contains approximately 8,4 g per 100 g protein.

α_{s2}-CN is known to be the most hydrophilic of the caseins. The C-terminal, consisting of 47 amino acid residues, is hydrophobic and has a relatively strong positive net charge at the normal pH of milk. The N-terminal consists of 68 amino acid residues, is hydrophilic and have a stronger negative charge than the positively charged C-terminal.

The polypeptide chain of α_{s2}-CN can be divided into four different sections due to their hydrophobicity and hydrophilicity. The N- and C-terminals are two of these, and in the middle of the polypeptide chain, there is a hydrophobic and hydrophilic region, once again with two anionic clusters. Due to this structure α_{s2}-CN is more sensitive to Ca^{2+} than α_{s1}-CN (Farrell et al. 2004).

β-casein

According to Walstra et al. (2006) the general protein profile of bovine milk contains approximately 26 g β-CN per 100 g protein. β-CN contribute to approximately 45% of the total content of casein in bovine milk. The reference variant for this group is the β-CN A2-5P, consisting of 209 amino acid residues (Farrell et al. 2004). β-CN is known to be the most hydrophobic casein and has a N-terminal that is negatively charged and a C-terminal that is neutral. The distribution of the charge and the hydrophobicity of the polypeptide chain is unusual. One third of the total charge of the whole protein, is concentrated to a small region of the polypeptide chain at the N-terminal. The N-terminal therefore has a strong negative charge caused by phosphoserine clusters. In contrast, the C-terminal is strongly hydrophobic and approximately 75% of the molecule is hydrophobic with a neutral charge. This results in a molecule structure that has a

very concentrated polarization at the N-terminal (Fox & McSweeney 2003; Farrell et al. 2004). According to Farrell et al. (2004) this unusual structure contributes to the dissociation of the β-CN from the casein micelles at low temperatures.

There is a total of 12 different genetic variants of β-CN and the two most common genetic variants are β-CN A1 and β-CN A2. The difference between these two variants is found at the 67th sequence position of the amino acids, where β-CN A1 has histidine and β-CN A2 has proline. The amino acid proline in the β-CN A2 variant will influence the structure of the protein, resulting in smaller micelle size (Van der Schaaf et al. 2022).

β-CN A1 and A2 have also been evaluated from a health-related perspective (Rashidinejad et al. 2017). When being digested, β-CN A1 releases a compound that will trigger the receptors in the human body causing allergically responses. The activation compound, named beta-casomorphin-7, is a degradation product of A1. The beta-casomorphin-7 is created since histidine in β-CN A1 allows cleavage of the polypeptide-chain at the 67th position. In contrast, this cleavage is inhibited by proline in the A2 variant (Pal et al. 2015). Due to large economic interests from researchers advocating the A2 variant, the European Food Safety Authority (EFSA), evaluated and later rejected the statements in lack of evidence (EFSA (2009). However, research is still in progress on this subject.

Another genetic variant is the β-CN B, which is not as common as A1 and A2. Nevertheless, according to Massella et al. (2017) all three genetic variants have been thoroughly researched during the years, based on their positive influence on the technological properties of milk and health benefits for humans. There is evidence that β-CN B has a positive effect on the cheese-yield in cheese manufacturing. A higher content of β-CN B has a beneficial influence on the total casein content, the micelle size and rennet coagulation properties pf bovine milk. (Massella et al. 2017).

κ-casein

The κ-CN is differentiating from the α_s-caseins, as it lacks the phosphoserine residues and moreover it is the only casein that is glycosylated. During the early work of defining the α_s-caseins, κ-CN was defined as the calcium-insensitive protein (Goulding et al. 2020). The genetic reference variant is κ-CN A 1-P which consists of 169 amino acid residues and the most common variants are A 1-P and B-1P (Farrell et al. 2004). Independently on temperature and Ca^{2+} concentration the κ-CN is soluble in milk and it has intramolecular disulfide bridges (Fox & McSweeney 2003). According to Walstra et al. (2006), a general protein profile of bovine milk contains approximately 9.3 g/ κ-CN per 100 g protein.

2.3.3. Whey protein profiles

α-lactalbumin

According to Walstra et al. (2006), the general protein profile of bovine milk contains approximately 3.7 g α-LA per 100 g protein. The physiological function of α-LA is located in the Golgi apparatus within the epithelial cells of the mammary gland where α-LA has an essential regulation function in the synthesis of lactose, together with the enzyme β-1,4-galactosyltransferase (Farrell et al. 2004). Due to α-LA regulating the function of the synthase enzyme, there is a direct correlation between the content of α-LA and lactose in milk. It consists of 123 amino acid residues and has intramolecular disulfide bonds. In contrast to the CN:s, the whey proteins does not contain phosphate (Goulding et al. 2020).

α-LA is known to have the ability to bind metals, for example calcium and zinc. Its ability to bind calcium has shown to be essential for the formation of its structure (Farrell et al. 2004). Each molecule can bind two Ca^{2+} at the position of four aspartic acid residues. (Goulding et al. 2020). The α-LA molecule contains four disulfide bonds, linked to cysteine residues, stabilizing the structure (Brew, 2003; see (Edwards & Jameson 2014).

Genetic variants of α-LA are the A and B variants. The difference between the variants is at the 10th position of the amino acid residue, which in the A variant consists of a glutamic acid and in the B variant an arginine. In *Bos taurus* cattle only the A variant occur, but in *Bos indicus* both A and B variants are found (Farrell et al. 2004).

β-lactoglobulin

Approximately 50% of the total whey protein is β-LG and it represents around 12% of the total protein content in bovine milk (Farrell et al. 2004). The most common genetic variants are the β-LG A and B, although 12 different genetic variants have been recognized. The genetic reference variant for the entire group is the β-LG B, which consists of 162 amino acid residues. The structure includes two intramolecular disulfide bonds and one free thiol group. This is of importance since a complex between the intramolecular disulphide bindings of β-LG and κ-CN can occur during thermal denaturation. (Goulding et al. 2020).
According to Walstra et al. (2006) the general protein profile in bovine milk contains approximately 9.8 g β-LG per100 g protein.

The biological function of β-LG is not yet totally determined, although it possesses the ability to bind to both hydrophobic and amphiphilic molecules, as e.g. vitamin D (Farrell et al. 2004). However, there are theories stated about β-LG, functioning as a retinol (vitamin A) transport protein (Fox & McSweeney 2003). The theory defines the binding of retinol to its more hydrophobic part, that is associated as a hydrophobic pocket. The idea of the theory is that the pocket works as a protector against oxidation of the retinol, this to be transported to the small intestine for nutrient uptake (Goulding et al. 2020).

2.3.4. Bovine protein profile in general

Little is known about the genetic background of the indigenous cattle in Sri Lanka (Silva et al. 2008; Silva et al. 2010; Silva et al. 2019) However, a study of the coagulation properties of milk from the indigenous cattle, TW and LC, as well as Friesian cattle, was performed by (Abeykoon et al. 2016). In this study the milk protein profiles were determined by the method capillary zone electrophoresis (see Table 1.).

Table 1. Protein profile and gross composition content in percentage of total measured protein, fat and lactose in milk from TW, LC and FR cattle according to the study by Abeykoon et al. (2016).

	Thamankaduwa White	Lankan Cattle	Friesian
α_s-CN	38.82	38.92	38.75
β-CN A1	0.00	0.49	14.96
β- CN A2	26.15	33.96	22.87
β-CN B	3.03	0.00	0.00
κ-CN	19.12	11.22	7.69
α-LA	0.77	1.23	2.96
β-LG	8.59	9.72	10.05
Total protein	3.48	3.47	3.27
Total fat	3.84	3.31	3.08
Total lactose	4.92	4.91	4.59

Abbreviations; TW=Thamankaduwa white cattle, LC= Lankan cattle, FR=Friesian cattle, CN=casein, LA=Lactalbumin, and LG=Lactoglobulin

The study reported that the milk from TW had better coagulations properties, likely explained by the higher content of κ-CN (19.12% of total protein), and the presence of β-CN B. This compared to the milk from LC and FR cattle which had lower content of κ-CN (11.22% and 7.69%, respectively) and 0.00% of β-CN B (see Table 1) (Abeykoon et al. 2016).

In the study of Gustavsson et al. (2014) capillary zone electrophoresis was used for identification and quantification of protein profiles in milk from Swedish Red, Danish Holstein, and Danish Jersey cows. The Holstein showed the following mean value percentages of total protein detected:
α-LA=2.56%, β-LG=10.12%, α_{S1}-CN=29.06%, α_{S2}-CN=6.56%, κ-CN= 5.05% and β-CN=38.54%.

The Jersey showed a protein profile of α-LA=2.14%, β-LG=8.76%, α_{S1}-CN =31.51%, α_{S2}-CN=7.32%, κ-CN= 6.39% and β-CN=36.41%. In this study all proteins of the Holstein and Jersey were significantly different ($p< 0,05$) to each other.

2.4. Factors affecting the composition and quality of bovine milk

As previously mentioned, the composition of milk varies. However, it can also vary within the same milking occasion from one single cow. The size of both fat globules and casein micelles can differ in the milk during the milking process. Therefore, milk is a solution in constant change of its composition. Factors that naturally can have an impact on the composition is genetic variation between breeds and individuals, stage of lactation, mastitis, and feed. When milk has left the udder, it will enter an environment of external factors where microorganisms and oxygen can change the composition. Climate and differences in management systems between farms are also of importance, which can affect the final composition. Therefore, the composition of milk from the same breeds from one region can be very different compared to another region, even when the geographical distance is small. All factors that affects the composition of the milk and how they interacts are not yet determined (Walstra et al. 2006).

There is both genetic variation between breeds, but according to Walstra et al. (2006) the variation between individuals within the same breed, can have an even greater effect on the composition. According to NRCC (1988) the genetic factors have the largest influence on the protein content and composition. In contrast, the feed has almost no influence on the protein composition in the milk. However, the feed has greater impact on the fat content and composition.

Nevertheless, according to NRCC 1988, age of the cows has shown to influence the protein composition and content in the milk. Furthermore, stage of lactation has more impact on the protein content, than the composition. Mastitis is another factor that has a small impact of the total content of protein but affects the protein composition to a larger extent.

The reason for changes in protein composition by increasing age of the cows, is partly due to that the incidence of mastitis increases in cows with age (NRCC 1988; Walstra et al. 2006).

Climate usually has a little influence of the milk composition. However, in more extreme conditions the cattle can suffer from heat stress (Walstra et al. 2006). Heat stress has been shown to influence the protein composition due to a variety of factors as reduction in feed intake and impact on the cow's metabolism. Nevertheless, the knowledge is scarce of the exact mechanisms which impact on the protein composition of the milk in temperate regions (Gorniak et al. 2014; Cowley et al. 2015; Gao et al. 2017). However, according to Cowley et al. (2015) the proportion of α_{s1}-CN and α_{s2}-CN in Holstein-Friesian cattle was affected, with α_{s1}-CN increasing and α_{s2}-CN decreasing during heat stress in their study.

2.4.1. Somatic cell count (SCC) and mastitis

Raw bovine milk always contains somatic cells that are secreted from the blood to the alveoli. A somatic cell count (SCC) below 10^5 cells/ml is considered as a low

cell count, representing milk from a healthy cow. It is an important factor due to its big impact on the milk quality. Which in turn, has a major impact on the milk's technological properties in further processing steps to different food products (Nilsson 2017). Somatic cells are not always of the same type of compound. It can be in form of polymorphonuclear leucocytes (PMN), macrophages and lymphocytes. The different somatic cells types normally appear in milk at amounts of 74%, 18% and 8% respectively (Fox & McSweeney 2003).

Mastitis is an inflammation of the mammary gland. It is typically caused by infection by pathogenic bacteria penetrating the teat canal, entering the mammary gland (N.C. 1977) and affecting both composition and yield of the milk. There are many different pathogenic bacteria species that causes mastitis, for example *Escherichia coli*, *Streptococcus agalactiae* and *Staphylococcus aureus* (Zadoks et al. 2011). The study by Johansson et al. (2013) showed that *Staphylococcus aureus* produced enzymes that lowered the concentration of α_{s1}-CN, by 2.5%, β-CN A1 by 3%, and β-CN A2 by 5%. *S. aureus* also affected κ-CN, which was hydrolyzed to para-κ-CN, resulting in a degradation of 7.4% of k-CN. The degradation of total CN was 21% after 6-hour incubation time compared to the control milk, without *S. aureus*.

During mastitis, the numbers of leukocytes and epithelial cells in milk increase. Thus, measuring these numbers indicates of how severe the mastitis is. Nevertheless, the general number of somatic cells varies between individuals and is highly correlated with the cow's age and lactation stage, which also increases the cell count. According to Walstra et al. (2006) a cell count at 3×10^6 indicates a severe mastitis. Mastitis can decrease the total casein content, lactose content, and fat content as well as the total milk yield. Nonetheless, how the different parameters will decrease in relation to the SCC is hard to predict, as the relations are not linear (Walstra et al. 2006).

Johansson et al. (2017), reported differences in total proteolysis in milk between automated and conventional milking systems. In the automated milking system (SCC 230×10^3 cells/ml) total proteolysis in milk was 73% higher than in the conventional milking system (SCC 182×10^3). In contrast, plasmin activity in the conventional group was 7 % higher than in milk from the automated milking group. A positive correlation between SCC and total proteolysis (TP) was observed in the study of Johansson et al. (2017). At lower SCC, most of the proteolysis in milk seemed to be caused by plasmin, but as SCC increased, other proteases had larger impact on the proteolysis in the milk (Kelly 2006; see (Johansson et al. 2017).

2.4.2. Proteolytic activity in milk

Proteolytic activity in milk occurs due to proteases, which are enzymes that degrade the proteins into smaller peptides or amino acid residues (Walstra et al. 2006). The degradation of the proteins will change their structure, affecting the composition and characteristics of the milk. High proteolytic activity is correlated with a higher degradation of the caseins, which has a negative effect on the production efficiency in the manufacturing of dairy products (Barbano et al. 1991).The proteases origin

from different sources and can be divided into indigenous proteases and exogenous proteases (Fox 2021).

2.4.3. Indigenous proteases

Indigenous proteases originate from different sources and a minimum of 60 different enzymes are known in bovine milk today (Fox 2021). Indigenous proteases can be divided into different systems depending on their origin. One of them is the plasmin/plasminogen system that origins from the bovine blood. The other system includes the proteases that enter the milk via somatic cells (Fox & Kelly 2006).

Plasmin is one of the most studied indigenous proteases according to Ismail & Nielsen (2011). Proteolysis caused by plasmin has a large impact on the quality of milk. It can both have positive, as well as negative effects on the characteristics of the milk quality, depending on what the final product will be. In cheese manufacturing, it can have a positive effect on texture and flavor. Although, in UHT milk with an expected long shelf life, it can have a negative effect, as it can gelatinize the milk during storage (Ismail & Nielsen 2011). Proteolysis in milk leads to losses of casein to the milk serum, which results in a lower cheese yield (Walstra et al. 2006).

The plasmin system consists of plasmin (PL), plasminogen (PG), plasminogen activators (PA) and inhibitors (PI) (Fox & Kelly 2006). These different variants have different functions in the plasmin system. PL is the active form of the enzyme and PG is the inactive form, that can be activated by a PA. As plasmin is a blood-derived enzyme it usually enters the milk in the form of PG through the mammary cells by leakage or increased permeability of the mammary gland tissue (Fox & Kelly 2006; Ismail & Nielsen 2011).

Plasmin activity depends on many factors in the milk and its optimal activity is observed at pH 7.5 and 37°C. Mastitis and late lactation stage are connected to increased activity of plasmin. This as the content of plasmin components that migrates from the blood into the milk increases during these conditions. Plasmin is very heat stable and usually survives the most intense heat processing steps in the dairy industry (Fox & Kelly 2006).

According to Fox & Kelly (2006) plasmin in milk is associated to casein micelles and has a big influence on α_{s1}-CN, α_{s2}-CN and β-CN. It has a high specificity for β-CN, which plasmin is degrading into several variants of γ-CN. Nevertheless, its influence on κ-CN is basically none and plasmin has no impact on the whey proteins α-LA and β-LG (ibid.). It has been shown that the hydrophilic residues of the N-terminal of α_{s2}-CN, including one of the anionic clusters, are exposed to plasmin activity. Cleavage by plasmin has shown to form a various of peptides from this domain (Farrell et al. 2004).

It is not only the plasmin system that contributes to the proteolysis of milk proteins, various proteinases also are released from the somatic cells. Cathepsin D is a

cellular protease (Fox & McSweeney 2003), an indigenous enzyme that enters the milk via the mammary epithelial cells (Graf et al. 2021). A correlation between a high SCC and Cathepsin D activity has been observed, but the exact origin of the enzyme is not known. The Cathepsin D system is similar to the plasmin system, which contains different activators of the enzyme. However, the knowledge of the Cathepsin D system is not studied to the same extent as the plasmin system (Campbell & Drake 2013). Cathepsin D can cleave α_{s1}-CN, β-CN and κ-CN in a similar way as chymosin, but is not in the vicinity of the coagulation properties of chymosin (Fox & Kelly 2006). Cathepsin D is an acid proteinase and therefore the system has an optimum at a pH of 4.0 and therefore is unusual in milk proteolysis (Walstra et al. 2006). In milk, Cathepsin D exists in the milk serum together with the whey proteins (Larsen et al., 1996; see Fox & McSweeney, 2003). Cathepsin D has shown to have little impact on the whey proteins. It cleaves α-LA at two different positions, but there is no known cleavage point of β-LG. Another type of proteinase is elastase, which origins from polymorphonuclear leucocytes (PMN) cells and have been shown to be active on α_{s1}-CN and β-CN (Fox & McSweeney 2003).

2.4.4. Exogenous proteases

In comparison to the indigenous proteases, there are proteases that enter the milk with microbes. Through the passage of the teat canal contamination occurs of lactic acid bacteria (LAB). The amount of LAB in milk is less compared to other microbes that can originate from the skin of the teats and from the environment (Graf et.al 2021). Good hygiene praxis is of importance since the teats normally are contaminated with dung, soil and dust that is rich in microorganisms, which also will increase the somatic cells in the milk (Walstra et al. 2006).

The environment in milk is beneficial for growth of bacteria, as it is rich in nutrients. The most common group of bacteria in cold stored milk are the psychrotrophs and more specifically bacteria from the genus *Pseudomonas* spp. The psychrotrophic bacteria are gram-negative rods that grow at refrigerated temperatures, even though their proteolytic activity has been found to be highest at temperatures between 20-30°C (Walstra et al. 2006).

There are different species of pseudomonads and the most abundant are *P. fluorescens, P. gessardii, P. fragi*, and *P. lundensis*. *Pseudomonas* spp. growth range is at 4 - 42°C and during growth the species produce proteases in form of peptidases. The peptidases are produced during storage at low temperatures and are heat stable. One of the most important peptidases is the alkaline metallopeptidase (AprX), that has been detected within various strains of the *Pseudomonas* spp. (Meng et al. 2017). According to Zhang et al. (2018) AprX protease from *P. fluorescens* hydrolyzes almost all of the κ-CN in ultrahigh temperature (UHT) treated milk.

The AprX protease is also active on the other casein variants (α_{s1}, α_{s2} and β-CN). According to Machado et al. (2017) some studies have shown that the AprX protease hydrolyzes the caseins in the order κ-CN, β-CN, αs_1-CN. According to

Baglinière et al. (2012) their study suggested a different order of hydrolysis, where β-CN was hydrolyzed before κ-CN. It is suggested that the reason for the different order of the AprX hydrolysis may depend on the species and strains that are used in the different studies. The AprX protease therefore can have an impact on all casein variants (α_{s1}-CN, αs2-CN, β-CN, and κ-CN) (Machado et al. 2017).

2.5. Analysis of total proteolysis and protein profile

2.5.1. Detection of total proteolysis by fluorescence

Detection of peptides by using a fluorescence detector, which measures the fluorescence at a certain wavelength, can be used to quantify the number of free peptides in milk. The basis for the analysis is the reaction that occur between the N-terminal of peptides and the reagent fluorescamine, creating fluorescence derivates. The structure of the formed derivates are highly fluorescent and the formed fluorescence is proportional to the concentration of free peptides (Udenfriend et al. 1972; González de Llano & Polo Sánchez 2003). For quantification, a standard curve of fluorescent derivates at known concentrations is produced, to be compared with the results of the derivates of unknown concentrations (Lorenzen & Kennedy 1993). In this study the standard curves were based on L-Leucine and the result from the fluorescence measurement was expressed as total proteolysis in *mM* of L-Leucine equivalents (*leucine eq.*).

Trichloroacetic acid (TCA) is not strong enough to break the peptide bonds in a protein, but strong enough to break the hydrogen bonds of the water molecules that surround the protein in the solution. After addition of TCA the proteins will be denatured, i.e. no longer soluble (Koontz 2014) and can be separated from the smaller peptides in the sample by centrifugation (Johansson 2020).

2.5.2. Capillary electrophoresis

The separation and characterization of the different variants of caseins was first possible when the electrophoresis method was invented (Walstra et al. 2006).
Capillary electrophoresis (CE) is a developed analytical method that originates from the first principle of electrophoresis. There are many different developed methods based on the electrophoresis principle, where the CE is the most automated of the methods. The principle of electrophoresis is based on the separation and migration of charged molecules in an electric field. The system is built up of two different electrodes (positive charged=anode, negatively charged=cathode) with a background electrolyte (run buffer). The separation of the charged molecules occurs depending on the migration velocity (v) which is described by the formula $\boldsymbol{v = mE}$. In this formula m *is* the mobility of the charged molecule, which is dependent on the size, shape and charge of the molecule, and E is the electric field strength (Frazier et al. 2000; Fritsch & Krause 2003).

When the electric field is applied, the solute ions will migrate through the buffer in the small capillary against their opposite charge (either against the cathode or anode). The capillary wall consists of negatively charged silanol groups and when the buffer is added into the capillary, they will attract positively charged molecules from the buffer that will form different layers in the capillary. This will create a mobile phase in the middle of the capillary, with an excess of cations. When the electrical field is connected to the capillary, this will create a flow in the capillary against the cathode, due to the excess of cations. This is called the electroosmotic flow (EOF). A strength of the CE method, compared with other separating methods of proteins e.g., HPLC, is that the EOF pressure is flat in the capillary. This compared to the added pressure with a pump, where the pressure profile is more parabolic within the capillary, due to air droplets of the column wall. This is important as the flat EOF pressure promote an overall higher precision in separation efficiency (Frazier et al. 2000).

The migration time will be different depending on the size, shape and charge of the molecule and the time for migration is recognized by a UV-vis detector. The UV-vis detector can measure absorbance at wavelengths between 190 and 800 nm. The detector information is presented in an electropherogram, which shows the peaks of the different molecules migrating at different time intervals (Frazier et al. 2000).

2.6. Statistical analysis

Principal Component Analysis (PCA)

The principal component analysis (PCA) is a multivariate statistical technique used to explore the variation from a big data set with several variables. The technique compresses all data and only keeps the most important information. This is possible since all variables are converted to new and fewer ones, called principal components. Finally, the most important information can be plotted on the principal components to get a better overview of the data and how variables are associated (Abdi & Williams 2010).

Analysis of variance (ANOVA)

One-way analysis of variance (ANOVA) can be used when a comparison between the mean values of two or more groups are of interest. The method compares the variance of the mean values between the groups (Kim 2014). The one-way ANOVA is usually structured with a null hypothesis that the group means are equal and with the alternative hypothesis that the null hypothesis is false (Weiss & Weiss 2005). Results will indicate if there is a statistically significant difference or not between the groups means of the parameter of interest.

Tukey pairwise comparison

ANOVA-test indicates if there is a significant difference or not between two groups or more. Nevertheless, the test does not identify the groups that differ, it only identifies if there is a statistical difference or not. Therefore, ANOVA is usually

followed by another statistical analysis to find out which groups that are statistically different from each other and which groups that are not. This can be done with a Tukey pairwise comparison test (Abdi & Williams 2010).

Pearson's correlation coefficient

By measuring statistical correlation, the linear relation between two different continuous variables can be determined. The strength of the linear correlation is measured with the correlation coefficient that is determined between the intervals of -1 to + 1. If there is no correlation, the value of the coefficient is zero (0). A negative value indicates a negative correlation, which implies that when one of the variables decreases, the other increases. The closer the coefficient is to ±1, the stronger is the correlation. A positive correlation, (close to +1) implies that when one variable increases, the other also increase. There are different variants of correlations coefficients, where the Pearson's correlation coefficient is one that is commonly used. One drawback with Pearson's coefficient is that the measure reliability is easily disrupted if there are values that do not follow the normal distribution (Mukaka 2012).

Using Pearson´s correlation coefficient, there are no exact defined limit values, which means that a correlation may not always be true. However, there are guidelines and usually a coefficient value of ±0.50-0.70 indicates a moderate correlation, ±0.70-0.90 a high correlation and 0.90-1.00 a very high correlation (Mukaka 2012).

3. Material and methods

3.1. Sample collection and preparation

Sample collection

Milk samples were collected from two different crossbreeds with a purity of approximately 70 % of Jersey (JE) and Friesian (FR). The remaining 30% of their genetic background was unknown, but they were probably mixtures of indigenous cattle types due to uncontrolled breeding. Milk samples were also collected from two indigenous cattle types, Thamankaduwa White (TW) and Lankan cattle (LC). In this study, all cattle are referred to as cattle types.

Milk samples from 15 different individuals were collected from each cattle type. The samples were randomly selected from different smallholders in the dry zone area in Sri Lanka. According to personal communication, there are no big cattle farms in the dry zone, only small holders with a few individuals on each farm (Weerasingha 2021). The sample collection was made by W.V.V.R Weerasingha, Rajarata University of Sri Lanka. A total of 60 milk samples were collected and the samples were from the morning milking and stored at -20°C for two days. Two days after the sampling all milk samples were packed in a styrofoam box with ice and then transported from Sri Lanka by flight transport to the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. After arrival, the samples were stored at -80°C to avoid any degradation of the milk. Factors as e.g., age and genetic background of the individuals in this study were not taken into consideration. All cows in the study were estimated to be in mid-lactation.

Data of the milk samples for gross composition, i.e., total SCC, total fat content, total lactose content, total protein content and total solid non-fat content, were received from the Rajarata University of Sri Lanka and University of Peradeniya in Sri Lanka. At SLU, the pH-value of the milk samples was measured, and the samples were analyzed for total proteolysis and protein profile. All raw data were then statistically compared to see if there were any significant differences between the cattle types based on gross composition, pH-value, protein profile and total proteolysis.

Measurement of pH-value

The raw milk samples were taken from the -80°C freezer and thawed in a 35°C water bath and thoroughly vortexed to solve all lumps that had occurred due to the freezing. When the raw milk samples had solved to a sufficient extent, they were cooled down to room temperature by storage on the bench and then the pH-value was measured for each sample separately by pH-meter (Mettler Toledo, SevenCompact pH, Switzerland).

Defatting of raw milk samples

Before the analyses of total proteolysis and protein profile, the milk samples were defatted. Directly after the measurement of the pH-value, 1.5 ml of a milk sample was pipetted into new Eppendorf tubes and the remaining milk sample was immediately re-frozen. The Eppendorf tubes were centrifuged in 10 000 rpm for 10 minuets at 4°C in a centrifuge (Himac CT 15RE, Hitachi Koki Co., Ltd., Tokyo, Japan). After the centrifugation step, the samples were stored on ice and the surface layer of milk fat was removed and discarded. The defatted milk samples were frozen at -20°C until further.

Milk controls

Arla EKO Lantmjölk, containing 3.8-4.5% fat was used as control. The milk was defatted by centrifugation (Sorvall Super T21, Sorvall Products L.P., Newton, Connecticut, USA), and 20 aliquots of 1 ml were transferred to 1.5 ml Eppendorf tubes and frozen at -20°C for future use. The controls were used in the total proteolysis measurements and in the capillary electrophoreses analysis during the study. The controls were handled and prepared according to the same procedure as the Sri Lankan milk samples in all methods below. One control was used in each measurement or analysis run.

3.2. Preparation for analysis of total proteolysis using fluorescence

Preparation of solutions for total proteolysis

24% w/v Trichloroacetic acid (TCA) solution was prepared by weighing 12 g of (TCA) see (Table 2) which was dissolved in a beaker with 50 ml of MilliQ water.

Fluorescamine solution was prepared by weighing 10 mg of fluorescamine which was dissolved in a beaker with 50 ml of 97 % acetone (Table 2).

0.10 M sodium tetraborate buffer pH 8 (TBB) solution was prepared by weighing 2.00 g of borax (Table 2) which was dissolved in a beaker with approximately 80 ml of MilliQ water. The borax was difficult to dissolve. Therefore, constantly stirring with a magnet and carefully heating was necessary. The solution was then titrated to pH 8 by using 1mM HCL and then filled with MilliQ water to a final volume of 100 ml.

All solutions were stored at RT and covered in aluminum foil to avoid any influence from light.

Table 2. Materials used to prepare the solutions needed in the analysis of total proteolysis in milk samples. All materials were from Sigma-Aldrich (Sigma-Aldrich, USA).

Trichloroacetic acid (TCA): Product number: T6399, CAS no: 76-03-9, Mw:163.4
Fluorescamine: Product number: F 9015, CAS no: 38183-12-9, Mw: 278.26
Sodium borate (borax): Product number: S-9640, CAS no:1303-96-4
Acetone: Merck KGaA (M-58.07 g/mol, CAS no: 67-64-1)
L-Leucine L8000-25 G

Preparation of samples for total proteolysis

The following procedure was carried out to determine total proteolysis (eq. mM leucine) in the milk. Procedures were performed according to Wiking et al. (2002) and modified according to Johansson *et. al* (2017). Directly after the defatting step of the milk samples, 300 µL from each milk sample was taken and transferred by pipetting it to a new 1.5 ml Eppendorf tube, where it was mixed with 300 µL of the TCA-solution. All samples which had been mixed with the TCA-solution were thoroughly vortexed and left on ice for 30 minutes.

After this, the samples were centrifuged at 16 000 rpm for 20 minutes at 4°C (Himac CT 15RE, Hitachi Koki Co., Ltd., Tokyo, Japan). The supernatant from each sample was transferred by carefully pipetting it to a new 1.5 ml Eppendorf tube and then frozen at -20°C where it was stored until the day for the analysis of total proteolysis.

The frozen supernatants were thawed at room temperature and thoroughly vortexed. The supernatant (20 µL) of a sample was pipetted into a new separate 1.5 ml Eppendorf tube and mixed with 600 µL of the TBB. The fluorescamine solution (200 µl) was added as the last compound to the samples that were thoroughly vortexed. The samples (200 µl) were then loaded in triplicate on a 96-microwell plate (Sarstedt, Nümbrecht, Germany) together with a blank consisting of TBB and fluorescamine. The measurement of each microwell plate was performed exactly 30 minutes after addition of the fluorescamine solution.

Fluorescence measurement

Each measurement consisted of three replicates of blank, three replicates of each concentration of the L-Leucine standards, three replicates of the milk control and three replicates of each sample from one cattle breed/type (a total of 15 different samples) for each run. The resulting fluorescence was determined with a fluorometer (POLARstar Omega, BMG LABTECH, Germany) using the program Omega and Omega Data analysis, software version 5.50 R4 and firmware version 1.51. All measurements were performed exactly 30 minutes after addition of the fluorescamine solution to a well plate. After loading of a 96-well microplate into the machine, 20 seconds shaking of the plate followed at a frequency of 300 rpm to avoid any air bubbles. All measurements were done using excitation at 370 nm and emission at 480 nm.

L-Leucine standard curve

The prepared L-Leucine standard curve concentrations were thawed in room temperature and thoroughly vortexed before 20 µL was taken from each concentration and transferred to new separate 1.5 ml Eppendorf tubes. TBB were added to each concentration in a volume of 620 µL and fluorescamine was added at the same time as it was added to the milk samples in a volume of 200 µL.

The L-Leucine standards were prepared from a 0.1 mM Leucine stock solution, and made in the concentrations 0.05, 0.30, 0.50, 0.75- and 1.00-mM L-leucine (Table 3).

The 0.1 mM L-Leucine stock solution was prepared by weighing 26.44 mg of L-Leucine (Sigma-Aldrich, L8000-25 G, USA), which was dissolved in a 2 ml Eppendorf tube with 1mM HCL in a total volume of 2 ml. To prepare the respective standards, smaller volumes from the stock solution (Table 3) were transferred to new separate Falcon tubes and diluted to a final volume of 10 ml in each tube by adding 1 mM HCL. The standards were then transferred from the Falcon tubes by pipetting to 1.5 ml Eppendorf tubes and stored at -20°C.

Table 3. Dilution of the L-Leucine solution to prepare standards for construction of the standard curve used in the analysis of total proteolysis in milk

Concentration	0.10 mM L-leucine stock solution
0.05	5.00 µL
0.30	30.00 µL
0.50	50.00 µL
0.75	75.00 µL
1.00	100.00 µL

3.3. Preparation for capillary electrophoresis (CE) analysis

Preparation of buffers for the CE

Two different solutions were needed to analyze the protein profile, the Sample buffer (SB) and the Run buffer (RB), according to the standard operating procedure for the CE-analysis. The SB and RB contained the solutions in Table 4. The amount of chemicals included in the SB and RB was calculated according to the formula:

$m\ (g) = M\ (g/mol) * c\ (m) * V\ (L)$

Table 4. Material used for the capillary electrophoreses buffers

Run buffer:
6M urea (Mw 60.06)
0.05M Hydroxypropylmethyl cellulose (MHEC)
0.02M Trisodium citrate dehydrate (Mw 294.14)
0.19M Citric acid monohydrate (Mw 210.14)

Sample buffer:
6M urea (Mw 60.06)
0.167M Tris[hydroxymethyl]aminomethane (Triss; Mw 121.14)
0.067M Ethylenediaminetetraacetic acid (EDTA; Mw 372.24)
0.042M 4-Morpholinopropanesulfonic acid (MOPS; Mw 209.26)
0.05M Hydroxypropylmethyl cellulose (MHEC)
0.0017M DL-dithiothreitol (DTT; Mw 154.25)

All material were from Sigma-Aldrich (Sigma-Aldrich, USA), if nothing else is specified.

The 6M urea stock solution was left to dissolve overnight, then filtered through a 0.45µm membrane. The RB was divided into 25 aliquots of 2 ml and stored in -20°C until further use. The SB was divided in 20 aliquots of 10 ml into 15 ml falcon tubes and were stored at -20°C until further use. The RB and SB were thawed and thoroughly vortexed on the day of analysis and then 0.0017M DTT (Table 4.) was added to the SB at the same time as the preparation of the samples occurred. This to disrupt the disulfide bridges of the proteins (Johansson et al. 2017).

Preparation of samples for the CE

For the analyses of milk proteins, the method described by Johansson et al. (2013) was used. In short: The defatted milk samples were thawed at room temperature and thoroughly vortexed. From each sample 200 µL was transferred by pipetting to a new 1.5 ml Eppendorf tube and mixed with 400 µL of SB. All samples were vortexed and left in RT for 1 hour. After that, all samples were defatted a second time by centrifugation in 10 000 rpm for 10 minutes at 4°C in the centrifuge (Himac CT 15RE, Hitachi Koki Co., Ltd., Tokyo, Japan). The small remaining amount of the milk fat was removed from the surface and discarded. All samples were then filtered through a 13mm 0.45µl Captiva Econofilter nylon membrane

(Agilent Technologies, USA) into new 1.5 ml Eppendorf tubes. Each filtered sample was then transferred by pipetting to a conic vial in a volume of 30 μL. All samples were carefully examined to ensure that no air bubbles were present at the bottom of the conical vials before the CE analyses.

Capillary electrophoresis analysis

Each analysis-run consisted of 10 milk samples and 1 milk control. The analysis was performed in the CE instrument (Agilent Technologies 7100, USA). The detector was based on UV-vis absorbance at a wavelength of 214 nm. The software used was Agilent 7100 CE, program version Rev.C01.08(210). The protein profile was determined by comparing the migration time for the different peaks on the electropherograms to the reference migration time according to the standard procedure. By using this method, the following proteins could be identified: α_{s1}-CN, α_{s2}-CN, β-CN B, β- CN A1, β-CN A2, κ-CN, α-LA, and β-LG. Each sample, representing the milk from one individual cow, was analyzed in one replicate.

3.4. Statistical analysis

In this study the PCA is presented as a score and loading plot to visualize the overall variation and patterns of the data. For this SIMCA 16.0.1. software was used.

One-way ANOVA was applied in this study, using Minitab 18.1. A p-value of $p<0.05$ was considered as statistically significant. Each parameter value (gross composition, pH-value, SCC, protein profile and TP) was analyzed against all cattle types for the significance. The null hypothesis was that the cattle types mean values are equal, and the alternative hypothesis was that not all means are equal.

The ANOVA was followed by a Tukey pairwise comparison test. This to identify where the statistical differences between the different groups could be seen. Minitab 18.1. was used and $p<0.05$ was considered as statistically significant.

Pearson´s correlation test was applied using the software Minitab 18.1, to identify if there were correlations between different parameters.

4. Results and discussion

4.1. PCA, gross composition, SCC, pH, protein profile and total proteolysis

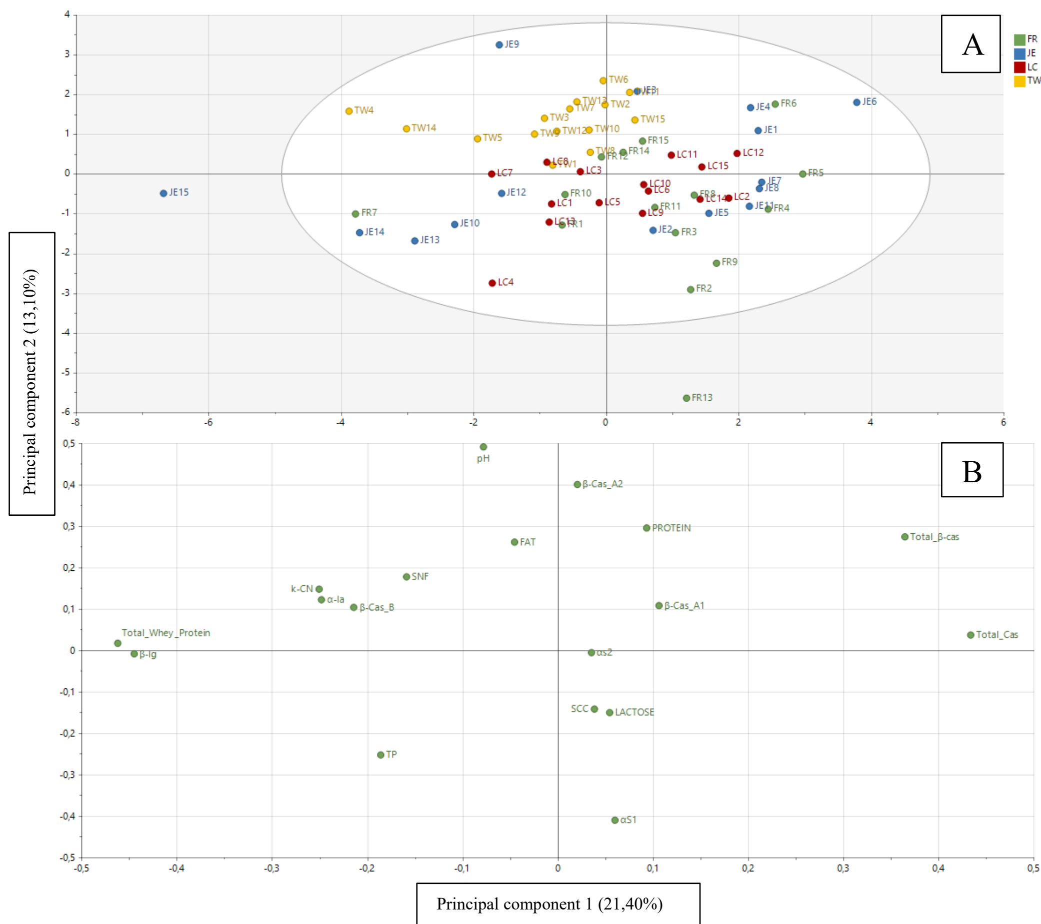

Figure 1. Principal component analysis (PCA) Principal component analysis score plot (A) and loading plot (B) of the overall variation in milk quality attributes used to characterize milk from the 4 cattle types. In the score plot, each dot represents a milk sample, colors indicating cattle type (TW, LC, JE and FR cattle). There were 15 individuals per cattle type and in total 60 milk samples were analyzed.

Abbreviations; TW=Thamankaduwa white cattle, LC= Lankan cattle, FR=Friesian cattle, CN=casein, LA=Lactalbumin, LG=Lactoglobulin, SCC=somatic cell count

The PCA in Figure 1 is presented in a two-dimensional score and loading plot which shows no obvious grouping of, or separation between milk samples based on the investigated parameters. However, TW cattle shows a tendency of grouping in the upper left marked in yellow, although there is no clear separation from the other cattle types. This indicates that there is no clear difference in milk composition from the cattle types based on the analysed parameters. The loading plot shows the different parameters that the data is based on. Groupings of the parameters indicate that they are related, whereas parameters on opposite sides of the principal components tend to be inversely related to each other.

The results in the Tables 5-8 summarize the results of the different analyses of the 60 samples that were collected and sent from the Rajarata University of Sri Lanka and the University of Peradeniya in Sri Lanka to SLU, in Uppsala, Sweden. There were 15 samples of each cattle type and each milk sample represented one individual. All pH-values of the different milk samples were measured directly after the milk samples were thawed and had reached RT and were properly solved. Gross composition and SCC were analyzed at the Rajarata University of Sri Lanka before the milk samples were sent to Department of Molecular Science, SLU, Uppsala, where they were analyzed for total proteolysis and protein profile.

Table 5. Average composition, protein profile and proteolytic activity of milk samples from the Thamankaduwa White cattle type. Standard deviation, minimum and maximum values are indicated. n=15

Parameter	Average	SD	Minimum	Maximum
Gross composition, SCC, and pH-value:				
Total protein %	3.20	0.17	2.95	3.47
Total fat %	4.56	0.35	4.13	4.99
Total lactose %	4.27	0.09	4.13	4.44
Total SNF %	7.89	0.06	7.74	8.02
SCC (10^3/mL)	108.13	196.73	3.00	788.00
pH-value	6.77	0.07	6.61	6.90
Protein Profile as % of total protein:				
α-LA	1.04	0.43	0.40	2.04
β-LG	6.57	1.31	4.99	10.15
αs1-CN	33.36	1.68	30.12	36.35
αs2-CN	5.12	1.40	3.41	8.16
β-CN B	(4.84)	1.15	0.00	7.24
β-CN A1	(21.73)	*	0.00	21.73
β-CN A2	40.54	4.99	24.13	44.79
κ-CN	6.03	1.76	3.64	9.15
Total β-CN	45.22	3.00	40.15	49.18
Total whey protein	7.61	1.28	5.74	11.11
Total CN	89.73	2.06	84.92	92.32
Proteolytic activity (eq. mM L-Leucine):				
Total proteolysis	23.11	5.70	15.20	33.04

The values within parenthesis ( ) are values only based on a few individuals that expressed the specific protein. The Asterisk * shows that no standard deviation can be calculated since the value is based on only one observation. 10 individuals within the cattle type expressed β-CN B and one individual expressed β-CN A1. Abbreviations; SD=standard deviation, CN=casein, LA=Lactalbumin, LG=Lactoglobulin, SCC=somatic cell count, SNF=solid nonfat and TP=total proteolysis

Table 6. Average composition, protein profile and proteolytic activity of milk samples from the Lankan cattle type. Standard deviation, minimum and maximum values are indicated. n=15

Parameter	Average	SD	Minimum	Maximum
Gross composition, SCC and pH-value:				
Total protein %	3.14	0.13	2.92	3.39
Total fat %	4.36	0.18	4.09	4.60
Total lactose %	4.29	0.14	4.09	4.52
Total SNF %	7.80	0.21	7.43	8.26
SCC (10^3/mL)	119.40	204.15	5.00	810.00
pH-value	6.54	0.07	6.40	6.67
Protein Profile as % of total protein:				
α-LA	0.95	0.40	0.30	1.52
β-LG	5.53	0.93	4.13	7.27
αs1-CN	34.02	2.23	31.43	39.53
αs2-CN	5.61	1.59	3.16	8.23
β-CN B	3.93	1.39	2.32	8.30
β-CN A1	(17.71)	5.67	0.00	21.04
β-CN A2	38.86	7.16	24.60	45.54
κ-CN	3.89	1.32	2.11	7.08
Total β-CN	46.33	2.95	38.19	50.06
Total whey protein	6.48	1.15	4.99	8.76
Total CN	89.85	2.26	84.91	92.96
Proteolytic activity (eq. mM L-Leucine):				
Total proteolysis	40.40	11.02	18.14	59.61

The values within parenthesis ( ) are values only based on a few individuals that expressed the specific protein. Three individuals expressed β-CN A1. Abbreviations; SD=standard deviation, CN=casein, LA=Lactalbumin, LG=Lactoglobulin, SCC=somatic cell count, SNF=solid nonfat and TP=total proteolysis

Table 7. Average composition, protein profile and proteolytic activity of milk samples from the Jersey cattle type. Standard deviation, minimum and maximum values are indicated. n=15

Parameter	Average	SD	Minimum	Maximum
Gross composition, SCC and pH-value:				
Total protein %	3.18	0.19	2.98	3.38
Total fat %	4.13	0.45	3.57	4.79
Total lactose %	4.11	0.63	3.68	4.48
Total SNF %	7.37	1.35	6.74	7.74
SCC (10^3/mL)	143.33	294.36	14.00	1176.00
pH-value	6.59	0.12	6.42	6.95
Protein Profile as % of total protein:				
α-LA	0.92	0.41	0.28	1.45
β-LG	6.35	2.61	3.08	12.43
αs1-CN	32.02	3.85	23.74	36.87
αs2-CN	5.44	1.06	3.56	7.05
β-CN B	5.07	1.32	3.47	7.81
β-CN A1	(18.59)	5.83	0.00	24.97
β-CN A2	36.25	8.92	24.15	49.80
κ-CN	3.46	1.80	1.64	6.73
Total β-CN	48.75	5.38	38.39	56.27
Total whey protein	7.26	2.81	3.36	13.88
Total CN	89.68	4.28	82.29	93.99
Proteolytic activity (eq. mM L-Leucine):				
Total proteolysis	38.97	20.08	15.02	91.27

The values within parenthesis ( ) are values only based on a few individuals that expressed the specific protein. Six individuals expressed β-CN A1. Abbreviations; SD=standard deviation, CN=casein, LA=Lactalbumin, LG=Lactoglobulin, SCC=somatic cell count, SNF=solid nonfat and TP=total proteolysis

Table 8. Average composition, protein profile and proteolytic activity of milk samples from the Friesian cattle type. Standard deviation, minimum and maximum values are indicated. n=15

Parameter	Average	SD	Minimum	Maximum
Gross composition, SCC and pH-value:				
Total protein %	3.06	0.12	2.90	3.18
Total fat %	3.15	0.76	2.29	4.11
Total lactose %	4.33	0.31	4.08	4.59
Total SNF %	7.29	0.19	7.13	7.46
Somatic cell count (10^3/mL)	495.93	487.27	15.00	1535.00
pH-value	6.57	0.14	6.32	6.79
Protein Profile as % of total protein:				
α-LA	0.87	0.40	0.31	1.55
β-LG	5.15	1.82	1.98	10.03
αs1-CN	33.63	3.84	28.96	45.11
αs2-CN	5.68	1.67	3.10	9.18
β-CN B	(4.43)	0.86	0.00	5.60
β-CN A1	(20.87)	2.44	0.00	24.51
β-CN A2	37.18	9.82	18.56	50.85
κ-CN	4.88	1.32	2.99	8.95
Total β-CN	46.58	3.92	38.54	52.93
Total whey protein	6.02	2.00	2.76	11.52
Total CN	90.78	2.56	84.86	94.10
Proteolytic activity (eq. mM leucine):				
Total proteolysis	29.37	6.16	16.49	40.12

The values within parenthesis ( ) are values only based on a few individuals that expressed the specific protein. 13 individuals within the cattle type expressed β-CN B and 4 individuals expressed β-CN A1. Abbreviations; SD=standard deviation, CN=casein, LA=Lactalbumin, LG=Lactoglobulin, SCC=somatic cell count, SNF=solid nonfat, TP=total proteolysis

SCC was in the same range for all cattle, except for the FR cattle in (Table 8). The FR cattle had an average value of SCC that was 71.10% higher than JE cattle, 75.92% higher than LC and 78.20% higher than TW cattle. The TW cattle had the highest average pH-value (Table 5.) between the different cattle types (Table 5-8.) The average pH-value for TW cattle was 3.40% higher than LC, 2.66% higher than JE cattle and 2.95% higher than FR cattle. The average total fat content was highest in the TW cattle (4.56%) which was 4,40% higher than LC, 9.40% higher than JE cattle and 30.92% higher than FR cattle.

The cattle types were significantly ($p<0.05$) different based on the parameter SCC. From the gross composition the total fat ($p<0.000$) and total SNF ($p<0.05$) were significantly different between the cattle types (Table 9). The pH-value was also significantly different ($p<0.000$) between the cattle types (Table 9).

According to the Tukey test the FR cattle was significantly different from the other cattle types (Table 10.) According to the parameter total fat in Table 11., the TW cattle, LC and JE cattle were significantly different from the FR cattle. However, the total SNF content that was significantly different according to the ANOVA-test, showed no significant difference between the cattle types in the Tukey-test (Table 10.). The pH-value was significantly different for the TW cattle, compared to the other cattle types (Table 10.).

Table 9. Effect of cattle type on the investigated milk quality parameters. Statistical significance of effects indicated by p-value, p<0.05 considered as significant. n=60 (n=15 for each cattle type)

Parameters significantly affected by cattle type	P-value	Not significant parameters	P-value
Total fat	0.000***	Total protein	0.086
SNF	0.043*	Total lactose	0.346
SCC	0.003**	α-LA	0.705
pH	0.000***	β-LG	0.106
κ-CN	0.000***	αs1-CN	0.315
TP	0.001***	αs2-CN	0.723
		β-CN B	0.075
		β-CN A1	0.792
		β-CN A2	0.472
		Total β-CN	0.108
		Total whey protein	0.107
		Total CN	0.706

* = P<0.05 ** = P<0.01*** = P<0.001
Abbreviations; CN=casein, LA=Lactalbumin, LG=Lactoglobulin, SCC=somatic cell count and SNF=solid nonfat, TP=total proteolysis

Table 10. Results were further analyzed with the Tukey comparison test to identify significant differences in the analysed milk quality parameters between the cattle types. Mean values ± SD are indicated. (n=15 for each cattle type).

Parameter	TW	LC	Jersey	Friesian
Total protein %	3.20 ± 0.17	3.14 ± 0.13	3.18 ± 0.19	3.06 ± 0.12
Total lactose %	4.27 ± 0.09	4.29 ± 0.14	4.11 ± 0.64	4.33 ± 0.31
Total fat %	4.56 ± 0.35 [a]	4.36 ± 0.18 [a]	4.13 ± 0.46 [a]	3.15 ± 0.76 [b]
Total SNF %	7.89 ± 0.06	7.79 ± 0.21	7.37 ± 1.35	7.29 ± 0.19
SCC (10^3/mL)	108.10 ± 196.70 [a]	119.40 ± 204.10 [a]	143.30 ± 294.40 [a]	496.00 ± 487.00 [b]
pH-value	6.77 ± 0.07 [a]	6.54 ± 0.07 [b]	6.59 ± 0.12 [b]	6.57 ± 0.14 [b]
α-LA %	1.04 ± 0.43	0.95 ± 0.40	0.92 ± 0.41	0.87 ± 0.40
β-LG %	6.57 ± 1.31	5.53 ± 0.93	6.35 ± 2.61	5.15 ± 1.82
α_{s1}-CN %	33.36 ± 1.68	34.02 ± 2.23	32.02 ± 3.85	33.63 ± 3.84
α_{s2}-CN %	5.12 ± 1.40	5.61 ± 1.59	5.44 ± 1.06	5.68 ± 1.67
β-CN B %	4.84 ± 1.15	3.93 ± 1.39	5.07 ± 1.32	4.43 ± 0.86
β-CN A1 %	21.73 ± *	17.71 ± 5.67	18.59 ± 5.83	20.87 ± 2.44
β-CN A2 %	40.54 ± 4.99	38.86 ± 7.16	36.25 ± 8.92	37.18 ± 9.82
κ-CN %	6.03 ± 1.76 [a]	3.89 ± 1.32 [b]	3.46 ± 1.80 [b]	4.88 ± 1.32
Total β-CN %	45.22 ± 3.00	46.33 ± 2.95	48.75 ± 5.38	46.58 ± 3.92
Total whey protein %	7.61 ± 1.28	6.48 ± 1.15	7.26 ± 2.81	6.02 ± 2.00
Total CN %	89.73 ± 2.07	89.85 ± 2.26	89.68 ± 4.28	90.78 ± 2.56
TP *(eq. mM leucine)*	23.11 ± 5.70 [a]	40.40 ± 11.02 [b]	38.97 ± 20.08 [b]	29.37 ± 6.16

Mean values that do not share a superscript are significantly different to each other. Mean values without any letter are not significantly different to each other. The Asterisk * shows that no standard deviation is indicated due to only one observation. Abbreviations; CN=casein, LA=Lactalbumin, LG=Lactoglobulin, SCC=somatic cell count and SNF=solid nonfat, TP=total proteolysis

4.1.1. Protein profile

Results from the CE analysis of the protein profile showed no significant differences between the cattle types, except on the protein κ-CN (Table 10). The content of κ-CN in TW cattle was 35.50% higher than in LC, 42.62% higher than

in JE cattle and 19.07% higher than in FR cattle. According to Table 9, there was a significant difference ($p<0.000$) in the parameter κ-CN between the cattle types. The Tukey-test showed that the TW cattle was significantly different to the LC and JE cattle in κ-CN content. Nevertheless, there was no significant differences between FR cattle and the other cattle types according to the Tukey-test (Table 10.).

The relative concentration of β-CN A2 varied in a range 36.25 – 40.54 % of total milk protein between the different cattle types. Milk from the indigenous cattle had the highest numerical percentage of β-CN A2, but there was no significant difference between the cattle types based on this parameter (Table 8). β-CN A1 varied in a range between 17.71 – 21.73 % of total milk protein between the different cattle types. However, there were very few individuals that produced type A1. TW only had one individual that produced the A1 variant, LC had three individuals, JE cattle had six individuals and FR cattle had four individuals. There were no significant differences between the cattle types based on the parameter β-CN A1. β-CN B was also less common in the milk, with 10 individuals in TW cattle and 13 individuals in FR cattle that produced variant B.

4.1.2. Total proteolysis

TW cattle was the group with the lowest average value of L-Leucine eq. mM, indicating the lowest total proteolysis (TP). The average value of TP for TW cattle was 42.80% lower than LC, 40.70% lower than JE cattle and 21.31% lower than FR cattle. TP results were significantly different between the cattle types according to the ANOVA-test. The Tukey-test showed that TW cattle were significantly different ($p<0.01$) compared to the LC and JE cattle with respect to TP. The LC and JE cattle were not significantly different from each other, and FR cattle was not significantly different from any of other cattle types with respect to TP.

4.2. Discussion

Gross composition and pH

According to Buchanan (2002) the Jersey breed is known to have a higher content of fat and protein than the Friesian. The results of this study were consistent with expectations, since Friesian cattle had the lowest content of total fat, protein and SNF. However, the lower values within these parameters were not significantly different to the other cattle types, except for total fat. The Jersey cattle had 23.73% higher fat content, 3.77% higher protein content and 1.09% higher SNF content compared to the Friesian breed. The fat and protein content of the Jersey and Friesian were within the general ranges according to Walstra et al. (2006). However, the SNF content for the Jersey and Friesian cattle types were lower than the general ranges according to Walstra et al. (2006). This raises the question if the SNF parameter is affected by the unusual genetic crosses of Jersey and Friesian with the indigenous cattle. In this study the crosses were estimated to be 70/30 respectively according to Weerasingha (2021).

The indigenous cattle were 30.92% (TW) and 27.75% (LC) higher in total fat than the Friesian cattle. The ANOVA-test (Table 9) indicated that there were significant differences in fat content between the investigated cattle types. The Tukey- test (Table10) showing a significantly lower fat content in milk from FR cattle. Total fat content in milk from FR was significantly different from the milk fat content of the other cattle types.

Lactose was within the normal range for all cattle types according to Walstra et al. (2006). There were no significant differences for this parameter according to (Table 9.) and/or between the groups (Table 10.) based on the parameter lactose.

The pH-value was a parameter which differed significantly between the cattle types, with TW cattle showing the highest pH of 6.77. However, according to Walstra et al. (2006) normal pH of milk is approximately 6.6. Therefore, it can be assumed that pH should be considered as relatively normal for milk for all cattle types. Differences in bacterial load in milk could be a reason for the lower pH in milk from the other cattle types. However, in this study the bacteria content was for practical reasons unfortunately not taken in consideration. It is interesting that TP was lower in the milk from TW cattle compared to the other cattle type, suggesting that the low pH could be related to high TP caused by microbial activities. However, in present study, no such correlation could be seen (see Appendix 1). According to Walstra et al. (2006), LAB can be responsible for a decreasing pH-value due to the degradation of lactose to lactic acid. Nevertheless, the amount of lactose between the cattle types showed no indication of differences (Table 5-8.).

Protein profile

There are many factors that can affect the protein profile of milk as e.g., age, climate, lactation stage, management systems, feed, genetics , hygiene praxis on the farm (Walstra et al. 2006) and heat stress (Gorniak et al. 2014; Cowley et al. 2015; Gao et al. 2017). In this study many of these factors were not considered, because of lack of information. Which makes it difficult to know which factors that could have influenced the results and in what way.

The proportion between the casein and whey protein in this study was 92:8 for the TW, 93:7 for LC, 92:8 for JE and 94:6 for the FR respectively. This differs from the general proportion of 80:20 respectively (Walstra et al. 2006; Nilsson 2017). According to the study of Abeykoon et al. (2016) the proportion of casein and whey protein in TW was 87:13, LC was 89:11 and FR 87:13. In the study of (Abeykoon et al. 2016), the protein profile was determined with capillary zone electrophoresis. As not all milk proteins are detected by this method the percentage is shifted. The percent proportion of the proteins in this study is based on the detected proteins, considered to be 100% in total.

Another reason to the relatively low whey proteins could be the human factor. The concentration of DTT that was added to the sample's during preparation, was lower than according to the standard procedure. According to Fox & McSweeney (2003), Farrell et al. (2004) and Goulding et al. (2020) α-LA and β-LG have intramolecular

disulfide bridges. The DTT is added to break the disulfide bridges of the whey proteins (Johansson et al. 2017). The lower content of these proteins in the result (Table 5-8), could be a reason of insufficient breakage of intramolecular disulfide bridges. This could have interfered and affected the results of the capillary electrophoresis negatively. According to Goulding et al. (2020) the synthesis of lactose is directly correlated with the presence of α-LA. Since the lactose content is within normal ranges (Walstra et al. 2006) this indicates that the results of α-LA in Table 5-8. probably is lower than the actual content in the milk.

According to the result, κ-CN was the only protein that was significantly different between the cattle types (Table 9. and Table 10.). Milk from TW was significantly different with respect to the relative K-CN concentration compared to the LC and JE cattle. However, the FR cattle was not significantly different compared to the other types. The content of κ-CN in TW cattle was 35.50% higher than in LC and 42.62% higher than in JE cattle. Nevertheless, the relative content of κ-CN was low within all the types compared with the study of (Abeykoon et al. 2016) Still, it is interesting that the TW deviates for this parameter, since the study of Abeykoon et al. (2016) also suggested that milk from TW had the highest content of κ-CN of the cattle types. This raises the question whether the κ-CN percentage in this study is low or normal. Then there is little known about the milk protein profile of these cattle types and the study of Abeykoon et al. (2016) is one of few, which showed a very high relative concentration, i.e. 19% of κ-CN for the TW cattle. According to the study of Gustavsson et al. (2014) the κ-CN percentage was 5.05% for Holstein cattle and 6.39% for the Jersey. Comparing these results with those observed in the current study, the results for the LC (3.86%), JE cattle (3.49%) and FR cattle (4.88%) may be considered a little low, and more normal for the TW cattle (6.03%). Although, it is difficult to compare these studies as the conditions between the cattle holding systems, climate, genetics, and other factors differentiates widely.

According to the study done by Abeykoon et al. (2016) the TW cattle produced 0.00% β-CN A1 and 26.15% β-CN A2, LC produced 0.49% β-CN A1 and 33.96% β-CN A2 and Friesian produced 14.96% β-CN A1 and 22.87% β-CN A2. In the present study the TW cattle only had one individual of 15, and LC had three of 15 that produced β-CN A1. This is consistent with the statement of (Rashidinejad et al. 2017) that the indigenous cattle produce less of β-CN A1 than β-CN A2, i.e. fewer individuals that produce the protein variant. However, comparing the results in Table 5-8 the highest amount of β-CN A1 (21.73%) was found in the single individual producing this protein in the TW cattle. This was unexpected and raises the question if it could be due to the unknown factors of the genetic background and possible uncontrolled crossbreeding. According to Walstra et al. (2006) the individual genetic differences within a breed can have an even larger influence of the composition of the milk and therefore this individual may have inherited genetic predisposition to produce β-CN A1. This can also be the case for the three individuals that produced β-CN A1 within the LC type. The crossbreeds of JE and FR also had few individuals that produced β-CN A1. This is possibly due to their 30% unknown genetic background, likely with a genetic influence from indigenous cattle due to uncontrolled crossbreeding. Overall, all cattle types in this study had

few individuals that produced β-CN A1, possibly due to the influence of the genetic background of the indigenous cattle.

The β-CN A2 was high in all cattle types, and within the ranges of 36.25 – 40.54% of total protein, with the highest content in the indigenous cattle, TW (40.54%) and LC (38.86). However, there were no significant differences between the cattle types in respect to β-CN A2. The content of β-CN B was not significantly different between the cattle types in this study. However, in TW the β-CN B was only produced by 10 individuals and in the FR only by 13 individuals. According to the study of Abeykoon et al. (2016), the β-CN B was present in the TW cattle (3.03%) but had 0.00% in LC and FR. The result in this study is contradictory to this, and the reason could lie concealed in the unknown genetic backgrounds of the individuals in this study.

Total proteolysis

There are many factors that can affect the activity of proteases in milk as e.g., age, health status (e.g. degree of mastitis), lactation stage, bacterial load, individual genetic differences and genetic differences between breeds (Walstra et al. 2006). In this study many of these factors were not taken into consideration.

Somatic cells release different variants of proteases into the milk as e.g., Cathepsin D. Bacteria in the milk can also contribute to protein degradation. Furthermore, there are many proteases which function and origin is unknown (Fox & McSweeney 2003). According to this study, there were significant differences between cattle types in TP, but also in SCC. The TW, LC and JE cattle were significantly different from FR cattle. The FR cattle had the average highest SCC, with a value of 495 930 cells/ml. SCC indicates the udder health of the cow and in turn, the quality of the milk. During mastitis and microbial infections, the number of somatic cells increases (Walstra et al. 2006). Plasmin activity is often higher in milk from cows suffering from mastitis and thereby increase the total proteolysis in milk (Fox & Kelly 2006). However, the standard deviation (SD) for the SCC was higher than the average value in all types except in the FR cattle, which are presented in Table 10. The minimum and maximum values of the parameter SCC in Tables 5-8 shows a wide individual variation within the cattle types. Therefore, it is difficult to find significantly differences when there are so few individuals representing each cattle type.

The level of total proteolysis was shown to be significantly affected by cattle type (Table 9). Milk from TW cattle had significantly lower TP compared to milk from LC and JE cattle. The average total proteolysis value in the milk from TW cattle was 42.8% lower than in LC and 40.7% lower than in JE cattle. However, milk from FR was not significantly different with respect to TP compared to the other cattle types. According to Johansson et al. (2017) a correlation between SCC and TP could be seen at a level of 230 000 cells/ml and an average value of 26.50 leucine eq. mM respectively. In this study no correlation could be seen between SCC and TP (see Appendix Table 1a). However, the individual differences within the cattle types were wide for TP. This is especially so, within LC and JE (Table 6. and 7.), where the range between the minimum and maximum values of TP was

18.10/15.00 – 59.60/91,.30, respectively. As the 15 cows within each cattle type are from different smallholders in the dry zone (Weerasingha 2021) the individual variation and differences in management procedure between the smallholders, could be a reason for the wide range between the individuals. According to Walstra et al. (2006) the individual differences can have a larger impact on the milk composition, than the differences between breeds. The differences in management systems between farms, can also affect the composition of the milk (Walstra et al. 2006), which also can be an explanation to the large variation between the cattle types for this parameter.

One reason for the lower content of κ-CN in the milk samples in this study compared to the studies of (Gustavsson et al. 2014; Abeykoon et al. 2016), could be due to exogenous proteases, such as peptidases that are produced by *Pseudomonas spp*. According to Meng et al. (2017) one of the most important of peptidases is the alkaline metallopeptidase (AprX), that has been detected within various strains of the *Pseudomonas spp*. It has been shown that the peptidase AprX can be active and in some cases have a high specificity for hydrolyzing κ-CN (Machado et al. 2017). The *Pseudomonas spp*. can grow and produce peptidases at temperatures between 4 - 42°C (Meng et al. 2017). When the milk samples that were sent from Sri Lanka were unpacked, some of the sample replicates were empty and open. This could be a sign of a broken freezing chain and that the samples could have been thawed during the transportation from Sri Lanka to Sweden, before they were frozen again. Therefore, temperatures within ranges of where proteolytic activity and microbial growth can have occurred, cannot been taken out of consideration. Plasmin is not known to be active on κ-CN (Fox & Kelly 2006) and therefore it is probably not plasmin. As TP was lowest in the TW cattle and TW cattle had the highest amount of κ-CN, this can be an indication that proteases have been active on the protein. By looking at the results in Table 5-8 and by comparing the parameters TP and κ-CN, the cattle with the higher TP have lower amounts of κ-CN. This supports the theory that other proteases than plasmin have been active in the milk. However, no correlation between these parameters could be seen (see Appendix 1. Table 1a.).

5. Conclusion

The objective of this study was to investigate if there were any significant differences between the milk of the different cattle types, based on the parameters of total proteolysis, protein profile, pH, SCC, and gross composition. There was a total of six parameters that were significantly different between the types in this study: total fat, SNF, SCC, pH, κ-CN, and TP. However, the significance was low for differences in SNF, and SCC had a high standard deviation. TW was the cattle type that differed the most against the others. The TW cattle type were significantly different to some of the other cattle types, based on the parameters pH, κ-CN and TP. However, there are many factors that can have affected the composition of the milk and many of those factors are not taken into consideration in this study. Factors as for example differences in feed, management systems on the different farms and age of the individuals, are not taken into consideration in this study. The knowledge about the different cattle types genetic background and differences, are also limited in this study. Factors like these would be preferable to take into consideration in further studies to get more information of which factors that lays the ground for the differences. Therefore, it is important to point out that the significant differences in this study only are based on the factor of genetic background. Also, the low number of individual cows in each group can be considered as a limitation. However, this study can be considered as a contributing part in the work of deeper investigations of the indigenous cattle types in Sri Lanka.

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Appendix 1- Pearson's correlation coefficients

Table 1a. Pearson's Correlation Coefficients (r-values), 95% confidence intervals of milk quality parameters, protein profile, total proteolysis, pH, and total protein from all cattle types (n=60)

	SCC	T Pro.	pH	TP	α-LA	β-LG	αs2	αS1	k-CN	B-CN B	β-CN A1	β-CN A2	T β-CN	TWP
T Pro.	-0.065													
pH	-0.196	0.247												
TP	-0.161	-0.041	-0.345											
α-LA	-0.008	-0.177	0.232	0.170										
β-LG	-0.178	-0.055	0.103	0.199	0.274									
αs2	-0.032	-0.056	0.078	0.116	0.173	-0.033								
αS1	0.037	-0.147	-0.296	-0.089	-0.204	-0.068	-0.181							
k-CN	-0.000	-0.033	0.261	-0.278	0.070	0.336	-0.296	0.003						
β-CN B	0.023	-0.217	0.061	0.170	0.188	0.141	-0.035	-0.344	0.283					
β-CN A1	0.071	-0.241	-0.058	**-0.566**	0.112	-0.371	0.012	-0.315	0.124	-0.086				
β-CN A2	-0.038	0.198	0.290	-0.173	0.064	-0.076	-0.104	-0.311	0.237	-0.048	**-0.580**			
T β-CN	0.018	0.223	0.063	-0.221	-0.146	**-0.523**	0.033	-0.496	**-0.528**	-0.153	0.366	0.183		
TWP	-0.167	-0.087	0.143	0.219	0.458	**0.980**	0.005	-0.105	0.325	0.170	-0.319	-0.058	**-0.513**	
TCN	0.047	0.108	-0.024	**-0.523**	-0.292	**-0.610**	0.160	0.282	-0.253	-0.389	0.372	0.024	**0.557**	**-0.623**

1. r-values +/- 0.5-0.7= positive/negative moderate correlation.
2. r-values +/- 0.7-0.9= positive/negative strong correlation
3. r-values +/- 0.9-1.0= positive/negative very strong correlation
4. Abbreviations: SCC=Somatic cell count, T Pro.=Total protein, TP=Total proteolysis, T β-CN=Total beta-casein, TWP=Total whey protein, TCN=Total casein

Appendix 2- Popular scientific summary

Jonathan Skager

Indigenous cattle from Sri Lanka; an investigation of differences in their milk composition

Sri Lanka is a country in south Asia, located on an island in the Indian Ocean. The climate in the country varies, and is divided in the up-country, mid-country, and lowland, depending on the height over the sea. These different types of land also can be divided into wet zone, intermediate zone, and dry zone. In the up-country, the temperature during the year varies between 10 - 32°C and consist of mostly wet zone, which means more rainfall. Therefore, this environment is quite suitable for cattle holding. However, this part of the country is relatively small, compared to the large mid- and lowland dry zone. In this zone the temperatures vary between 21-38°C during the year, with little rainfall and therefore it is a challenging environment for cattle holding.

The national milk demand in Sri Lanka is higher than the production of milk, therefore the country is dependent of milk import. During the beginning of 21st century until 2016 the importation of milk almost doubled.

So why is the milk production lower than the demand?
As mentioned, one reason is the climate, where a large part consists of dry land with high temperatures and little rainfall. Another reason is the indigenous cattle that can be traced back to ancient times when cattle first was recognized on the island. These cattle are known as very poor producers of milk and are estimated to represent around 60% of the total cattle population in the country. But the indigenous cattle also have advantages, since they during century's have adapted to the severe environment and therefore can resist heat, diseases and manage on a relatively low feed intake. However, when it comes to genetics, the knowledge about the indigenous cattle is insufficient and therefore, they are referred to as cattle types instead of breeds. Two types of the indigenous cattle are the Thamankaduwa White cattle and Lankan cattle. These types are the most common within the dry zone and Thamankaduwa White cattle are especially found in the dry zone of the eastern part of the country.

It is not only milk that has been imported during the years. To become more self-sufficient, the government has also imported cattle breeds with European origin, known for their good milk production properties. However, the European breeds are used to live at more temperate conditions, at lower temperatures. The environment, especially in the dry zone, affects the European breeds negatively. This can result in a lower milk production and disease. Also, the European breeds can have problems to adapt to the feed. Back in the 1970^{ths}, breeding programs were financed to create crossbreeds of the indigenous types, with the European breeds,

Jersey and Friesian. This to get a crossbreed with better milk production properties than the indigenous cattle and at the same time maintain the indigenous types resistance to the severe environment. However, the crossbreeding programs failed, since it was hard to preserve the characteristics from the different cattle types during the crossbreeding and therefore the programs were shut down.

Is there a solution?

Since there is no simple solution to increase the milk production problem in Sri Lanka, it is of interest to get more knowledge about these indigenous types. As mentioned, there is not much known about their genetic differences.

The aim of this study was to investigate if there are any differences between milk from the indigenous types Thamankaduwa White and Lankan cattle, as well as two types of crossbreeds of Jersey and Friesian. This study was possible, thanks to the contribution from the Rajarata University of Sri Lanka and University of Peradeniya in Sri Lanka. Which contributed with milk samples and associated data of gross composition and somatic cell counts of the milk. The milk samples were analyzed, and the composition compared between the cattle types using the data from Sri Lanka, as well as measurements of pH-value, protein profile and total proteolysis at the Swedish University of Agricultural Sciences in Uppsala. The result showed that the Thamankaduwa White cattle, was the cattle type that was differed significantly on most parameters compared to the other cattle types, i.e., pH-value, protein profile and total proteolysis. Other significant differences between the cattle types, that were also observed in the study, included total fat, somatic cell count and solids nonfat. The milk composition of the Friesian crossbreed differed the most compared to the others. However, results related to the somatic cell count and solid nonfat content had a lower significance level and there was no difference in the solid nonfat content observed between the cattle types.

This study can be seen as a contributing part in assembling information about the indigenous cattle types in Sri Lanka.

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