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Relationship Between Kappa Casein Genes (CSN3) and Industrial Yield in Holstein Cows in Nariño-Colombia

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Carlos Eugenio Solarte-Portilla and Carol Yovanna Rosero-Galindo

Additional information is available at the end of the chapter

1. Introduction

Most dairy farms in Colombia are grouped into four geographical areas, termed "competitive regions", according to Resolution 000017 of 2012, issued by the Ministry of Agriculture and Rural [1] Development. The majority of the high-tropics herds, which are mostly specialized dairy farms, are located in Antioquia, Cundinamarca and Nariño provinces, while dual-purpose dairy farms are more abundant in the basins of the low tropics [2].

One of the most limiting factors affecting the competitiveness of specialized dairy farms in Nariño is the low quality of the product, in terms of milk composition. According to [3], the low protein content in milk negatively affects the efficiency of industrial processes to produce curd and cheese. Therefore, the improvement of the milk composition in this area is an important matter to take into account when planning nutrition and the genetic variables for optimal animal production [4]. The compositional quality of milk depends not only on environmental factors, but also on genetic traits such as the Kappa Casein gene (CSN3), which has been widely studied in order to establish relationships between its polymorphisms with the percentage of total milk protein and industrial yield [5].

Research conducted in various regions has reported mixed results regarding the associations between the AA, AB and BB genotypes of CSN3 and milk yield [6 - 8] However, given the complexity of gene expression, it is necessary to compare these results with those obtained under environmental conditions typical of this area in Colombia.

Therefore, in this study we established the relationships between the CSN3 genotypes and curd yield and the percentage of milk protein in Holstein cows, which are the predominant breed in the Andean region of Nariño.

2. Genetic improvement of the dairy cattle in colombia

2.1. General review

The main purpose of animal breeding is to obtain, by selection, future offspring with superior performance in relation to their predecessors. To achieve this objective, the frequency of desirable genes should be increased from one generation to another, which leads to an increase of genotypes with higher productivity. The most important tool with the greatest impact on animal breeding has been the control of production and the reliable permanent recording of pedigrees as well as of all the productive and reproductive events of the herds. Having this information, it has been possible to quantify genetic variability and identify the superior genetic merits of animals through mathematical models, through more and more refined means. This procedure corresponds to the so-called classical breeding schemes, which have greatly contributed to increased production and productivity in virtually all domestic species.

The classical methods of animal breeding have experienced a rapid development since the last decade of the twentieth century thanks to molecular techniques, which have allowed, among other things, the more precise quantification of genetic variability, the better understanding the phenomena of genes and the diagnosis of a great number of diseases [9]. DNA molecular markers are expressed in various forms and can always be located in the same site of the genome from generation to generation, which is an advantage of genetic analysis [10].

Today, the use of molecular markers is almost routine, following the development of PCR [11]. This is a technique for the direct amplification of DNA from tissue samples, such as blood, skin, hair and - in general - any tissue. PCR is based on the use of polymerizing enzymes and the initiation of oligonucleotides, known as primers.

Nowadays, studies of genetic polymorphism in cattle and other domesticated species are closely related to production traits and health and, for this purpose, techniques capable of detecting small variations in the DNA molecule are used [12]. One of the biggest advantages of genetic studies using PCR and DNA molecular markers is to reduce the generation interval, as can be identified in very early life stages, including embryonic individuals with superior genotypes, which increased efficiency in breeding programs.

2.2. Overview of milk production in Colombia

Colombian dairy has excelled over the past 30 years in terms of its dynamics, reflected in high rates of output growth. In the 1970s, milk production grew at a rate of 4.7% on average per year. Over the next decade, it accelerated its expansion, reaching an annual rate of 6.5%, while in the 1990s growth decreased, but succeeded in satisfactory rates of 3.8% per year, reaching a figure of 5.877 billion litres of milk fluid in 2001 [13]. The FAO estimate for 2005 indicates that the country produced 6.77 billion metric tons of milk, with the Atlantic region contributing 40% of total production, the Occidental adding 17%, and with Central Pacifica contributing 34% and 9%. FEDEGAN [14] (2006) estimated that the percentage growth

between 2004 and 2005 was 3.2%, which for the same period was lower than that achieved in countries with little experience in cattle, such as Costa Rica, Peru, Ecuador and Bolivia. However, Colombia ranked as the third largest producer in Latin America with 6.02 million tons in 2003, after Brazil and Argentina with 23 million and 7.7 million tons respectively.

The Agrocadenas observatory [2] stands out in that milk production has had a tendency to grow and which seem likely to be sustained over the next five years. This fact requires the search for new domestic and international markets and, therefore, the improvement of all links in the dairy chain, so as to increase the productivity and profitability of milk production systems.

The milk produced in Colombia comes from a specialized and dual purpose. It is estimated that the country has 25 million cattle, of which 11 million are engaged in milk production, 10 million in dual purpose and one million in the specialized system [14]. 88% of production is assigned for the dairy industry and 12% for the marketing of raw milk and the feeding of calves [16].

According to data from DANE and FEDEGAN [17, 14] in the living areas of the High Tropics 80% of the production system consists of specialized dairy breeds, with Holstein, Normando Brown Swiss, Jersey and Creole crossbreeds. In the dairy of the Tropic High of Nariño, also predominantly Holstein breed, which was introduced in the region for over a century, primarily considering its high production capacity? However, nowadays a need to consider different traits - especially those related to the compositional quality of milk, fertility and longevity - has been detected [18].

According to Agronet [19] and the Monitoring Unit Price of milk, from the Ministry of Agriculture and Rural Development-MARD in Colombia [1], in the region where the Department of Nariño is included, the average of fat is 3.63%, the protein is 3.10%, the total solids is 12.00% and the colony forming units vary within a range between 25,000 and 175,000. These figures are calculated on a permanent basis in the Animal Breeding Program at the University of Nariño - Meg@lac - for their importance in the selection process, which has been taking place since late 2006. Meanwhile, Agronet [19] indicates that in this region the protein content is below the national average. This weakness in compositional quality negatively affects the competitiveness of the livestock in Nariño, because the protein content is directly related to the biological value of milk, and their capacity for industrialization, making it mandatory to design and implement strategies leading to its improvement.

2.3. Milk composition

Milk is the normal product of the secretion of the mammary gland of female mammals. Physically, it is a complex fluid with more than one hundred substances in different stages, such as suspension, emulsion and true solution, which are the determinants of the nutritional quality and properties that mark it as a feedstock for the production of a large number of products [20]. As to the average composition of bovine milk among different breeds, Table 1 shows the composition of milk from the main dairy breeds in Colombia.

Breed	Fat(%)	Protein (%)	Lactose (%)	Ash(%)	Total solids (%)
Holstein	3.54	3.29	4.68	0.72	12.16
Brown Swiss	3.99	3.64	4.94	0.74	13.08
Ayrshire	3.95	3.48	4.60	0.72	12.77
Guernsey	4.72	3.75	4.71	0.76	14.04
Jersey	5.13	3.98	4.83	0.77	14.42
Shorthom	4.00	3.32	4.89	0.73	12.9

Table 1. Composition of milk from different breeds of cattle [21]

The variation in the constituents of milk is due to several factors that alter their composition, structure and properties. Among the most influential factors, the species, breed, lactation number, age, pathological conditions, and environmental factors such as diet, climate and the milking system, may be mentioned [22].

Such variations are observed more easily in components such as fat, lactose, ash and - more importantly - in proteins [23]. Usually, milk proteins have been divided into two groups, depending on their behaviour by acidification to pH 4.6 and isoelectric point at room temperature. Such groups refer to a soluble fraction containing whey proteins, namely alpha-lactalbumin (α-LA) and Beta-Lactoglobulin (β-LG), which represent 20% of the total proteins. The other fraction is insoluble, which is where we can find the caseins alpha s1 (αs1-Cs), alpha s2 (αs2-Cs), Beta (β-Cs) and Kappa (κ-Cs), which represent 80% of total milk proteins. The protein content in milk is especially affected by the influence of nutritional and genetic factors, considering the latter to be the most important, because they are the genes that allow the animal to produce milk of a certain quality, in an environment that provides the conditions for this to happen [24].

The average protein content in cow milk is 3.5% and an effective way to increase it is the selection of animals with the best genotypes, which, once identified, can be spread extensively using reproductive biotechnologies such as artificial insemination, multiovulación and embryo transfer. In species with a long generation interval, such as cattle, the genetic progress is relatively slow compared with that achieved in other species. This is one reason as to why in the last two decades use has been made of molecular markers as an option to increase genetic progress, as it facilitates the identification of desirable genotypes at an early age and - even in embryos - decreases the generation interval thereby increasing the genetic gain each year [25].

2.4. Genetic bases of kappa-casein (K-Cs)

2.4.1. Overview of K-Cs

Proteins are the major milk components though they differ in function and biological value, so it is necessary to study them individually in order to determine their physico-chemical and functional differences. In bovine milk, proteins represent approximately 3.5% of its total components. Caseins (Cs) constitute 80% of the protein fraction while whey proteins account for 20% [26].

Among the casein fractions are included αs1-Cs, αs2-Cs, β-Cs and κ- Cs casein. In the whey proteins, which are synthesized in higher concentrations, are α-LA and β-LG and lower concentrations of lactoferrin (LFE) and defensins (DFS), among others [26].

Several studies have confirmed that casein and whey proteins have an influence on the properties of milk for industrial processing, especially in terms of coagulation time, curd firmness and performance [27 to 30]. The κ-Cs has a clearly different structure from other caseins, since it is smaller, being comprised of 169 amino acids, and it is phosphorylated by only one phosphate group, which causes fewer interactions with calcium ions over other caseins. However, it shares with β-Cs the property of having predominantly hydrophilic and hydrophobic areas clearly marked and separated.

A peculiarity of this casein is the presence of a net positive charge zone between amino acids 20 and 115. This area allows the interaction of casein with polysaccharides that are negatively charged. The chain also has two groups of cysteine and is the only casein which has partly glycosylated molecules. The carbohydrate group is comprised of a trisaccharide or a tetrasaccharide or else is linked to a threonine residue.

Besides the presence of hydrophilic and hydrophobic areas, caseins interact together to form a colloidal dispersion which consists of spherical particles called micelles. The casein micelle is a very stable colloidal system in milk. This fact has important practical implications related to both the formation of casein gels and the stability of dairy products during thermal treatment, concentration and storage. For these reasons, the microstructure of the casein micelle has been intensively studied during the past five decades, since their knowledge is crucial in cheese making [31].

The formation of the casein micelle may be affected by changes in pH, salt concentration, temperature and hydrophilic regions. Under normal conditions of pH and salt concentration, casein micelles are well hydrated, having about 3.7 grams of water connected per gram of protein. When these conditions change, the casein micelles are destabilized by the acidity and proteolysis of the κ-Cs, which is also related to the allelic variant for this protein fraction.

As to the acid, there are two effects: first, a decrease in pH generates the breaking of the bonds between the phosphate and calcium ion by reducing the ionisation of the phosphates. Second, the repulsion between the micelles is reduced when the pH approaches the isoelectric point of casein. At a pH of about 4.5 and a temperature above 20 ^{O}C, casein curds are added forming a slightly mineralized.

A clear example of how changes in the hydrophilic regions and temperature affect the stability of the micelle is its treatment with chymosin. By adding chymosin, the κ-Cs loosens, by proteolysis, its hydrophilic region thereby facilitating aggregation. At low temperatures of refrigeration, the hydrophobic forces which hold together the molecules of the β-Cs are weakened, causing them to expose their hydrophilic region to the outside, increasing micelle hydration and volume. As a consequence, at refrigeration

temperatures, there is no aggregation of casein, neither by the action of the acidity nor by that of chymosin.

Other factors which greatly affect micelle aggregation are the calcium and salt content. The loss of Ca++ leads to the dissociation of β-Cs without the disintegration of the micelle. Furthermore, the salt content affects the activity of serum calcium and the amount of calcium phosphate of micelles.

2.5. Molecular basis of polymorphisms

The milk proteins are synthesized in Lactocytes, and their expression is co-dominant. Moreover, not all of the alleles encode exactly the same protein, due to the alteration or substitution of one or more amino acids in the polypeptide chain; some alleles are even null, i.e. they do not code for any protein. Individuals heterozygously synthesize a protein, one for each allele that they possess, in the homologous chromosomes [32].

The caseins, meanwhile, are encoded by four characteristic genes called polymorphic autosomal CSN1S1, CSN1S2, CSN2 and CSN3, physically located in a 250 Kb region of chromosome 6, very close together, particularly at position 6q31-33 [33]. Genes αs1-Cs, αs2-Cs and β-Cs are within the 6q31 locus in a region of 140 Kb, while the gene of the κ-Cs is located in a region between 95 and 120 Kb.

Figure 1 shows the primary structures of the caseins. The αs1-Cs consists of 199 amino acids and has a molecular weight of 23,000 Dalton [34]; of these amino acids, 17 correspond to proline, with two hydrophobic regions separated by a polar region which contains the phosphate groups (Figure 1a). For its part, has the αs2-Cs 207 amino acids in its conformation (Figure 1b) and has a molecular weight of 25,000 Daltons and 10 amino acids of a proline type. Finally, the κ-Cs has 169 amino acids in conformation (Figure 1c) and its molecular weight ranges from 19,006 to 19,037 Daltons.

The primary structure of the β-Cs is formed by a chain of 209 amino acids with five phosphate groups, which provides for the property of strongly binding calcium ions. The molecular weight ranges from 23,983 to 24,000 Daltons (Fig. 1d), depending on the allelic present variation t [35]. As with the κ-Cs, Cs has a β-polar and a hydrophobic area. In the polar end, the phosphates' attached groups are concentrated on the amino acids serine and are more hydrophilic. However, the β-Cs are the most hydrophobic casein fractions, containing a larger number of proline compared to any other type of casein [36]. The amino acid sequence of the β-Cs was established in 1972 by Ribadeau & Dumas and a review of the same part by Yan and Wold (1984) found four differences in the original sequence, where three of them corresponded to a changing of glycine by glutamic acid at positions 117, 175 and 195, and a fourth in a reversal of the amino acids proline to leucine at positions 137 and 138 respectively [37].

Figure 2 describes the sequences and nucleotide positions for the proteins αs1-Cs, Cs αs2-, β and κ-Cs-Cs available on the NCBI database [41] through the Genbank.
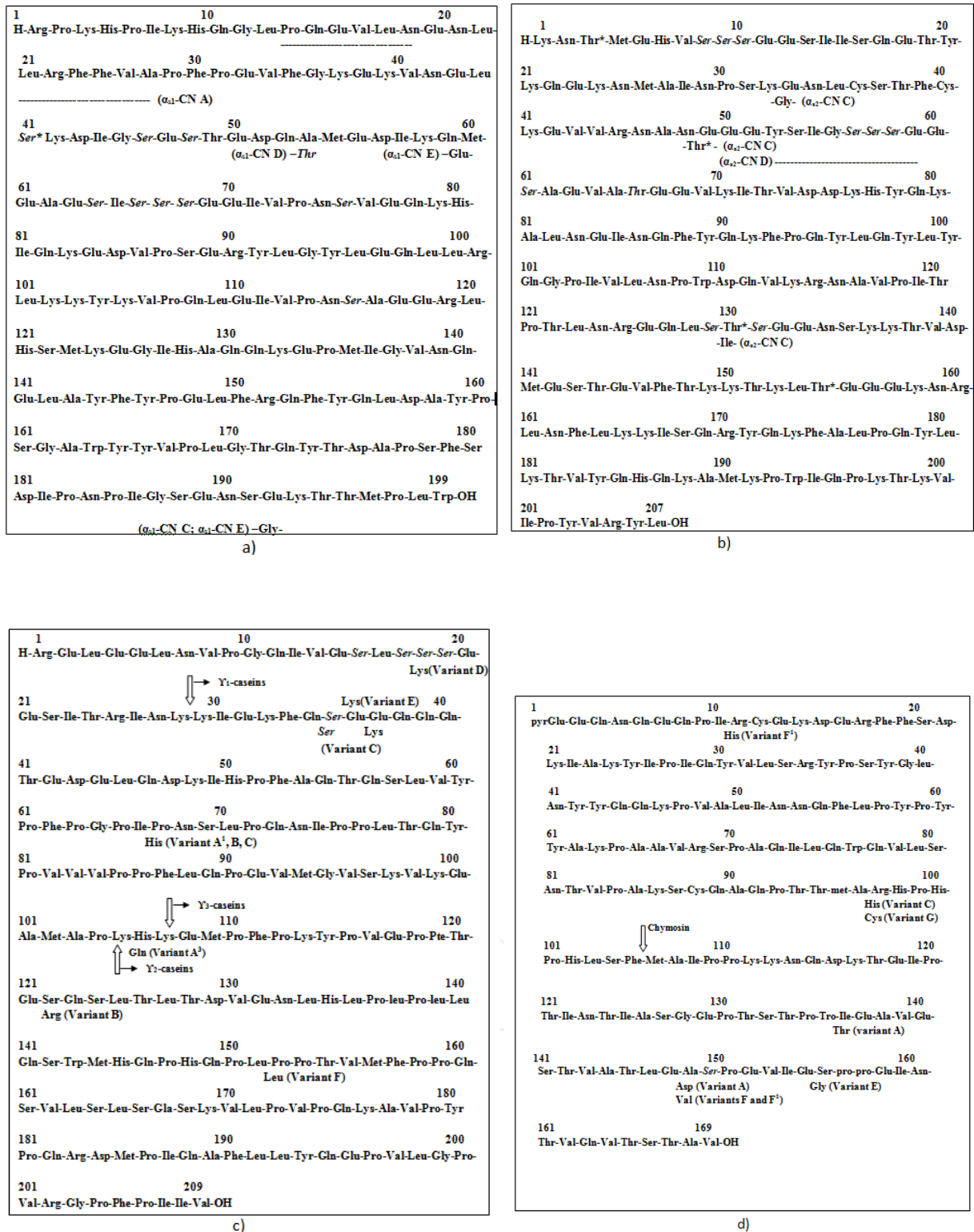
```
1                          10                    20
H-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-Leu-Asn-Glu-Asn-Leu-
                                            ------------------------

 21                    30                    40
Leu-Arg-Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu
----------------------- (αs1-CN A)

 41                    50                    60
Ser* Lys-Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met-
                  (αs1-CN D) −Thr          (αs1-CN E) −Glu-

 61                    70                    80
Glu-Ala-Glu-Ser- Ile-Ser- Ser- Ser-Glu-Glu-Ile-Val-Pro-Asn-Ser-Val-Glu-Gln-Lys-His-

 81                    90                   100
Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-

101                   110                   120
Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-Ser-Ala-Glu-Glu-Arg-Leu-

121                   130                   140
His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-

141                   150                   160
Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-

161                   170                   180
Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser

181                   190                   199
Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp-OH

         (αs1-CN C; αs1-CN E) −Gly-
                        a)
```

```
 1                          10                    20
H-Lys-Asn-Thr*-Met-Glu-His-Val-Ser-Ser-Ser-Glu-Glu-Ser-Ile-Ile-Ser-Gln-Glu-Thr-Tyr-

 21                    30                    40
Lys-Gln-Glu-Lys-Asn-Met-Ala-Ile-Asn-Pro-Ser-Lys-Glu-Asn-Leu-Cys-Ser-Thr-Phe-Cys-
                                         -Gly- (αs2-CN C)

 41                    50                    60
Lys-Glu-Val-Val-Arg-Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-Ser-Ser-Ser-Glu-Glu-
                                -Thr* - (αs2-CN C)
                                  (αs2-CN D) -----------------------------------

 61                    70                    80
Ser-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys-Ile-Thr-Val-Asp-Asp-Lys-His-Tyr-Gln-Lys-

 81                    90                   100
Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe-Tyr-Gln-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr-

101                   110                   120
Gln-Gly-Pro-Ile-Val-Leu-Asn-Pro-Trp-Asp-Gln-Val-Lys-Arg-Asn-Ala-Val-Pro-Ile-Thr-

121                   130                   140
Pro-Thr-Leu-Asn-Arg-Glu-Gln-Leu-Ser-Thr*-Ser-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-
                                    -Ile- (αs2-CN C)

141                   150                   160
Met-Glu-Ser-Thr-Glu-Val-Phe-Thr-Lys-Lys-Thr-Lys-Leu-Thr*-Glu-Glu-Glu-Lys-Asn-Arg-

161                   170                   180
Leu-Asn-Phe-Leu-Lys-Lys-Ile-Ser-Gln-Arg-Tyr-Gln-Lys-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-

181                   190                   200
Lys-Thr-Val-Tyr-Gln-His-Gln-Lys-Ala-Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys-Thr-Lys-Val-

201           207
Ile-Pro-Tyr-Val-Arg-Tyr-Leu-OH
                        b)
```

```
 1                          10                    20
H-Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Gln-Ile-Val-Glu-Ser-Leu-Ser-Ser-Ser-Glu-
                                                 Lys(Variant D)
          ⇓  Y1-caseins
 21              30          Lys(Variant E)  40
Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile-Glu-Lys-Phe-Gln-Ser-Glu-Glu-Gln-Gln-Gln-
                                         Ser      Lys
                                       (Variant C)
 41                    50                    60
Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-

 61                    70                    80
Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Tyr-
                  His (Variant A¹, B, C)
 81                    90                   100
Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val-Ser-Lys-Val-Lys-Glu-
                  ⇓  Y3-caseins
101                   110                   120
Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Glu-Pro-Pte-Thr-
                  ⇑  Gln (Variant A³)
                  ⇑→ Y2-caseins
121                   130                   140
Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-leu-Pro-leu-Leu
     Arg (Variant B)
141                   150                   160
Gln-Ser-Trp-Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln-
                   Leu (Variant F)
161                   170                   180
Ser-Val-Leu-Ser-Leu-Ser-Gla-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-Ala-Val-Pro-Tyr

181                   190                   200
Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-

201           209
Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH
                        c)
```

```
 1                          10                    20
pyrGlu-Glu-Gln-Asn-Gln-Glu-Gln-Pro-Ile-Arg-Cys-Glu-Lys-Asp-Glu-Arg-Phe-Phe-Ser-Asp-
                                               His (Variant F¹)
 21                    30                    40
Lys-Ile-Ala-Lys-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg-Tyr-Pro-Ser-Tyr-Gly-leu-

 41                    50                    60
Asn-Tyr-Tyr-Gln-Gln-Lys-Pro-Val-Ala-Leu-Ile-Asn-Asn-Gln-Phe-Leu-Pro-Tyr-Pro-Tyr-

 61                    70                    80
Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg-Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu-Ser-

 81                    90                   100
Asn-Thr-Val-Pro-Ala-Lys-Ser-Cys-Gln-Ala-Gln-Pro-Thr-Thr-met-Ala-Arg-His-Pro-His-
                                                     His (Variant C)
                                                     Cys (Variant G)
       ⇓  Chymosin
101                   110                   120
Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys-Thr-Glu-Ile-Pro-

121                   130                   140
Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro-Thr-Ser-Thr-Pro-Tro-Ile-Glu-Ala-Val-Glu-
                                                     Thr (variant A)
141                   150                   160
Ser-Thr-Val-Ala-Thr-Leu-Glu-Ala-Ser-Pro-Glu-Val-Ile-Glu-Ser-pro-pro-Glu-Ile-Asn-
                                Asp (Variant A)        Gly (Variant E)
                                Val (Variants F and F¹)
161           169
Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val-OH
                        d)
```

Figure 1. Primary structure of the casein from milk; a) αs1-Cs [34], b) αS2-Cs [38], c) β-Cs [39] y d) κ-Cs [40].

a)

Genbank sequence
Accession number: X59856, M38641
Definition: *Bos taurus* gene.
Accession: X59856
Version: X59856.1 GI: 91
Secuencia parcial: Gen de la Alfa s1 caseina bovina

TTGCAGGAAA AAGATTAGAC CACATATAAT GTAACTTATT TCACAAGGTA AATAATTATA
ATAAATAATA TGGATTAACT GAGTTTTAAA AGGTGAAATA AATAATGAAT TCTTCTCATG
GTCTTGTATG TTAATAAAAA TTGAAAAATT TTGAAGACCC CATTTTGTCC CAAGAATTTC
ATTTACAGGT ATTGAATTTT TCAAAGGTTA CAAAGGAAAT TTTATTGATA TAATAAATGC
ATGTTCTCAT AATAACCATA AATCTAGGGT TTTGTTGGGG TTTTTTTTTG TTTGTTAATT
TAGAACAATG CCATTCCATT TCCTGTATAA TGAGTCACTT CTTTGTTGTA AACTCTCCTT
AGAATTTCTT GGGGAGAGGAA CTGAACAGAA CATTGATTTC CTATGTGAGA GAATTCTTAG
AATTTAAATA AACCTGTTGG TTAAACTGAA ACCACAAAAT TAGCATTTTA CTAATCAGTA
GGTTTAAATA GCTTGGAAGC AAAAGTCTGC CATCACCTTG ATCATCAACC CAGCTTGCTG
CTTCTTCCCA GTCTTGGGTT CAAGGTATTA TGTATACATA TAACAAAATT TCTATGATTT
TCCTCTGTCT CATCTTTCAT TCTTCACTAA TACGCAGTTG TAACTTTTCT ATGTGATTGC

PRIMERS:
Forward: 5' TGCATGTTCTCATAATAACC 3'
Reverse: 5' GAAGAAGCAGCAAGCTGG 3'

b)

Genbank sequence
Accession number: X14711
Definition: *Bos taurus* gene.
Accession: X14711
Version: X14711.1 GI: 120
Secuencia parcial: Gen de la beta caseina bovina

GATGAACTCCAGGATAAAATCCACCCCTTTGCCCAGACACAGTCTCTAGTCTATCCCTTCCCTGG
ACCCATCCATAACAGCCTCCCACAAAACATCCCTCCTCTTACTCAAACCCCTGTGGTGGTGCCGC
CTTTCCTTCAGCCTGAAGTAATGGGAGTCTCCAAAGTGAAGGAGGCTATGGCTCCTAAGCACAA
AGAAATGCCCTTCCCTAAATATCCAGTTGAGCCCTTTACTGAAAGCCAGAGCCTGACTCTCACTG
ATGTTGAAAATCTGCACCTTCCTCTGCCTCTGCTCCAGTCTTGGATGCACCAGCCTCACCAGCCT
CTTCCTCCAACTGTCATGTTTCCTCCTCAGTCCGTGCTGTCCCTTTCTCAGTCCAAAGTCCTGCCT
GTTCCCCAGAAAGCAGTGCCCTATCCCCAGAGAGATATGCCCATTCAGGCCTTTCTGCTGTACCA
GGAGCCTGTACTCGGTCCTGTCCGGGGACCCTTCCCTATTATT

PRIMERS
Forward 5' CCAGAC ACAGTCT CTAGTC TATCCC 3'
Reverse 5' CAACATCAGTGAGAGTCAGGCTCCG 3'

c)

Genbank sequence
Accesion number: X14908, NM_174294
DEFINITION Bovine gene for kappa-casein exons 3-5.
ACCESSION X14908 X14326
VERSION X14908.1 GI:180
Exons 3-5
>gi|27881411|ref|NM_174294.1| Bos taurus casein kappa (CSN10), mRNA
1   TTCACTTACAGTGGAAAGGCCAACTGAACCTACTGCCAAGCAAGAGCTGACGGTCACAAGGAAAGGTGCA
73   ATGATGAAGAGTTTTTTCCTAGTTGTGACTATCCTGGCATTAACCCTGCCATTTTTGGGTGCCCAGGAGC
147 AAAACCAAGAACAACCAATACGCTGTGAGAAAGATGAAAGATTCTTCAGTGACAAAATAGCCAAATATAT
201 CCCAATTCAGTATGTGCTGAGCAGGTATCCTAGTTATGGACTCAATTACTACCAACAGAAACCAGTTGCA
272 CTAATTAATAATCAATTTCTGCCATACCCATATTATGCAAAGCCAGCTGCAGTTAGGTCACCTGCCCAAA
345 TTCTTCAATGGCAAGTTTTGTCAAATACTGTGCCTGCCAAGTCCTGCCAAGCCCAGCCAACTACCATGGC
419 ACGTCACCCACACCCACATTTATCATTTATGGCCATTCCACCAAAGAAAAATCAGGATAAAACAGAAATC
591 CCTACCATCAATACCATTGCTAGTGGCGAGCCTACAAGTACACCTACCACCGAAGCAGTAGAGAGCACTG
664 TAGCTACTCTAGAAGATTCCTCCAGAAGTTATTGAGAGCCCACCTGAGATCAACACAGTCCAAGTTACTTC
735 AACTGCAGTCTAAAAACTCTAAGGAGACATCAAAGAAGACAACGCAG---------INTRON IV------
-----------TCTGTTACAGAGAAGGCGAAATGGGC--------------------------------
783 GTCTAGCTGAAACCAAATGACTACTTCAAACTTTCCTTTGGCCAGTTGTCTGCCTTCGGTGAACAGAGAAT
ATGATTTTCACAGATCTGGCTCCTTCCTCGTCTCCTCTTACATTTTACTTTTATGCCAGATTTAATTTTTTGAT
TCCTGCATAATAAAGCCAATCAAATGCA

Figure 2. Figure 2 Nucleotide sequence of the genes of caseins present in bovine milk: a) Sequence αs2 αs1-Cs-Cs and, b) Sequence of the β-Cs c) Sequence of the κ-Cs.

2.6. Allelic variants of κ-Cs

The gene for κ-Cs has been studied extensively in order to establish the relationship between their polymorphisms or allelic variants and the industrialization of milk, especially in relation to cheese processing. Several findings emerged from the study of the genetic polymorphism of milk proteins which have been conducted with the purpose of improving the different sectors of the dairy industry [42]. Animal breeding contributes to the genetic basis of knowledge on which the production characters are based [43]. In research aimed at determining the relationship between various DNA markers and production traits, a special emphasis has been placed on the association between the genetic polymorphism of proteins and the physicochemical properties of milk [44].

Milk protein polymorphism is caused by mutation, which produces the genetic variants detected by electrophoresis [42]. These variants are identified by the letters A, B and C and in bovine species it is observed that the frequency of each allelic form varies between breeds [20].

The polymorphism of milk proteins in cattle was first described more than 50 years ago [45]. The first studies were based on protein polymorphism from milk samples, but thanks to technical advances in the field of molecular genetics, until 2005 DNA polymorphisms had

already been reported, of which 8 corresponded to variants different for αs1-Cs [46 - 48], 4 for αs2-Cs, 12 for β-Cs, 11 for κ-Cs, 11 for β-LG, and 3 for α-LA in Bos taurus [49].

From 1983 to 2007, 11 allelic variants have been reported for κ-Cs (A, B, C, E, F, G, H, I, A1, A2, A3), but in *Bos taurus* variants A and B are the most frequent alleles and not all of them appear in all races [33, 50, 51, 47, 52, 53].

Both in cattle and in goats, associations between certain protein polymorphisms and production traits have been shown, especially those related to the composition of protein and fat, although the results of several studies do not coincide because, in some cases, associations have been found but not in others [54, 55]. Therefore, it is important to confirm these findings under the conditions of the Tropical High Nariño.

Other studies have found lower industrial yields of cheese for the A allele when compared with allele B. The results for this variable have not been matched in all the investigations and, also, it is necessary to confirm them under our conditions. In some studies, it has been established that there is a significant effect on the conversion of milk into cheese, according to the specific genotype κ-Cs, given that in most cases the milk from animals with the BB genotype is homozygous and increased cheese yield; however, the milk from animals with the AA genotype has lower yields. This has been explained by a lower percentage of casein genotypes AA and as a result a greater proportion of large micelles [56].

In the case of the Tropic High Nariño, research conducted by the Genetic Improvement Program of the University of Nariño in southern Colombia, South America [18], shows gene frequencies close to fixation for allele A of the αs2-Cs, making it impossible to establish a direct association between the genotypes of the protein fraction with productive and reproductive performance variables [57].

These sample studies for the αs1-Cs show the absence of statistically significant differences on the above characteristics. These results do not agree with those obtained by Haenlein [58] and Graml [59] in relation to the stained brown bavárico and Guernsey, who claim that cows carrying the AB genotype reach a higher milk production compared to those with the homozygous genotype AA. On the contrary, [6, 60, 61] they reported the highly significant effect of genotype BB on this feature in the Holstein breed.

For the β-Cs protein fraction it was observed that the genotypes AA and AB have a significant effect on milk production. These results are similar to those described by several investigators on the same race, where the A allele is closely linked to an increased volume of milk per lactation [42, 62, 56, 63, 64].

Finally, our studies on the average figures for the production of a kilogram of curd for κ-Cs indicate that milk from animals with the genotype BB produce better yields of curd, requiring only 5.46 litres of milk [28]. This is explained by the formation of more stable micelles and smaller in the milk from these animals. Similar results are reported by [65], indicating that the B allele has better heat resistance, a shorter clotting time and produces a more consistent curd.

3. Materials and methods

3.1. Type of study

The present study was conducted in the municipality of Pasto, in south-western Colombia, located at north latitude 1°13'22'', west longitude 77°16'22'', at a height of 2690 m, with an average temperature of 12 °C and a relative humidity of 82% [66]. A total of 348 cows were sampled to determine their CSN3 genotype using PCR-SSCP. Once identified by this criterion, lactating cows were selected and classified according to their lactation stage, i.e., initial (first), mid (second), and final (third).

3.2. Identification of genotypes

The molecular identification of AA and BB homozygous and AB heterozygous genotypes for the CSN3 gene was conducted at the Animal Breeding laboratory of the University of Nariño, following the methodology described by [12] and modified [4].

3.3. Collection of the milk samples

Milk samples were obtained from animals whose output was adjusted to 305 days and an adult equivalent (PL) using the factors indicated by [24] for Colombian Holstein cattle. The experimental unit was each animal in its corresponding lactating stage. Four litres of milk were obtained from each cow during the morning milking and immediately transported under refrigeration (4 °C) to the processing plant. Upon receipt of the sample, three litres of milk were used for the assessment of the curd yield and one litre for physicochemical, microbiological and compositional analysis. An EKOMILK (KAM Milkama 98-2A) industrial milk analyser was used to assess milk composition. The values obtained for acidity and fat using the analyser were confirmed by manual testing. Acidity was determined by a qualitative change in colour after mixing one volume of milk with an alkaline solution of sodium hydroxide, adding phenolphthalein as an indicator (0.1 N). Fat content was determined using [67]. The number of colony-forming units (CFU) and the degree of environmental contamination of the milk were included in the microbiological analysis.

3.4. Curd

A protocol was established at the processing plant for the evaluation of curd yield. The equipment and implements used to obtain the curd were a centrifuge (Funke Gerber, Berlin Germany), autoclave (All American, Wisconsin, USA), stainless steel containers, electric stoves, burettes (Schott, Mainz, Germany), pipettes (Schott , Mainz, Germany), thermo-hydrometer (Funke Gerber, Berlin Germany), analytical balance (Mettler Toledo, Mexico DC), thermometers (B & S, Germany), curd knives to cut 1 x 1 cm, industrial moulds (500 g), and bags for packaging. The curd obtained was classified as "peasant type", according to the processing plant. This product is characterized by 60% moisture content. The fat and protein contents were not standardized prior to milk processing.

3.5. Evaluation of the curd yield

After 10 hours of cooling, the curd yield was calculated taking into account the volume of processed milk and the final weight of the curd. The calculations were based on the following formula:

$$RC = \frac{VL}{WC}$$

Where:

- RC = curd yield, expressed in litres of milk required to produce one kg of curd;
- VL = volume of milk;
- WC = final weight of curd.

Statistical analysis.

Analyses were performed using a linear model which included the fixed effects of the lactation stage (lactation was divided into thirds), genotype, and the interaction effect of the lactation stage by genotype. The percentage of fat in milk was included as a covariate. The statistical model is expressed as:

$$y_{ijk} = \mu + \tau_j + \alpha_k + (\tau,\alpha)_{jk} + \beta_1(x_{1i} - \overline{x}_1) + \varepsilon_{ijk}$$

Where:

$$y_{ijk}$$

= curd yield, associated with the jth genotype, the kth lactation stage, and the interaction of the jth genotype with the kth lactation stage, taking into account the percentage of fat in the milk.

- μ= Media comon to all observations;
- τ_j = Effect of the jth genotype. J= 1, 2, 3;
- αk = Effect of the kth lactation stage. k = 1, 2, 3;
- $\tau\alpha$ = Interaction effect of the jth genotype with the kth lactation stage;
- $\beta_1(x_{1i} - \overline{x}_1)$ = Lineal effect of the covariate "percentage of fat in the milk";
- ε_{ijk} = Experimental error associated with the jth genotype in the kth lactation stage and the interaction between the jth genotype with the kth lactation stage.

Each genotype in each lactation stage was regarded as a treatment. Each treatment had three replicates and thus, in total, 27 experimental units were evaluated. The age was not included in the model as a covariate because it was not statistically significant. The analysis was performed with the GLM procedure of SAS statistical software version 9.20 and Enterprise SAS Guide version 4.2. (2009).

4. Results

Kappa - Casein (CSN3) genotypes: According to [12], the PCR-SSCP molecular technique identifies more than four allelic variants for the CSN3 gene. In our studied population, only two allelic variants were found (A and B), identifying the homozygous genotypes AA, BB and heterozygous AB, in accordance with the electrophoretic pattern described by [12] (Figure 3). These results are consistent with those reported by other researchers, in which the A and B alleles have the highest frequency for the CSN3 gene in dairy breeds [8, 33, 50, 47].
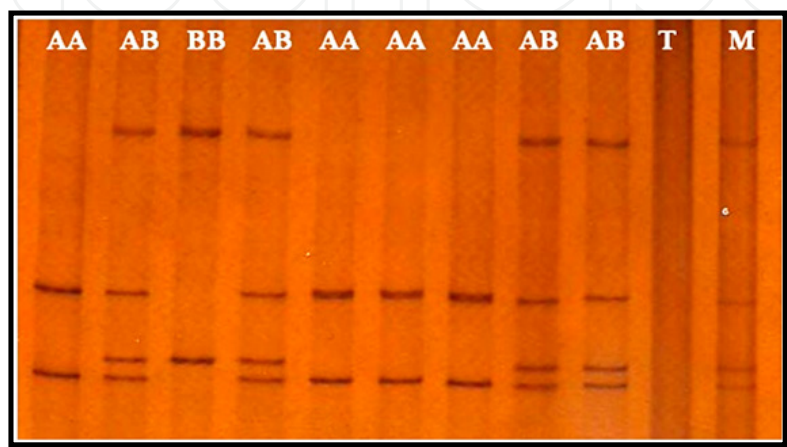
Figure 3. Electrophoretic bands generated by PCR-SSCP for the CSN3 gene in Holstein cattle of the High Tropics in Nariño.

Table 2 presents the percentages of protein (PP), body fat (BF), total solids (PST), and litres required to produce one kg of curd (L / Kg), separated for each genotype.

Genotype	Variable	Average	Standard deviation	Minimum	Maximum
AA	L/Kg	6.722	0.712	5.500	7.700
	PP	3.076	0.105	3.000	3.320
	PG	3.117	0.582	2.310	4.120
	PST	11.699	0.756	10.670	13.290
AB	L/Kg	6.111	0.653	5.000	7.000
	PP	3.062	0.114	2.900	3.260
	PG	3.718	0.659	2.510	4.470
	PST	12.269	0.774	11.140	13.470
BB	L/Kg	5.456	0.615	4.300	6.400
	PP	3.188	0.080	3.080	3.340
	PG	3.924	0.367	3.330	4.500
	PST	12.730	0.477	11.950	13.330

PP: protein (%); PG: fat (%); PST: total solids (%); L/Kg: litters of milk required to make one kilogram of curd

Table 2. Descriptive statistics of the variables evaluated according to the CSN3 genotype.

4.1. Analysis of variance for curd yield

The genotype, lactation stage and the linear effect of the fat percentage of the milk (included as a covariate) were statistically significant (p <0.05) in the ANOVA. The coefficient of determination was 0.624, indicating that the effects included in the model explain curd yield by 62.4%.

The least square means for curd yield were compared using the Tukey – Kramer test, concluding that the BB genotype had the highest yield compared to genotypes AA and AB, and no significant differences were found between AA and BB, as shown in Figure 4.
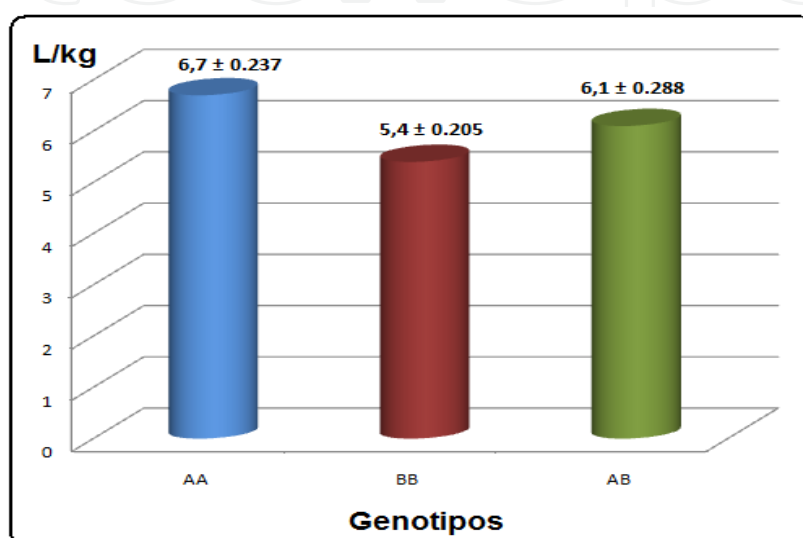
Figure 4. Number of milk litres required to produce one kilogram of curd for three κ-Cs genotypes of the Holstein breed in the Nariño High Tropic.

Finally, according to the Tukey – Kramer multiple comparison test, the only differences found for protein percentage were between the first and the second third of lactation. This is in agreement with reports by [68]. However, no differences were observed among the different genotypes.

The results of this study are consistent with those reported by [6, 7], who concluded that the BB genotype for κ-Cs determines the best milk properties for cheese production because of the greater firmness in the curd and smaller time required for the formation of small micelles. With regard to the homozygous AA genotype, these animals had lower casein contents and, as a consequence, a higher proportion of large micelles, which reduces the curd yield efficiency. Furthermore, according to [12], there is a positive relation between the κ-Cs genotype and the milk protein content, and this protein content influences the clotting time required by rennet as well as the firmness and the cheese yield, showing higher values in milk from cows with genotype BB with respect to the homozygous AA [69, 64], reaching differences that, in some cases, amount to up to 3% [70].

The study of [71] using Limonero cattle indicated that variant B of the κ-Cs could be used to improve the efficiency for transforming milk into cheese. A similar conclusion was arrived at

in [5] for the same variant of the gene in the Harton del Valle cattle breed. In summary, as stated by [4], these studies found that bovine milk from cows with the BB genotype for κ-Cs has greater stability in relation to heating and freezing, requiring less clotting time, producing a more consistent curd, and increasing cheese yield. Those results are in agreement with the present study. These results confirm the need to reorient the selection process in the Nariño High Tropics, where κ-Cs genotype should be considered as an important criterion for defining selection objectives. This trait should be included along with other factors that are relevant to the region, such as the functional type, the longevity and the somatic cell count, consistent with current selection trends for the Holstein breed in many countries [72].

5. Conclusions

The use of molecular techniques - an unprecedented event in the region - to determine the genotypes of the κ-Cs and its implementation is useful for the correct identification of individuals according to their genetic constitution and allows for the development of other studies for measures, such as population structure and genetic diversity for the gene CSN3 in the High Tropic of Nariño.

In the Holstein breed and under the conditions of the High Tropic of Nariño, the highest yield was obtained with curdled milk from animals with the genotype BB.

The highest content of total protein was obtained in animals with the genotype BB and in the last third of lactation, factors that did not produce an interaction effect.

The results of this research provide are a good guide to reviewing and developing breeding programs in the Tropic Alto de Nariño for Holstein cattle, since in order to improve the compositional quality of milk and in order to increase industrial performance, it is necessary to include the feature of the genotype for κ-Cs in a comprehensive genetic evaluation which includes, along with other important factors, the increasing of the gene frequency of the B allele for kappa casein.

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