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TÍTULO: GENETIC CHARACTERIZATION OF BETA-CASEIN, KAPPA-CASEIN AND BETA-LACTOGLOBULIN

Trabajo de integración curricular presentado como requisito para la obtención del título de Biólogo

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RESUMEN

Existen 24 biomarcadores o genes asociados con características esenciales para la producción de lácteos. Tres de esos genes son CSN2, CSN3 y LGB.

CSN2 tiene dos alelos comunes, A1 y A2. En la digestión gastrointestinal de los monómeros de A1 se libera un bio péptido, la beta casomorfina-7(BCM-7). BCM-7 es un opioide peptídico que tiene un rol en algunas enfermedades y la intolerancia a la leche. La BCM-7 liberada de beta-caseína A1, ha sido relacionada como un factor de prevalencia de diabetes, enfermedades autoinmunes y otros. Por otra parte, la beta caseína A2 no libera BCM-7 porque es más estable que A1

CSN3 está asociado a la formación de micelas y la coagulación de la leche, lo cual es una característica fundamental para la producción de lácteos. Algunos estudios han mostrado que la variante BB de CSN3 tiene una coagulación más rápida y firme, lo cual es esencial para producir lácteos. LGB está asociando al contenido de grasa, proteína y caseína en la leche, lo que es fundamental en el rendimiento de queso.

El objetivo de este proyecto de investigación fue determinar la frecuencia genética de estos 3 genes en 4 rebaños lecheros mediante diferentes técnicas de biología molecular.

PALABRAS CLAVE: Beta caseína, kappa caseína, beta lactoglobulina, polimorfismo genético.

ABSTRACT

There are 24 molecular markers or genes associated with essential milk traits for the production of dairy. Three of those molecular markers are *CSN2*, *CSN3*, and *LGB.*

CSN2 has two common alleles, namely A1 and A2. In the Gastrointestinal digestion of the A1 monomers release a bioactive peptide, beta casomorphin-7 (BCM-7). BCM-7 is an opioid peptide plays a role in several human diseases and milk intolerance. BCM-7, derived from A1 beta-casein, has been linked is linked as a prevalence factor of diabetes, autism, autoimmune disease and others. On the other hand, the beta-casein A2 does not release BCM-7 because beta-casein A2 is more stable than A1.

CSN3 is implicated in the micelle formation, and therefore in milk coagulation, which are fundamental characteristics for dairy production. Some studies have shown that the BB *CSN3* variant has a faster and firmer gelling ability, which is essential in the production of dairy. *LGB* is associated with fat, protein, and casein content, which is fundamental in cheese yield. The research project aim was determining the genotype frequency of these three genes in four dairy cattle herds by different molecular biology techniques.

KEY WORDS: Beta-caseín, kappa-caseín, beta-lactoglobulin, genetic polimorphism.

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INTRODUCTION-JUSTIFICATION

An overview of genetic improvement of cattle

Cattle improving is necessary to increase competitiveness, animal production (e.g., milk yield and composition), and adaptation to climate change. Cattle improvement requires the understanding of the biology of the animal through different biological sciences such as Genomics, Molecular Biology, and Genetics, to determine the expression of genes, and how they interact with each other (Kiplagat et al., 2012; Rexroad et al., 2019). Additionally, it is also required the development of techniques for animal reproduction (e.g., cloning and artificial fecundation) and animal welfare (e.g., nutrition, stressors). Both are essential as environmental or epigenetic factors due to their impact on gene expression (Boutinaud et al., 2019; Herve et al., 2019; Siqueira et al., 2017).

The study of genotypic and phenotypic characteristics complemented with environmental factors allows to determine, characterize, and predict the effects of different gene sequences (MacHugh et al., 2017). The translation of genotypes into phenotypes, which are mainly studied by genetic characterization, are the adaptation of cattle breeds to different environments, better resistance, or related to particular diseases and genotypes fundamental to economically important traits (König & May, 2019; Ramírez-Rivera et al., 2019). Other advantages are the reduction of costs of production, reduction of venereal diseases, sex selection, and better control and record of the production for future crosses (Garcés et al., 2018).

Genetic characterization programs require three fundamental steps: identification and performance recording, selection, and multiplication (Kiplagat et al., 2012). The first step is the identification and performance recording; an area or population is selected based on the availability of data of each individual about the productivity or physical characteristics (e.g., milk yield). The following step is selection. The data is essential in this step before and after the genetic characterization since it allows the measurement of the effectiveness of the selection, thus choosing the potential individuals, and identifying potential individuals that do not express the evaluated traits. For example, bulls do not produce milk; the

selection of potential bulls for milk production is on the yields of the relatives. The evaluation of data and genes allows us to identify relations; for example, the genotype BB of the *CSN3* gene is related to the production of milk with faster and firmer coagulation (Bonfatti et al., 2010b) (Poulsen et al., 2013). The identification and analysis of the molecular markers (i.e., genes) contribute to making a precise and faster selection. Finally, during the multiplication, the selected animals are used in breeding programs. In the multiplication, it is avoided the crossing of close relatives due to the deleterious effects of inbreeding (Kiplagat et al., 2012). In this study, we are focus on selection.

Genetic improvement of cattle in Ecuador

The first national programs of genetic improvement allowed us to increase the yield of milk and meat. During the last years, the programs are centered on the use or generation of new knowledge to study our native breeds, as well as to establish and evaluate programs in the long term (Rexroad et al., 2019; Toalombo et al., 2019). During the 70s, they have implemented the first programs of genetic improvement. Before this decade, Ecuador was an importer of powdered milk due to its deficient production (IICA, 1977). The above programs had as a primary objective the importation of pure breeds and embryos for artificial insemination.

Since 2015, a genetic improvement policy has been promoted by the Ministry of Agriculture and Livestock (MAGAP, by its initials in Spanish) through the establishment of the "*Centro de Mejoramiento Genético del Ganado Vacuno El Rosario*" (El Telégrafo, 2015). In that center, the national programs of cattle genetic improvement complemented with studies about cattle nutrition, the adaptation to different environments, and artificial insemination will allow to achieve the objectives of the programs (Gutiérrez & Quilligana, 2016). However, more excellent coverage of these programs is necessary since they are only studied breeds that are adapted to the Andean region. Therefore, it is necessary to expand studies nationwide and focus it to improve milk quality and study of native breeds.

Biomarkers implicated in genetic improvement

There exist many biomarkers associated with mammary gland development and function and therefore associated with milk production and its related traits. The studies of biomarkers around the world pave the way for improving milk quality, production, and

traits. The datasets of the National Animal Genome Research Program (www.animalgenome.org) shows a summary of the evidence from different studies on the role or relationship of each cattle gene.

There are at least 943 genes related to mammary gland development and function. From that gene set, only 24 are related to milk traits according to genetic association studies (Ogorevc et al., 2009). Some of the milk facts include milk yield, milk composition (mainly protein and lactose percentages), and coagulation properties (Singh et al., 2014). This research was focused on the genetic characterization of three cattle milk genes to investigate their genotypic diversity and establish a genetic improvement program in the near future. The genes that were characterized included *CSN2, CSN3*, and *LGB.* These genes are deemed to be essential for health and milk quality.

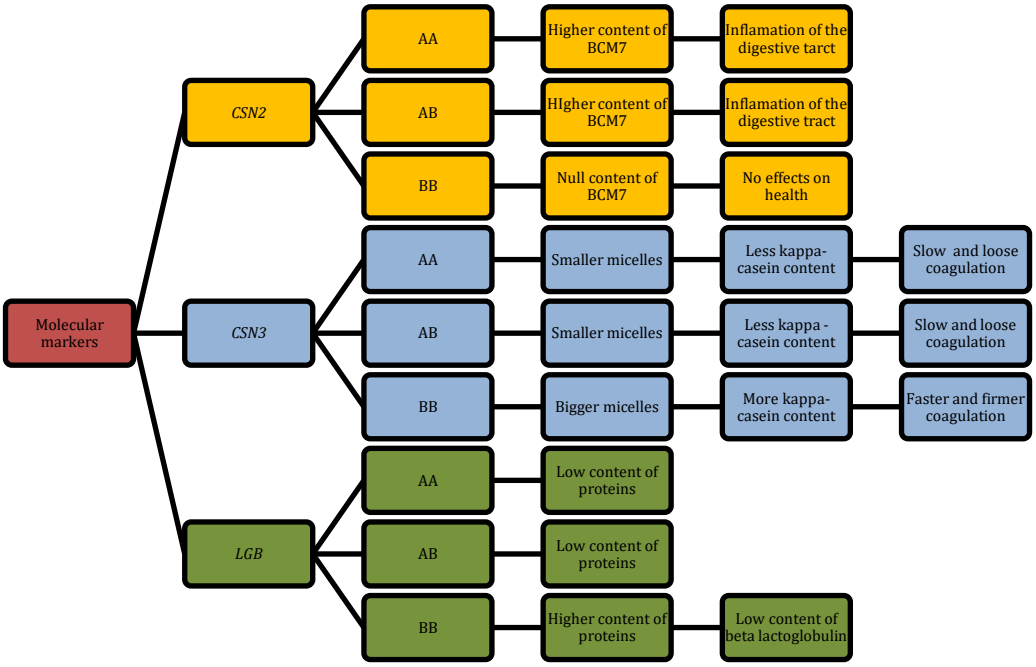

Figure 1. Summary of the effects of the molecular markers analyzed.

***CSN2* biomarker: beta-casein**

CSN2 is the gene of the bovine beta-casein, which has identified 13 alleles, as reported by Kamiński et al. and Rahimi et al. (Kamiński et al., 2007; Rahimi et al., 2014). The most common are A1 and A2, while the rarest are A3 and C (Farrell et al., 2004). A1 appeared

from an A2 mutation in the European cattles since it has a high prevalence in European herds (Jianqin et al., 2016; Rahimi et al., 2014, p. 1). The A1 arose from a single nucleotide polymorphism mutation at the position 67 of the CSN2 biomarker, consisting of a change of adenine for a cytosine (Figure 2). This mutation causes the exchange of histidine for proline in the amino acid chain (Kamiński et al., 2007).

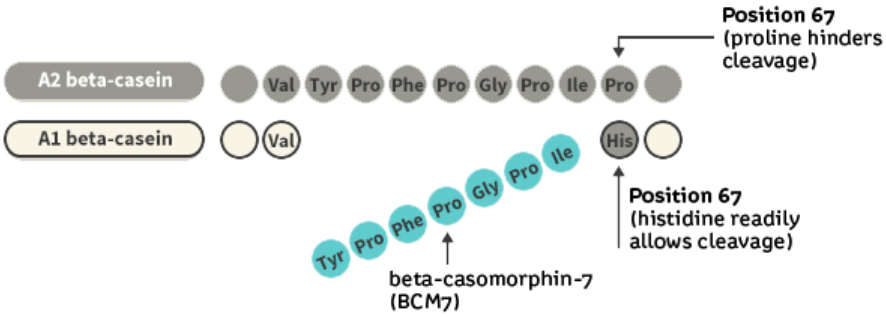

Figure 1. A1 and A2 beta-casein proteins. A1 digestion produces the release of BCM7.

The beta-casein genotypes are essential in dairy production and human health since they are related to milk traits and diseases such as diabetes, ischemic heart, and milk intolerance (Jianqin et al., 2016; Laugesen & Elliott, 2003, 2003; Pal et al., 2015). On the one hand, A2 is related to higher milk and protein production (Fontanesi et al., 2014; Nilsen et al., 2009). On the other, A1 is related to adverse effects on the curd firming rate and the time of coagulation, which are vital for cheese production (Poulsen et al., 2013). The A1 genotype is related to milk intolerance because of the production beta casomorphin-7 (BCM-7) after consumption (Sheng et al., 2019). BCM-7 is an opioid that regulates digestion by reducing the intestinal movements and the release of digestive enzymes (Ho et al., 2014; Jianqin et al., 2016). Slower digestion causes incomplete digestion. Commonly milk intolerance is confused with lactose intolerance after milk consumption because they cause similar symptoms such as diarrhea, gas, and bloating after milk consumption (Pal et al., 2015). Pure herds of A2A2 cattle have been established to yield A2 milk for milk intolerants.

CSN3 biomarker: kappa-casein

CSN3 is the bovine gene of the kappa casein, which has 12 alleles, as it is reported by Kour et al. (Kour et al., 2018). The most common and essential for the dairy industry are the alleles A and B because they are related to the time and firmness of coagulation. These alleles change two amino acid positions: threonine by isoleucine at position 136, and aspartic acid by alanine at position 148 (Prinzenberg et al., 2008). The B allele is better than A allele for cheese production because the cheese produced with milk containing kappa casein B present firmness and faster coagulation. Kappa casein A produces cheese with opposite characteristics at the molecular level (Bartonova et al., 2012; Bonfatti et al., 2010a). This polymorphism makes the kappa casein B protein smaller, which is caused by a change in the tertiary structure of the protein. As kappa casein B is smaller than A, it produces greater micelles with more content of kappa casein and other milk proteins responsible for cheese firmness (Bonfatti et al., 2010a; Prinzenberg et al., 2008).

LGB biomarker: beta-lactoglobulin

LGB gene is the bovine gene of beta-lactoglobulin, which has 15 alleles, the most common A and B. Both alleles differ by two single-nucleotide polymorphic mutations at the positions 64 (adenine by guanine) and 118 (guanine by thymine) (Ganai et al., 2009). These mutations produce an exchange of aspartic acid by glycine and valine by alanine in the protein chain, respectively (Patel et al., 2007). *LGB* gene is related to the yield of milk and the total amount of solids in milk (Zepeda-Batista et al., 2017). Allele B is related to the production of milk with better quality, as well as higher fat and protein contents (Bonfatti et al., 2010a; Soyudal et al., 2018; Strzalkowska et al., 2002; Zepeda-Batista et al., 2017). Additionally, B allele is also related to the production of milk with lower content of whey proteins, such as beta-lactoglobulin, and a higher content of caseins, which are essential for dairy production (Bedere & Bovenhuis, 2017; Robitaille et al., 2002). Beta-lactoglobulin is the major allergen in milk, given that it is not present in human milk (Høst, 2002). Therefore, it is guaranteed the selection of cattle that could produce milk with low content of beta-lactoglobulin.

PROBLEM STATEMENT

The national programs of cattle genetic improvement have increased the quantity of milk produced; that has, however, disregarded the improvement of milk quality and healthy consumption. It is necessary to establish new genetic improvement programs to improve the milk quality, in terms of the content proteins, lipids, lactose, and other solids. The genetic characterization of biomarkers might help to establish new cattle herds to produce milk of better quality. The milk of better quality will contribute to manufacture products with an extra value like the A2 milk, increase the yield of milk and dairy products, and have healthier milk for human consumption.

The objective of the study was to characterize three biomarkers to determine the allelic and genotypic frequencies of four herds of the primary dairy breeds in Ecuador. The aim in the long term is to characterize all the individuals to establish new herds to produce milk with the desired characteristics. The new herds might produce milk with better coagulation traits or a2 milk, which is healthier for human consumption.

GENERAL AND SPECIFIC OBJECTIVES

General objectives

- To determine the allelic and genotypic frequency of the following milk-related genes: *CSN2, CSN3*, and *LGB* in four herds from the main breeds for milk production in Ecuador, by molecular biology, means, which allow the genetic selection of new herds with improved traits for milk production.

Specific objectives

- Protocol standardization of the following techniques: restriction fragment length polymorphism (RFLP), sequencing, and TaqMan™ Real-Time PCR.
- Determine the genotyping frequency of the selected genes in the four herds under study.

METHODOLOGIES

Reference revision

It was crucial to determine the molecular biology techniques to be used in the genetic characterization of the herds, considering the equipment and reagents available in the research laboratories of the Universidad de las Américas (UDLA). We searched for papers in the databases or searched of Pubmed, Scopus, and Google Scholar. For the search, the were used the next keywords alone or combined were used: “kappa casein”; “beta-casein”; beta-lactoglobulin; “beta-casein” AND “polymorphism”; “kappa casein” AND “polymorphism” and “lactoglobulin” AND “polymorphism.”

Study sample

We select four cattle herds of the most frequent breeds for milk production in Ecuador. The four herds were selected because they were made up of purebred individuals, have individual both identification and control of the milk production. Moreover, the selected herds were implemented with periodic veterinary controls and artificial insemination programs, which would help establish new cattle herds amenable for future genetic evaluations. Table 1 compiles the information of the cattle herds analyzed.

Table 1. Summary of the characterized herds

Herd	Region	Location	Breed	Population Size	Sample Size
Herd 1	Andean	Pifo, San Javier Farm	Holstein	397	93
Herd 2	Coast	Santo Domingo, San Pedro farm	Jersey	16987	185
Herd 3	Andean	Aloag, the commune of Guagrabamba	Creole	232	92
Herd 4	Galapagos	Eight small farms around the Santa Cruz island	Creole	8000	93

Standardization of the molecular techniques

The genetic diagnosis requires the standardization of protocols and testing of reproducibility to obtain accurate results. To standardize the different techniques, we used as positive controls a small lot of samples (between 8 and 16), which genotypes were previously identified by DNA sequencing. We tested the reproducibility of the techniques

by successive characterizations of our lot of standardization. The standardization was performed until the time of performance of each technique was kept to a minimum (e.g., elimination of steps, reduction of time of incubation, increasing the number of processed samples), and the reproducibility reached 100%.

DNA extraction and purification

Blood samples were collected from the coccygeal tail vein in 4 ml EDTA vacutainers. The samples were transported to the laboratory at room temperature and stored at 4 °C until DNA extraction. DNA extractions were performed within a maximum of 48 h after blood withdrawal. For extractions, we used a set of buffers and protocols previously developed and tested in the UDLA research facilities, which is a modification of the Gene Clean® SPIN Glassmilk method. DNA isolation was evaluated through gel electrophoresis in order to determine the quantity of isolated DNA.

Gene amplification

Gene amplification for all the techniques used was performed by PCR where necessary. The gene fragments of interest, *CSN2* (362bp), *CSN3* (379bp), and *LGB* (252bp) were amplified using primers tested in previous studies (Table 2) (Nikšić et al., 2018; Rangel et al., 2017).

Each PCR reaction was performed using a final volume of 25 uL containing: 5,0 uL of genomic DNA (15-75ng/ul), 12,5 uL of GoTaq Green Master Mix (2X), 0,5 uL forward primers (10 uM), 0,5 uL reverse primers (10uM) and 6,5 uL of free nuclease water.

Table 2. Primers pairs used in the amplification by PCR.

Target	Direction	Sequence 5' 3'	Amplicon
CSN2	Forward	CTGGCTTTCAGTAAAGGGCTCAACTG	362 bp
	Reverse	TGACCCCAATTTCTTAACCAAACCAA	
CSN3	Forward	CACGTCACCCACACCCACATTTATC	379 bp
	Reverse	TAATTAGCCCATTTCGCCTTCTCTGT	
LGB	Forward	GTCCTTGTGCTGGACACCGACTACA	252 bp
	Reverse	CAGGACACCGGCTCCCGGTATATGA	

Table 3. Reagent concentrations and volume for PRC reactions

Reagent	Dissolution	Final Concentration	Volume

GoTaq® Green Master Mix	2x	1X	12.5uL
Primer Forward	10 uM	0.20 uM	0.5 uL
Primer Reverse	10 uM	0.20 uM	0.5 uL
Free DNAse water	-	-	6.5 uL
DNA sample	(17-75 ng/uL)	-	5.0 uL
Final Volume			25.0 uL

For the *CSN2* molecular fragment, we followed the thermal cycling parameters reported the table 3. For the molecular markers *CSN3* and *LGB*, we followed the thermal cycling parameters reported in Table 4. The amplified PCR products were visualized by electrophoresis on 1% agarose gels at 100 V and 70 A for 15 min using a 100-bp standard mass ladder (Invitrogen) in order to verify the concentration and quality of DNA existing in each sample.

Table 4. Thermal cycling conditions for PCR amplification of CSN2

Number of Cycles	Step	Temperature	Time
1	Initial denaturation	94°C	2 minutes
40	Denaturation	94°C	30 seconds
	Annealing	56°C	30 seconds
	Extension	72°C	30 seconds
1	Final extension	72°C	5 minutes

Table 5. Thermal cycling conditions for PCR amplification of CSN3 and LGB

Number of cycles	Step	Temperature	Time
1	Initial denaturation	94°C	2 minutes
40	Denaturation	94°C	30 seconds
	Annealing	60°C	30 seconds
	Extension	72°C	30 seconds
1	Final extension	72°C	5 minutes

Kappa-casein and lactoglobulin characterization

Restriction fragment length polymorphism

Restriction Fragment Length Polymorphism (RFLP) was the technique used to characterize the *CSN2* and *CSN3* milk molecular markers. In RFLP, DNA was digested using restriction enzymes, which are useful to recognize single nucleotide polymorphism (SNP) mutations and, therefore, different genotypes. The RFLP was performed in a final volume of 20 uL, containing: 10 uL of PCR product, 1,5 uL of restriction enzyme buffer, 7,5 ul of distilled water and 0,5 uL of restriction enzyme Hinf I or Hae III (10 U/uL), for *CSN3* or *LGB* respectively. An incubation for an hour at 37 °C allowed a suitable reaction. Finally, the fragments were analyzed by electrophoresis on 3% agarose gels at 100 V and 75 amperes for 35 min using a 100-bp standard mass ladder in order to determine the size of the bands and then the genotype. For each sample, it was observed different band patterns according to each genotype. For *CSN3* and *LGB,* we obtained the band patterns described in tables 6 and 7, respectively.

Table 6. Band patterns of the genotypes of CSN3.

Genotype	Band Pattern
AA	156bp, 132bp, 91bp
AB	288bp, 156bp, 132bp, 91bp
BB	288bp, 91bp

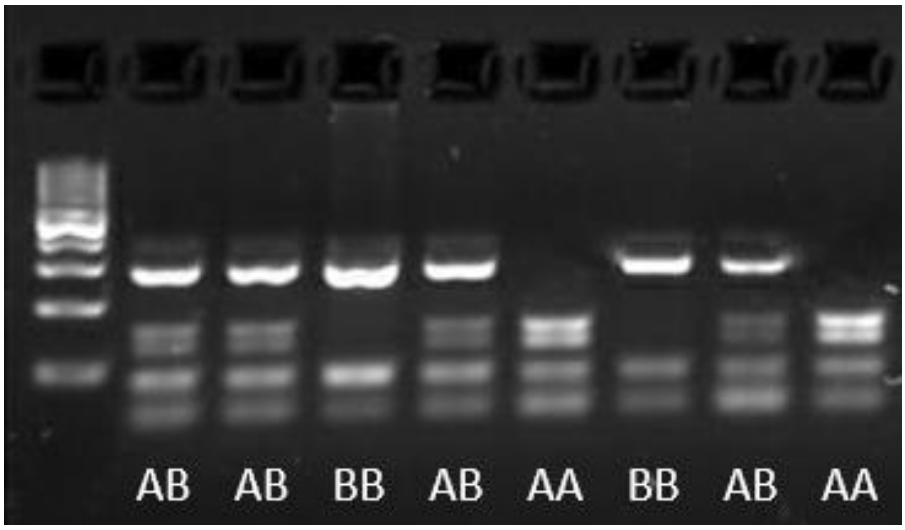

Figure 3. Gel electrophoresis of CSN3 gene digestion with a 100-bp ladder.

Table 7: Band patterns of the genotypes of the LGB. An example of gel electrophoresis is presented in Figure 4

Genotype	Band Pattern
AA	144bp,108bp
AB	144bp, 108bp, 74 bp, 70 bp
BB	108bp, 74bp, 70bp

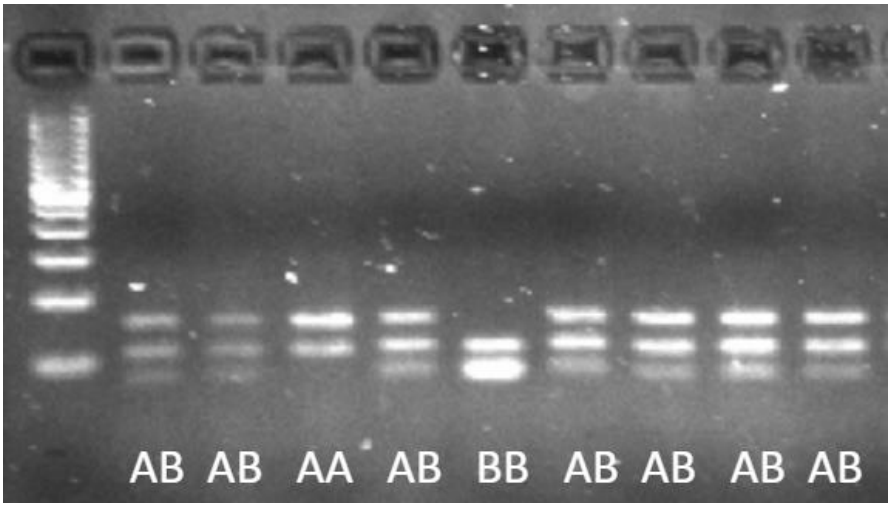

Figure 4. Gel electrophoresis of LGB gene digestion with a 100-bp ladder.

Beta-casein characterization

TaqMan assay and sequencing by capillary electrophoresis methods were selected to characterize the *CSN2* molecular marker.

Sanger sequencing by capillary electrophoresis

An initial purification step was run to eliminate the primer dimers that may affect the next PCR reaction step. A total of 10 ul of PCR product were purified by the AGENCOURT® AMPURE® XP magnetic beads kit following manufacturer instructions. The purified PCR product was amplified by PCR using the kit BigDye®Terminator v3.1, which is a PCR kit that includes fluorescent ddNTPs detected by the optical sensor of the sequencing equipment. The PCR was conducted using the reagent concentrations and volumes recommended by the kit manufacturer and under the thermal cycler parameters standardized by the sequencing service of the UDLA research laboratory. We used the kit AGENCOURT®

CLEANSEQ® Dye-Terminator Removal to eliminate ddNTPs, dyes, salts, and other reagents that would affect the reading of the optical sensor of the sequencing equipment, following the manufacturer directions as well as the protocol previously standardized by the sequencing service of the UDLA research laboratory. The protocol is not described because that information is confidential information of the UDLA research laboratory.

Finally, purified PCR products were sequenced by capillary electrophoresis (Sanger sequencing) in the Applied Biosystems® 3130 Genetic Analyze sequencer. We programmed the Genetic Analyzer Data Collection Software, according to the sequence analysis and the parameters (e.g., injection time, voltage) recommended by the manufacturer of the used PCR kits. The software Geneious prime 12 was chosen to determine alleles. In the option "Map to reference," we used the NC_037333.1 sequence (A2 allele) as the reference sequence for the alignment. The substitution C/A at the position 67 was used to classify the sequences in the different genotypes.

TaqMan Assay

The TaqMan Assay is a molecular biology technique used to amplify and detect specific alleles. We used primers and fluorescent probes previously designed by others (Manga & Dvorak, 2011) (Table 8). The TaqMan assay was run in the equipment CFX96 Touch Real-Time PCR Detection System in a final volume of 15 ul using the following reagents: 4.225 ul of ultra-pure water, 0.30 ul of forward primers (0.2uM), 0.30 of reverse primers (0.2uM), 0.225 ul of A1 probe (0.15uM), 0.45 ul of A2 probe (0.30 uM), 7.5 ul of Taqman Universal Master Mix II (1X), and 2 ul of DNA sample (PCR product) (Table 9).

Table 8. Primers and probes used for genotyping the CSN2 gene.

Name	Sequence 5' to 3'
Primer forward	CTTTGCCCAGACACAGTCTCTAGT
Primer reverse	GCACCACCACAGGGGTT
Probe A1	FAM-CTGGACCCATCCATAACAGCCTCCCABBQ-3
Probe A2	ROX-TGGACCCATCCCTAACAGCCTCCC-BBQ

To standardize the TaqMan Assay, we started with the final concentrations per reaction and thermal cycling parameters recommended by the manufacturer of the Taqman Universal

Master Mix II. The TaqMan assay was performed according to the thermal cycler parameters recommended by the manufacturer of the TaqMan Universal Master Mix II.

Table 9. Reagent concentrations and volume for the TaqMan assay.

Reagent	Dissolution	Final Concentration	Volume
Ultrapure water	-	-	4.225 uL
Primer forward	10 uM	0.20 uM	0.300 uL
Primer reverse	10 uM	0.20 uM	0.300 uL
A1 probe	10 uM	0.15 uM	0.225 uL
A2 probe	10 uM	0.30 uM	0.450 uL
TaqMan Universal Master Mix	2X	1X	7.500 uL
DNA sample	-	-	2.000 uL
Final Volume			15.000 uL

The A1 and A2 probes have the FAM and HEX dyes, respectively. In the Taq-Man assay, it was proceeded with amplification and union of specific probes according to the genotype of each sample. In the heterozygote samples, both fluorescences were detected because of the amplification of both A1 and A2 alleles. In homozygotes samples, just one single fluorescence was detected because of the amplification of two identical alleles. The Figure 3 shows the allelic discrimination chart. The X-axis represents the Relative Fluorescence Units (RFU) measured in the FAM spectra, and the Y-axis, the RFU measured in the HEX spectra. The software processed the values of RFU by cluster analysis, thus giving the genotype of each sample. The figure 5 shows four clusters: A2A2 samples (blue), green A1A2 samples (green), A1A1 samples (orange), and a no template control or NTC (black).

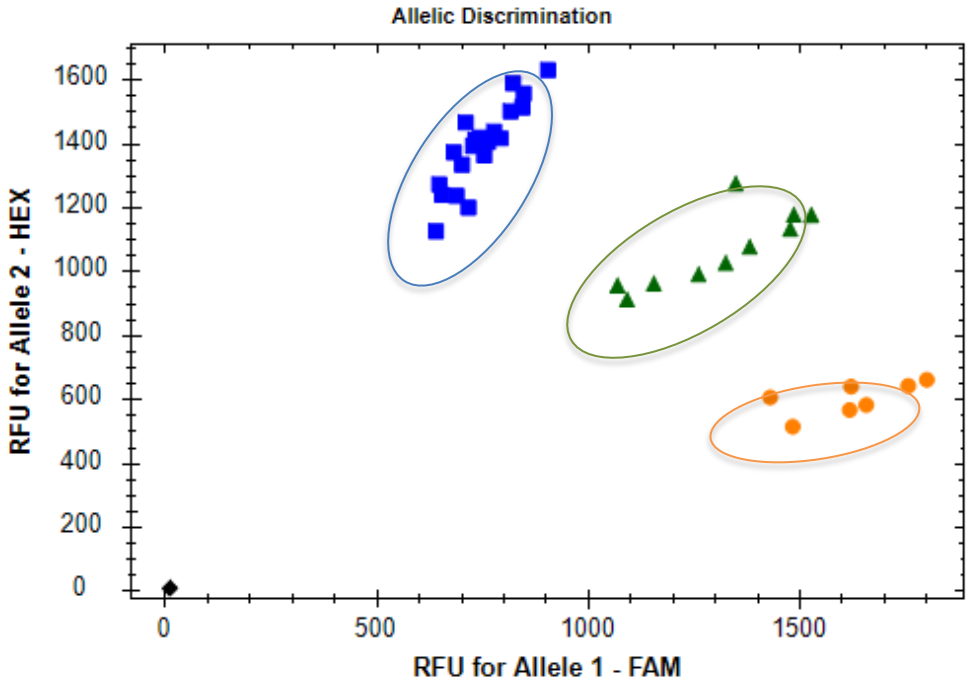

Figure 5. TaqMan Assay allelic discrimination

Statistical analysis

The Hardy-Weinberg Equilibrium (HWE) was applied to the analysis of the final genotypic frequencies to determine whether the genetic variation would be kept constant (equilibrium) from one generation to another, or by contrast, they were distributed by mutations, natural selection, non-random mating, genetic drift, and gene flow. The different allelic and genotypic frequencies obtained from each cattle herd were analyzed using an online software (http://www.husdyr.kvl.dk/htm/kc/popgen/genetik/applets/0.htm; Christensen, 2020). A confidence level of 95% and one degree of freedom were used for all the HWE analyses (alpha value set at 0.05).

RESULTS AND DISCUSSION

***CSN2* biomarker**

The results of the characterization of *CSN2* are summarized in tables 10, 11, and 12. All the herds analyzed showed a higher genotypic frequency of individuals with the heterozygote A1A2 genotype except for the herd 2, which had high numbers of individuals with genotype A2A2, as expected by previous studies reporting a higher percentage of individuals with the

genotype A1A2 in Jersey populations (Demirel & Çak, 2018; Zepeda-Batista et al., 2015). With the exemption of herd 1, the rest of the herds had a higher percentage of individuals with allele A2. The allele frequency varied from 0.4881 to 0.7222. Expected and observed genotype frequencies were determined by HWE and are summarized in table 7. The HWE analysis showed that all the herds were in equilibrium ($p > 0.05$), which means that the next generation might have similar genotypic frequencies (see table 8).

Table 10. Allelic and genotypic frequencies of the CSN2 gene

Herd	Breed	Observed frequency				Allelic frequency	
		A1A2	A1A1	A2A2	Total	A1	A2
Herd 1	Holstein	23	10	9	42	0,5119	0,4881
Herd 2	Jersey	43	11	63	117	0,2778	0,7222
Herd 3	Creole	21	9	13	43	0,4535	0.5465
Herd 4	Creole	21	5	20	46	0,3370	0,6630

Table 11. Observed and expected genotype frequencies of the CSN2 gene

Herd	A1A2		A1A1		A2A2	
	Observed	Expected	Observed	Expected	Observed	Expected
Herd 1	23	20,9881	10	11,0060	9	10,0060
Herd 2	43	46,9444	11	9,0278	63	61,0278
Herd 3	21	21,3140	9	8,8430	13	12,8430
Herd 4	21	20,2228	5	5,2280	20	20,2280

Table 12. Values calculated for Hardy-Weinberg Equilibrium of the CSN2 gene.

Herd	Chi-squared value	p-value	Hardy Weinberg Equilibrium
Herd 1	*0,3859*	*0,5344*	*YES*
Herd 2	*0,8260*	*0,3634*	*YES*
Herd 3	*0,093*	*0,9200*	*YES*
Herd 4	*0,0216*	*0,8830*	*YES*

CSN3 biomarker

The results of the HWE of *CSN3* are summarized in tables 13, 14, and 15. All the populations showed a high genotypic frequency of the heterozygote genotype AB, as expected from previous studies (Patel et al., 2007)(Zepeda-Batista et al., 2015). The herd 4 originally from San Cristobal showed 100% of the heterozygote genotype AB, which is particular. 100 % of heterozygote individuals were strikingly impossible because these particular cows were inseminated artificially using semen from different sources. We performed the test twice and obtained the same results. We just found a report of a whole population of heterozygote individuals (Mehta et al., 2007). The only plausible explanation was a failure in the performance of the PCR-RFLP, so we propose to use different reagents or another method for alternative analysis for future studies. The bioinformatics analysis of some sequenced samples from herd 4 shows that will exist an allele different to A or B; we can determine which is the allele because it is necessary to sequence a larger fragment of CSN3. There is a significant difference between the expected and observed genotypes values of the herds 2 and 4. The HWE shows that herd 2 and 4 do not follow the HWE ($p < 0.05$), which means that these populations will not have the same genotypic frequency in the next generation.

Table 13. Allelic and genotypic frequencies of the CSN3 gene.

Herd	Breed	Observed genotypes				Allelic frequency	
		AB	AA	BB	Total	A	B
Herd 1	Holstein	43	37	8	88	0,6648	0,3352
Herd 2	Jersey	117	35	32	184	0,5082	0,4918
Herd 3	Creole	47	27	18	92	0,5489	0,4511
Herd 4	Creole	85	0	0	85	0,5000	0,5000

Table 14. Observed and expected genotype frequencies of the CSN3 gene.

Herd	AB		AA		BB	
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Herd 1	43	39,2216	37	38,8892	8	9,8892
Herd 2	117	91,9755	35	47,5122	32	44,5122
Herd 3	47	45,5598	27	27,7201	18	18,7201
Herd 4	85	42,5000	0	21,2500	0	21,2500

Table 15. Values calculated for Hardy-Weinberg Equilibrium of the CSN3 gene.

Herd	Chi-squared value	p-value	Hardy Weinberg Equilibrium
Herd 1	0,8167	0,3661	YES
Herd 2	13,6207	0,0002	NO
Herd 3	0,0919	0,7617	YES
Herd 4	85,0000	0,0000	NO

***LGB* biomarker**

The results of the HWE of the *LGB* gene are summarized in tables 16, 17, and 18. All the herds had a higher genotypic frequency than 0.45 of the heterozygote genotype AB, as reported in previous studies (Čítek et al., 2019) (Bonfatti et al., 2010a)(Barbosa et al., 2019). Our results differ from previous studies were the genotypic frequencies of the Homozygotes B were higher (Karimi et al., 2009) (Zepeda-Batista et al., 2015). Expected and observed genotypic frequencies are significant differences, then we expected that the four populations did not follow the HWE rule. The calculated chi-square values were high and p-values < 0.05 and closed to zero, which means that the four populations do not follow the HWE rule. In other words, these populations may not have the same genotypic frequency in the next generation.

Table 16. Allelic and genotypic frequencies of the LGB gene.

Herd	Breed	Observed genotypes				Allelic frequency	
		AB	AA	BB	Total	A	B
Herd 1	Holstein	76	5	12	93	0,4624	0,5376
Herd 2	Jersey	122	22	41	185	0,4486	0,5514
Herd 3	Creole	52	9	24	85	0,4118	0,5882
Herd 4	Creole	79	3	11	93	0,4570	0,5430

Table 17. Observed and expected genotype frequencies of the LGB gene.

Herd	AB		AA		BB	
	Observed	Expected	Observed	Expected	Observed	Expected
Herd 1	76	46,2366	5	19,8817	12	26,8817
Herd 2	122	91,5243	22	37,2378	41	56,2378
Herd 3	52	41,1765	9	14,4118	24	29,4118
Herd 4	79	46,1559	3	19,4220	11	27,4220

Table 18. Values calculated for Hardy-Weinberg Equilibrium of the LGB gene.

Herd	Chi-squared value	p-value	Hardy Weinberg Equilibrium
Herd 1	38,5370	0,0000	NO
Herd 2	20,5119	0,0000	NO
Herd 3	5,8730	0,0154	NO
Herd 4	47,0915	0,0000	NO

CONCLUSIONS AND RECOMMENDATIONS

The standardization and test of the reproducibility of methodologies are necessary to make an accurate genetic diagnosis. All the techniques used in this investigation require standardization. In addition, it is also necessary to have the necessary reagents and equipment complemented with minimally trained people

The TaqMan assay for the characterization of SNPs is the better option to characterize larger populations. *CSN2* polymorphism can be used to characterize more individuals and establish new herds of A2A2 cows for the production of A2 milk. The TaqMan assay would be useful to characterize *CSN3* and *LGB* but may require the design of four probes due to both have at least two poymorphisms.

In the case of β-Cas, a high frequency of the A2 allele was found in the Jersey herd (herd 2), which allows the farmer to breed a new herd to produce healthier milk without gastrointestinal effects caused by the BMC-7 molecule. The rest of the herds have little cows with the A2A2 genotype. It is recommended to increase the number of individuals in a further genotyping characterization to establish new pure breeds.

A higher frequency of the AB allele was found in the genetic characterization of *CSN3* and *LGB*. In order to improve the milk quality of these cows for cheese production, the semen of bulls with BB genotype and the cows with the BB genotype should be used to establish a genetic improvement program.

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ANNEXES

Anexxe A: List of the genotype of each individual

Herd 1

Plate ID	Sample ID	Kappa	Lacto	Beta
6	85RITA	AB	AB	
6	73 LINDA	AB	AB	
6	61 COCA	BB	AB	
6	49 IRMA	AA	AB	
6	37 OLIVA	AB	AB	
6	25 TUCA	AA	AB	
6	13 POLA	AA	AB	
6	1 LIA	AA	AB	
6	86 TINA	AA	AB	
6	74 MACA	AB	AB	
6	62 NICOL	AB	AB	
6	50 PATA	AB	AB	
6	38 RODIO	AB	AB	
6	26 NELLY	AB	AB	
6	14 MORA	BB	AB	
6	2 TUNA	BB	AB	
6	87 FIEL	R	AB	
6	75 ALBA	AA	AB	
6	63 NONA	AB	AB	
6	51 MARTH	AB	AB	
6	39 BACHA	R	AB	
6	27 ALIZ	AB	AB	
6	15 PANA	AB	AB	
6	3 SALI	AB	AB	
6	88 JULIA	AA	AB	
6	76 JUCA	AA	AB	
6	64 DALIA	AB	AB	
6	52 FIFI	AB	AB	
6	40 CORA	AB	AB	
6	28 NOVIA	AA	R	
6	16 MINA	AB	AB	
6	4 NENA	BB	AB	
6	89 RAYA	AA	AB	
6	77 AIDA	AB	BB	
6	65 FELIX	AA	BB	
6	53 DORA	BB	AB	
6	41 RENE	R	AB	

Plate ID	Sample ID	Kappa	Lacto	Beta
6	29 FANNY	R	AB	
6	17 EVA	BB	AB	
6	5 ODA	AA	AB	
6	90 BETA	AB	AB	
6	78 FIERA	AA	AB	
6	66 RUBI	AA	AB	
6	54 GUIDA	AA	AB	
6	42 POCHA	AB	AB	
6	30 LAZY	AA	AB	
6	18 AZUL	AA	AB	
6	6 BERTHA	AB	AB	
6	91 DONA	AB	AB	A1A1
6	79 LISA	AA	BB	A2A2
6	67 HILDA	AB	AB	A2A2
6	55 LULI	AB	AB	A1A2
6	43 FAUNA	AB	AB	A1A2
6	31 PUCA	AA	AB	A1A1
6	19 IRENE	AB	AB	A1A2
6	7 NORA	AB	AB	A1A2
6	92 WAKY	AB	AB	A1A1
6	80 DANI	AA	AB	A2A2
6	68 CITA	AB	AB	A1A1
6	56 ROCIO	AA	AB	A1A2
6	44 LUCY	AB	AB	
6	32 ANABE	AA	AB	A1A2
6	20 BOLA	R	AB	A1A1
6	8 ELI	R	AB	A1A2
6	93 KIRKY	BB	AB	A1A1
6	81 LOJA	AA	AA	A1A2
6	69 ISABEL	AB	BB	A1A2
6	57 FLOR	AA	AB	A2A2
6	45 MARU	AB	AB	A2A2
6	33 ALIZ	R	BB	A1A2
6	21 SAMI	AA	AB	A1A2
6	9 TOÑA	AB	AB	
6	94 VIVA	AA	AA	
6	82 DOLI	AA	AB	A2A2
6	70 MATY	AA	AB	A1A2
6	58 JORA	AA	AB	A1A2
6	46 LEA	AB	BB	A1A2
6	34 RANA	AB	AB	A1A2
6	22 ISLA	BB	AB	A1A2
6	10 JAMA	AB	AB	A1A2
6	95 INES	AA	AB	A1A2
6	83 MICHI	AA	BB	A1A1

Herd 2

Sample ID	ID in plate	Kappa	Lacto	Beta
1	D9	AB	BB	
2	B9	AA	AB	
3	B10	AA	AB	
3	D12	AB	AB	A2A2
4	G9	AB	AB	
5	G4	AB	AB	
7	A11	BB	BB	A2A2
7	H7	AB	AA	
8	B12	AB	BB	A2A2
8	F7	AB	AB	
9	B11	AA	AB	A1A1
9	E8	AA	BB	
10	C7	AB	BB	
11	E9	AA	AB	
12	C9	AA	AB	
13	A10	AB	BB	
14	H9	AA	AB	
15	G5	AA	AB	
16	E7	AB	AB	
17	D7	AA	AB	
17	H10	AB	AB	
17	H2	AB	AB	A2A2
18	A8	AA	BB	
18	C12	AB	AB	A2A2
19	C11	AB	AB	
19	C8	AA	BB	
20	C10	BB	AB	
20	G8	AB	AB	
21	H4	AB	AA	
22	H8	AA	AB	
23	F9	AB	AB	
24	A9	AA	AB	
24	E12	AB	AB	A2A2

Sample ID	ID in plate	Kappa	Lacto	Beta
25	E10	AB	AB	
27	G7	AB	AB	
28	A1	BB	AB	A2A2
29	F8	AB	AB	
30	B8	AB	AB	
31	B5	AB	AB	
32	A5	AA	AB	
33	F4	AB	BB	A1A2
34	D5	BB	AB	
35	B7	AB	BB	
36	H6	AB	AB	
37	H5	AB	AB	
38	E4	BB	AA	
39	C4	AB	AB	A2A2
40	D11	AB	AB	
40	D4	AB	AB	
41	F5	AA	BB	
43	C5	AA	AB	
44	E5	AB	AB	
45	G10	AB	AB	
46	H11	BB	AB	
47	F11	AB	AB	
53	G11	AB	BB	
55	A12	AA	BB	A2A2
56	F10	AB	BB	
57	H1	AA	AB	
58	A1	BB	AB	
59	A2	AB	BB	
60	A3	BB	BB	
61	A4	BB	AB	
62	A5	AB	AB	
63	A6	AB	AB	
64	A7	BB	AB	
65	F2	BB	AA	A1A1
67	F3	AB	AA	A2A2

Sample ID	ID in plate	Kappa	Lacto	Beta
69	A9	AB	AB	
70	A10	AB	AA	
71	F7	AB	BB	A2A2
72	A12	AB	AB	
73	B1	AB	BB	
74	B2	AB	AB	
75	A8	AB	AB	A1A2
76	A9	AB	AB	A2A2
77	B5	AB	AB	
78	A11	AB	BB	A2A2
79	F10	AB	AB	A2A2
80	E12	AB	AB	A2A2
81	F1	AB	BB	A2A2
82	F5	AB	BB	A2A2
83	A3	BB	AB	A1A2
84	A4	BB	BB	A1A1
85	A6	AB	AA	A1A2
86	A5	AB	AA	A2A2
87	A2	AA	AB	A2A2
88	A1	AB	AB	A1A2
89	B10	AB	AB	A2A2
89	B2	AB	AB	A1A2
90	B11	AB	AB	A2A2
90	B1	BB	AB	
91	B3	AB	AB	A1A2
91	F6	AB	AB	
92	B12	AA	BB	A2A2
92	E6	AB	AB	
93	C1	AB	AB	A1A2
93	A6	AB	BB	
94	C3	BB	AB	A1A2
95	C2	AB	AB	A2A2
95	G6	AA	AA	
96	C5	AB	AA	A2A2
96	D8	AB	BB	

Sample ID	ID in plate	Kappa	Lacto	Beta
97	C4	AA	BB	A1A2
97	D2	AB	BB	
98	C1	AB	BB	A2A2
99	A12	AB	AA	A2A2
99	C2	BB	AB	A2A2
100	B6	AB	AB	A1A2
100	D10	AB	AB	
101	B2	BB	AB	A2A2
101	C6	BB	AB	
102	B5	AB	AB	A1A2
102	B6	BB	AB	
103	B4	AB	BB	A1A2
105	B7	AA	AB	A1A2
105	D6	AA	BB	
106	B6	BB	AB	A1A2
107	B8	BB	AB	A1A2
109	B9	AB	BB	A1A2
110	C7	AB	AB	A1A2
111	D11	AB	AB	A2A2
112	C9	AB	AB	A2A2
113	C6	AB	AB	A2A2
114	C11	BB	BB	A1A2
116	C8	AA	BB	A2A2
117	D1	BB	AB	A2A2
118	C10	AB	AB	A2A2
119	C12	AB	BB	A1A1
120	F1	AB	BB	A2A2
122	D1	AA	AB	A2A2
123	D3	AB	AB	A2A2
123	G2	AB	AB	
124	D2	AB	AB	A1A1
125	D5	BB	AB	A2A2
125	G3	AB	AB	
126	D4	AA	BB	A2A2
126	E2	AA	AB	A1A1

Sample ID	ID in plate	Kappa	Lacto	Beta
127	D7	AB	BB	A1A2
127	A4	AB	AA	
128	D6	BB	AA	A2A2
128	H3	AB	BB	
129	D9	AB	AA	A1A2
129	A3	BB	AB	A2A2
129	E3	AB	AA	A2A2
130	D8	AA	AB	A1A2
130	D3	AB	AA	A2A2
131	F3	BB	AB	A2A2
132	D10	AB	AB	A2A2
132	B4	BB	AB	
133	D12	AB	AB	A1A2
133	F2	AA	BB	A1A2
134	E1	AA	AB	A1A2
135	E6	BB	AB	A2A2
135	B3	AB	AB	A2A2
136	E5	AB	AB	A1A2
136	C3	AB	AA	A1A2
137	G1	AA	AB	A1A2
138	E1	AB	BB	
139	E2	AB	AB	A2A2
139	A2	AB	AA	A2A2
140	E3	AA	AB	A1A2
141	E4	BB	AB	A2A2
142	E8	BB	AB	A2A2
143	E11	AB	AB	A2A2
145	E9	AB	AB	A1A2
147	E10	AB	AB	A1A2
148	E7	AB	AA	A2A2
149	F11	AB	AB	A1A2
150	G7	AB	AB	
152	G2	BB	AA	A2A2
153	G3	AB	AB	A2A2
155	G4	BB	AB	A1A1

Sample ID	ID in plate	Kappa	Lacto	Beta
156	G5	AB	AA	A2A2
157	G6	AB	AB	A1A1
158	G7	BB	AB	A2A2
159	G8	AB	AB	A2A2
160	G9	AB	AA	A1A2
161	G10	AB	AB	A2A2
162	G11	AB	AB	A2A2
163	G12	AB	AA	A2A2
164	H1	AB	BB	A1A2
164	A7	BB	AB	
165	H2	AB	AB	A1A1
166	H3	AB	AB	A1A2
167	H4	AB	BB	A1A2
168	H5	AB	AB	A1A2
169	H6	AB	AB	A2A2
170	H7	AB	AB	A1A2
171	H8	AA	BB	A1A2
172	H9	AB	AB	A2A2
173	H10	AB	AB	
175	H11	AB	BB	A1A1
407	D9	AB	AB	
15C	A7	AB	AB	A2A2
25C	A10	AB	AB	A1A2
35C	F4	AA	AB	A2A2
45C	F6	AB	AB	A1A2
55C	F8	AB	AB	A1A1
65C	F9	AB	BB	A2A2
75C	F12	BB	BB	
85C	G12	AB	AB	

Herd 3

Sample ID	ID in plate	Kappa	Lacto	Beta
5	72 Esperar	AA	AB	A2A2
5	61 Esperar	AB	AB	A1A2
5	54 Chiquita	AA	AB	A1A2
5	49 Morena	AA	AB	A1A2
5	38 Quiteña	AB	AB	A1A2
5	21 Niña	AB	AB	A1A2
5	09 Tortuga	AB	R	A1A2
5	01 Morocha	AB	AB	A2A2
5	73 Cori	BB	AB	A1A2
5	64 Bambi	AB	AB	
5	55Rosa	BB	AB	
5	49 Canela	AB	AB	
5	38 Romina	AB	AB	A1A2
5	23 Bella	AB	R	A2A2
5	10 Fortuna	BB	AB	A1A1
5	02 Bermeja	AB	AB	A1A1
5	65 Xfavor	AA	BB	A1A2
5	56 Lluvia	AA	AB	A1A2
5	49 Gitana	AB	AB	A1A2
5	39 Karla	BB	AB	A2A2
5	24 Ines	BB	AB	A2A2
5	02 Ilusion	AB	R	A2A2
5	74 Rafaela	AB	AA	A1A1
5	65 Negra	AA	AA	A1A2
5	57 Princesa	AA	AB	A1A2
5	49 Cristal	AB	R	A2A2
5	39 Marisol	AB	AB	A1A1
5	26 Lunes	BB	AB	A1A2
5	10 Tañita	AB	R	A1A1
5	02 Cariño	BB	R	A1A2
5	77 Cocinera	AB	BB	A2A2
5	65 Esperar	BB	AB	A1A2
5	57 Zafiro	AA	AB	A1A1
5	49 Bonita	AB	AB	A2A2
5	40 Canela	AA	AB	A1A1
5	27 Milagros	BB	BB	A1A2
5	12Estrella	BB	BB	A1A1
5	02 Linda	AA	AA	A1A2
5	77 Pulguita	AB	BB	A2A2
5	66 Juliana	BB	BB	A1A2
5	57 Azucena	AA	AB	A1A2
5	49 Margarit	AB	BB	A2A2

Sample ID	ID in plate	Kappa	Lacto	Beta
5	40 Junaly	AA	BB	A1A2
5	28 Sami	BB	BB	A2A2
5	14 Mary	BB	AB	A2A2
5	03 Chavela	AA	AA	A1A1
5	77 Princesa	AA	BB	
5	67 Preciosa	AB	AB	
5	57 Troya	AA	BB	
5	50 Ploma	AA	AB	
5	41 Amore	AB	AB	
5	30 Shakira	AB	AA	
5	14 Karla	AB	AB	
5	04 Julieta	AA	AB	
5	77 Toñita	AB	BB	
5	68 Chola	BB	AB	
5	58 Cholita	AA	AB	
5	50 Negra	AA	BB	
5	41 Mishel	AB	BB	
5	30 Marques	BB	AB	
5	16 Pataqui	AA	AB	
5	5 Linda	AB	AB	
5	78 Genoa	AB	BB	
5	68 Azucena	AB	BB	
5	58 Colorada	AA	BB	
5	52 Vicenta	AB	BB	
5	41 Cariño	AB	AB	
5	33 Diana	AB	AA	
5	16 Vanda	AB	AB	
5	5 Dorada	AB	AA	
5	79 Gardeña	AA	AB	
5	59 Estrella	BB	BB	
5	52 Bonita	AB	AB	
5	46 Victoria	AB	BB	
5	33 Esperan	AA	AB	
5	17 Zapatera	AB	AA	
5	7 Blaki	AB	AB	
5	80 Colorada	AB	R	
5	69 Carame	BB	BB	
5	59 Blanca	AB	AB	
5	53 Josefa	R	AA	
5	48 Bony	AA	AB	
5	36 Purita	AB	BB	
5	20 Estrella	AA	AB	
5	05Chola	AB	BB	
5	80 Campec	AB	AB	
5	71 Martina	AB	AB	

Sample ID	ID in plate	Kappa	Lacto	Beta
5	60 Cielo	AA	R	
5	54 Margarit	AB	AB	
5	49 Lulu	AB	AB	
5	38	AB	BB	
5	20 Canela	BB	AB	
5	7 Manuela	AA	AB	

Herd 4

Plate ID	Sample ID	Kappa	Lacto	Beta
6	85RITA	AB	AB	A2A2
6	73 LINDA	AB	AB	A2A2
6	61 COCA	AB	AB	A2A2
6	49 IRMA	R	AB	A2A2
6	37 OLIVA	AB	AB	A2A2
6	25 TUCA	AB	AB	A1A2
6	13 POLA	AB	AB	A1A2
6	1 LIA	AB	BB	A1A2
6	86 TINA	R	AB	A1A2
6	74 MACA	AB	AB	A2A2
6	62 NICOL	AB	AB	A1A1
6	50 PATA	AB	AB	A1A2
6	38 RODIO	R	AB	A1A2
6	26 NELLY	AB	BB	A1A1
6	14 MORA	AB	AB	A1A2
6	2 TUNA	AB	AB	A1A2
6	87 FIEL	AB	AB	A1A2
6	75 ALBA	AB	AB	A2A2
6	63 NONA	AB	AB	A1A2
6	51 MARTH	AB	AB	A1A2
6	39 BACHA	R	AB	A2A2
6	27 ALIZ	AB	R	A2A2
6	15 PANA	AB	AB	
6	3 SALI	AB	AB	A1A2
6	88 JULIA	AB	AB	A1A2
6	76 JUCA	R	R	
6	64 DALIA	AB	AB	A2A2
6	52 FIFI	AB	AB	A2A2
6	40 CORA	R	AB	A1A2
6	28 NOVIA	AB	AB	A2A2
6	16 MINA	AB	AB	A2A2
6	4 NENA	AB	AB	A2A2
6	89 RAYA	AB	AB	A2A2
6	77 AIDA	AB	AB	A1A1
6	65 FELIX	AB	AB	A2A2
6	53 DORA	AB	BB	A2A2
6	41 RENE	AB	BB	A1A2
6	29 FANNY	AB	AB	A1A1
6	17 EVA	AB	AB	A2A2
6	5 ODA	AB	AB	A1A2
6	90 BETA	AB	BB	A2A2

6	78 FIERA	R	AB	A1A2
6	66 RUBI	AB	BB	A1A2
6	54 GUIDA	AB	AA	A1A2
6	42 POCHA	AB	BB	A1A2
6	30 LAZY	AB	AB	A1A2
6	18 AZUL	AB	AB	A1A1
6	6 BERTHA	AB	AB	A2A2
6	91 DONA	AB	BB	
6	79 LISA	AB	AB	
6	67 HILDA	AB	AB	
6	55 LULI	AB	AB	
6	43 FAUNA	AB	AB	
6	31 PUCA	AB	AB	
6	19 IRENE	AB	AB	
6	7 NORA	AB	AB	
6	92 WAKY	AB	AB	
6	80 DANI		AB	
6	68 CITA		AB	
6	56 ROCIO	AB	AB	
6	44 LUCY		AB	
6	32 ANABE	AB	AB	
6	20 BOLA	AB	AB	
6	8 ELI	AB	AB	
6	93 KIRKY	AB	AB	
6	81 LOJA		AB	
6	69 ISABEL	AB	AB	
6	57 FLOR	AB	AB	
6	45 MARU	AB	AB	
6	33 ALIZ	AB	AB	
6	21 SAMI	AB	AB	
6	9 TOÑA	AB	AB	
6	94 VIVA	AB	AB	
6	82 DOLI	AB	BB	
6	70 MATY	AB	AB	
6	58 JORA	AB	R	
6	46 LEA	AB	AB	
6	34 RANA	AB	AB	
6	22 ISLA	AB	AB	
6	10 JAMA	AB	AB	
6	95 INES	AB	AA	
6	83 MICHI	AB	AA	
6	71 TERE	AB	AB	
6	59 ANGEL	AB	AB	
6	35 GRIS	AB	AB	
6	47 YUCA	AB	AB	

Plate ID	Sample ID	Kappa	Lacto	Beta
6	3 LAURA	AB	AB	
6	11 VITA	AB	AB	
6	95 INES	AB	AB	
6	84 NANCI	AB	AB	
6	72 ROSI	AB	BB	
6	60 ASIA	AB	AB	
6	48 PINTA	AB	AB	
6	36 MAYTE	AB	AB	
6	24 ONIX	AB	BB	
6	12 CHULA	AB	AB	