ORIGINAL ARTICLE

Frequency distribution of polymorphisms on κ-casein and DGAT1 genes in dairy cattle used in Chilean milk production

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ABSTRACT. Milk solids are very important to transform milk in dairy products like cheese. Several genes and polymorphic variants had been associated with this process, increasing the milk fat and/or protein content or regulating the coagulation milk properties. In the present study, we evaluate the frequency distribution of two major polymorphisms present in *DGAT1* and *CSN3* genes in 6 dairy biotypes commonly used in southern Chile: Holstein-Friesian (HF), Jersey (JE), Montbeliarde (MB), Overo Colorado (OC), Frisón Negro (FN) and hybrids (HYB). For *CSN3* the results revealed that the A variant was predominant (0.57 to 0.71) in all breeds except Jersey which showed a high frequency of the B variant (0.73), one that favors milk transformation, and in MB (0.58). For *DGAT1* a similar trend was seen. The A variant predominated (0.64 to 0.96) in all populations except Jersey, which displayed a high frequency (0.70) of the K variant that favors milk solids. The analysis of FIS for both markers gave negative and non-significant values in all populations some exception of OC and HYB, which are not in Hardy-Weinberg equilibrium, suggesting an excess of heterozygotes. By another hand, FST analysis suggest a high genetic structure for Jersey. The simultaneous analyses of both markers produced 16 combined genotypes with hybrid animals displaying the highest number (11) whereas Jersey showed a favorable combination (*DGAT1* KK and *CSN3* BB) renewing their productive orientation to transform milk to cheese. Also, some Holstein animals displayed a heterozygous combined genotype (*DGAT1* A/K–*CSN3* AB). For decades, Holstein-Friesian has been an important breed used for milk production in southern Chile and during the last time, several farmers have introduced Jersey in order to improve milk quality. In this sense, our results show the presence of potential favorable genotypes for the transformation of milk to dairy products.

Keywords: Dairy cattle, milk solids, molecular markers, kappa-casein, dgat1.

INTRODUCTION

Bovine milk is one of the major foods included at healthy recommendations for human consumers. With a wide composition, it contains macro and micronutrients that are fundamental for our health and development. Within these, milk protein and milk fat are key components due to contribution of essential aminoacidic and fatty acids (Gibson, 2011). Both of them make up the majority of the well-known milk solids and are important for the technological transformation of milk to cheese and other dairy products (Cipolat-Gotet *et al*., 2018).

The content of milk solids is determined by two major factors: nutrition and genetics (Osorio *et al*., 2016). Thus, currently one of the more important dairy breeding's objectives at world and national level is the increase in the milk solids content (Uribe *et al*., 2017).

From a genetic perspective, the presence of variants in several milk proteins and major enzymes regulating milk fat synthesis and secretion has generated several studies related to the identification of molecular markers, mainly SNPs but also candidate genes to modulate milk composition (Caroli *et al*., 2009). This relationship between markers and production traits has been widely strengthened with the arriving of genomics, which allows the association of thousands of markers along the cattle genome (Gebreyesus *et al*., 2019; Sanchez *et al*., 2019).

An important milk protein is kappa casein (κ-casein), which is related to milk production, composition, and coagulation properties, playing a direct role in the manufacturing process of milk products like cheese (Bonfatti *et al*., 2010; Vallas *et al*., 2012). This protein is encoded by *CSN3* gene, which is highly polymorphic and being the A and B variants the most important ones. Those animals carrying the BB genotype show a milk with increased levels of κ-casein and a lower micelles size, reducing the clotting time and promoting the firmness of the rennet (Cipolat-Gotet *et al*., 2018). On the other hand, animals with AA genotype are related to higher milk production but lower clotting time (Caroli *et al*., 2009).

Respect to milk fatty acids, *DGAT1* gene codes for diacyglicerol:acyl CoA acyltransferase 1 (EC 2.3.1.20; Cases *et al*., 1998), the limiting enzyme for triglycerides biosynthesis at mammary gland. This gene shows a polymorphism (K232A) with a major effect on milk composition (Grisart *et al*., 2004). The mutation corresponds to an aminoacidic change of lysine (K) to alanine (A) which can explain up to 50% of variation of milk fat content. It is widely described that those cattle carrying the K allele show a high yield and content of milk fat, whereas those with the A variant display a high milk yield (Grisart *et al*., 2004; Carvajal *et al*., 2016).

In southern Chile several cattle breeds are used for milk production including worldwide and local adapted breeds. In addition, today exists a growing interest to increase milk solids. This is given by a greater incentive from industry in the payment schedule. Therefore, the

objective of this study was to evaluate the distribution of allelic frequencies of *CSN3* and *DGAT1* polymorphisms in representative cattle breeds used for milk production.

MATERIAL AND METHODS

ANIMAL SAMPLES

The study included 263 dairy cattle distributed in six different herds located in Los Ríos and Los Lagos regions, Chile. These animals had 2-4 parturitions and belonged to five different dairy breeds: Holstein-Friesian (HF; n= 56), Jersey (JE; n= 41), Montebeliarde (MB; n= 39), Overo Colorado (OC; n= 44) and Frisón Negro (FN; n= 46) plus other group called hybrid (HYB; n=38) corresponding to crosses between HF and OC. Individual blood samples from each animal were obtained by caudal puncture using a venojet system including the anticoagulant EDTA (Vacutainer, USA) and transported to laboratory on ice for their analysis. All procedure with animals were performed in accordance with protocols approved by the Institutional committee for use and care of animals in research of INIA (CICUA 04/2022).

GENOTYPING

Genomic DNA was purified from 200 µL of blood using the GeneJet Whole blood Genomic DNA Purification Mini Kit (Thermofisher Scientific, USA) following the manufacturer protocol. Integrity of DNA was assessed by agarose gel electrophoresis and its quantification by measuring DNA absorbance at 260 and 280 nm and evaluating its ratio using a plate-reader (Infinity M200 Pro, TECAN, Switzerland). Genotyping of the K232A *DGAT1* marker was performed by PCR-RFLP according to Carvajal *et al.* (2016), using the primers F-TGCCGCTTGCTCGTAGCTTTGGCC and R-ACCTGGAGCTGGGTGAGGAACAGC where a 414 amplicon was digested with *Eae*I enzyme (Thermo Scientific, USA). For *CSN3* (GenBank Y380228.1) we designed primers using the Primer-BLAST tool

(NCBI; F- GCGCTGTGAGAAAGATGAAAG and R-CCCATTTCGCCTTCTCTGTAA) to amplify a 631 bp product (T° annealing 59 °C) containing the polymorphisms rs43703015 and rs43703016 which determine the A, B, C and E variants. Then, amplicons were purified by column using the E.Z.N.A Pure cycle kit (Omega Bio-tek, USA) and sent to commercial sequencing (Macrogen, Korea) to reveal the variant.

DATA ANALYSIS

The analysis contemplated the estimation of allelic and genotypic frequencies for each of the markers by means of direct count. In addition, the Hardy-Weinberg equilibrium (HWE) was performed by Chi-test with a $P<0.05$ as significant (Falconer & MacKay, 1996).

Likewise, the genetic diversity of populations was determined using the Wright-F statistics Ho, He, FIS and FST. All calculations were performed using the GenePop 4.7.5 (Rousset, 2008).

RESULTS

Table 1 shows the allelic frequencies for *CSN3* marker. It showed that the A variant was predominant in four herds (HF, OC, FN e HYB) in a range between 0.569 to 0.709. At the same time, the results revealed that the B variant was best represented in JE (0.729) and MB (0.577) herds. The E variant, was poorly represented in HF, OC, FN, and HYB (between 0.022 and 0.071), and absent in JE and MB. Additionally, the C variant was only found in a few HYB animals with a frequency of 0.17. The analysis of these distributions showed that populations are in HWE, except for OC which displayed a χ^2 value of 6.812 (Table 1; p<0.05).

For K232A *DGAT1*, we found that frequency of the A allele was predominant (0.645 to 0.962) for all populations except JE (Table 1). The highest frequency was observed in MB and FN, and then OC, HF and HYB. Holstein-Friesian showed a higher frequency of AK genotype which was

Table 1. Allelic frequencies (%) and Hardy-Weinberg equilibrium for *DGAT1* and *CSN3* markers.

Breed	*DGAT1*				*CSN3*				
	n	A	K	χ^2	A	B	C	E	χ^2
HF	56	0.661	0.339	0.743	0.709	0.251	0	0.040	0.781
JE	41	0.305	0.695	1.781	0.271	0.729	0	0	0.650
MB	39	0.962	0.038	0.062	0.423	0.577	0	0	1.692
OC	44	0.750	0.250	1.980	0.569	0.360	0	0.071	6.812*
FN	46	0.967	0.033	0.049	0.658	0.320	0	0.022	3.171
HYB	38	0.645	0.355	5.149*	0.612	0.170	0.169	0.049	1.803

HF: Holstein-Friesian; JE: Jersey; MB: Montebeliarde; OC: Overo Colorado; FN: Frisón Negro; HYB: Hybrid; n, number of animals; χ^2: Chi-square test; *Indicates Hardy-Weinberg equilibrium deviation (p<0.05).

present in half of the animals (28 of 56 animals, data not showed). On the other hand, MB and FN did not show the KK genotype. Jersey, as expected, showed the highest frequency of the K variant (0.695; Komisarek *et al*., 2011; Anton *et al*., 2012). For this marker, all populations were in HWE with exception of hybrids animals (Table 1; $\chi^2 = 5.149$).

The analysis of FIS for *CSN3* (Table 2) gave negative and non-significant (p>0.05) values in almost all populations except for OC (-0.429; MB showed a positive value). Likewise, for *DGAT1* we observed the same tendency, except for HYB, which displayed a positive value (0.418). Regarding inbreeding, the FIT values for *DGAT1* and *CSN3* were non-significant (0.241 and -0.028, respectively). In relation to genetic differentiation, the paired FST values by breed were between 0.010 and 0.650 for *DGAT1,* and between 0.001 and 0.342 for *CSN3*, with significant

differences in most of the populations (Table 3). For *CSN3* it was possible to observe high (FST 0.15-0.25) or very high values (FST>0.25) for JE/HF and JE/HYB pairs, and low values with a low differentiation for JE/MB. For *DGAT1*, the FST analysis yielded very high values (JE/FR and JE/MB), and low values as observed for HF/OC and HYB/OC pairs.

The simultaneous analysis of both markers (*DGAT1* and *CSN3*) gave 16 combined genotypes (Table 4) with hybrid animals displaying a high number of combinations (11). The Jersey breed showed five combinations with exactly the favorable (*DGAT1* AA/AA and *CSN3* BB) in the highest frequency (44%). The other populations including MB, OC and FN barely exhibited the favorable combination, but only Holstein-Friesian displayed a few animals with this combination and high frequency (38%) of heterozygous genotype *DGAT1* AK – *CSN3* (AB).

Table 2. Values of observed (Ho) and expected (He) heterozygosity and FIS index for *DGAT1* and *CSN3* markers.

Breed	DGAT1				CSN3			
	Ho	He	FIS	P-value	Ho	He	FIS	P-value
HF	0.281	0.253	-0.106	0.867	0.26	0.231	-0.129	0.909
JE	0.212	0.175	-0.197	0.955	0.14	0.165	-0.142	0.294
MB	0.030	0.029	-0.027	1.000	0.23	0.192	0.196	0.940
OC	0.203	0.166	-0.201	0.971	0.30	0.211	-0.429	0.005*
FR	0.034	0.029	-0.023	1.000	0.26	0.204	-0.276	0.990
HYB	0.102	0.171	0.418	0.014*	0.16	0.147	-0.083	0.822
Total			-0.030	0.967			-0.177	0.868

HF: Holstein-Friesian; JE: Jersey; MB: Montbeliarde; OC: Overo Colorado; FN: Frisón Negro; HYB: Hybrid; Ho: Observed heterozygosity; He: Expected heterozygosity; FIS: Inbreeding coefficient; (*) P<0.05.

Table 3. FST values for *DGAT1* and *CSN3* markers between different breeds pairs.

	Breed	HF	JE	OC	FR	MB
DGAT1	JE	0.216*				
	OC	0.010	0.326*			
	FR	0.248*	0.650*	0.172*		
	MB	0.227*	0.626*	0.152*	-0.011	
	HYB	-0.012	0.201*	0.011	0.287*	0.261*
	Total FST	0.263*				
CSN3	JE	0.324*				
	OC	0.015	0.208*			
	FR	-0.004	0.275*	0.001		
	MB	0.154*	0.040*	0.063*	0.112*	
	HYB	-0.010	0.342*	0.021	-0.003	0.166*
	Total FST	0.125*				

HF: Holstein-Friesian; JE: Jersey; MB: Montbeliarde; OC: Overo Colorado; FN: Frisón Negro; HYB: Hybrid; FST: Proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance; (*) P<0.05.

Table 4. Combined genotypes (%) for *DGAT1* and *CSN3* markers.

	Combined genotypes		Frequency					
Nº	*DGAT1*	*CSN3*	HF	JE	MB	OC	FN	HYB
1	KK	AA	0	0	0	0	0	4
2	<u>KK</u>	<u>AB</u>	1	0	0	0	0	0
3	KK	ABC	0	0	0	0	0	3
4	KK	AE	1	0	0	1	0	1
5	<u>KK</u>	<u>BB</u>	3	18	0	0	0	0
6	AK	AA	4	2	0	12	0	4
7	AK	AB	21	14	0	1	0	1
8	AK	ABC	0	0	0	0	0	3
9	AK	AE	3	0	0	1	1	2
10	<u>AK</u>	<u>BB</u>	0	5	3	2	1	0
11	AK	BE	0	0	0	4	1	0
12	AA	AA	23	2	5	0	18	9
13	AA	AB	0	0	23	23	24	0
14	AA	ABC	0	0	0	0	0	8
15	AA	ABCE	0	0	0	0	0	1
16	AA	BB	0	0	8	0	1	1

HF: Holstein-Friesian; JE: Jersey; MB: Montbeliarde; OC: Overo Colorado; FN: Frisón Negro; HYB: Hybrid; Favorable genotypes are highlighted with underlining.

DISCUSSION

CSN3 and *DGAT1* markers are major polymorphisms related to cheese manufacturing and milk quality, respectively. They have been evaluated in several breeds in different production systems but there is little or no information available in our country. Thus, we evaluate the frequency distribution of these markers in five dairy breeds usually milky in southern Chile (three of them with worldwide presence and two local adapted breeds, Overo Colorado and Frisón Negro), and a hybrid population. For *CSN3* the most representative allele in almost all populations was A as have been reported in several studies including Holstein-Friesian and some crosses (Volkandari *et al*., 2017; Miluchová *et al*., 2018; Gurses *et al*., 2018; Adamov *et al*., 2020) and several local breeds worlwide (Akyüz & Çinar, 2014; Djedovic *et al*., 2015; Barbosa *et al*., 2019). For FN, a local crossed breed genetically related to Holstein we observed a high presence of the A variant but the percentage of animals with the B variant (0.32) was higher than that reported by Felmer & Butendick (0.18) (1998), suggesting a higher selection to milk quality during the last years. In turn, the B allele was more frequent in Jersey as expected. It is well known that Jersey cattle is highlighted because of their potential to transform milk to cheese, and it presents a higher frequency of B variant (Zepeda-Batista *et al*., 2015). Other alleles such as E and C were less represented according to reports in different breeds (Zepeda-Batista *et al*., 2015; Gurses *et al*., 2018).

The analysis of heterozygosity showed that all populations were in HWE except for OC (Table 2; p<0.05). Although OC is well-known as dairy cattle, this biotype was in its origin a double purpose cattle (milk and meat) and today it is breeding with distinct schemes and the pursue of higher levels of milk solids could be affect their genetic distribution.

It was interesting that, for *DGAT1*, Holstein-Friesian, which is characterized by a high milk production, showed a higher frequency of AK genotype, which was present in half of the animals (data not showed). It is widely recognized that selection of milk production has a negative effect on milk fat content (Uribe *et al*., 2017), which is explained by a lower occurrence of the K variant. However, it has been described that the managing of animals can induce higher frequencies of K allele (Ardicli *et al*., 2018) and there is a margin to select animals for milk production and solids content. Jersey, as expected, showed the highest frequency of the K variant (0.70; Komisarek *et al*., 2011; Anton *et al*., 2012). This breed is characterized by a high milk solids content.

In relation to heterozygosity, the analysis of FIS estimator for *CSN3* gave negative and non-significant values in all populations except for OC. This indicates an excess of heterozygotes. Likewise, for *DGAT1* we observed the same tendency, except for HIB, which displayed a positive value suggesting a deficit of heterozygotes. These results are correlated with the absence of HWE in both populations (OC and HYB). Regarding genetic

differentiation, the analyses of FST by breed for *CSN3* gave positive values with significant differences in most of the populations displaying high or very high values for JE/HF and JE/HYB pairs, suggesting a high differentiation according to Wright (1984), and also low values with a low differentiation for JE/MB.

These results must be considered cautiously because, though important, only two markers associated to milk composition yield were assessed. If a greater number of markers were included, the very high values of differentiation would be reduced to high values (in a range of FST between 0.15-0.25). However, these markers respond to an intraspecific diversity correlated to the differentiation of milk traits of every breed, determining a high genetic structure for Jersey biotype compared to the other breeds (Huson *et al*., 2020). Other studies using genomics tools, had described moderate values of FST (0.142) between Holstein and Jersey biotypes (Melka & Schenkel, 2012). On other hand, Brown Swiss cattle which display favorable traits for cheese manufacturing, shows a genetic differentiation trend, and recent studies confirm this with high values of FST (0.156) between Brown Swiss and Holstein (Signer *et al*., 2017).

The simultaneous analyses of both markers (*DGAT1* and *CSN3*) gave 16 combined genotypes. Hybrid animals displayed a high number of combinations (11) probably as a result of multiple crosses, according to breed records between HF and OC, but those didn't favor the milk solids or cheese manufacturing. The Jersey breed showed five combinations but with the favorable genotype (*DGAT1* KK and *CSN3* BB) in the highest frequency, renewing their productive orientation to transform milk to cheese or dairy products.

Since 2010 the national dairy chain set itself the goal of reaching a 7.6% of milk solids yield for 2020. Although progress has been made on this way, it has not been fully achieved, in part, by the utilization of genotypes mainly oriented to milk production but no milk solids. Holstein-Friesian and Overo Colorado are the main biotypes used in milk production in Chile, reaching together more than 60% of dairy cattle, whereas Jersey is less-represented reaching only a 5% (ODEPA, 2019). Holstein-Friesian and their crosses had been used worldwide including Chile as dairy cattle for milk production since the mid-twentieth century. Nevertheless, more recently milk solids have been incorporated to breeding through selection index (O'Sullivan *et al*., 2019). Although the results of previous (Carvajal *et al*., 2016) and current work show an important percentage of the *DGAT1* genotype associated to milk solids (K allele), their distribution in the total mass of dairy cattle is unknown. On the other hand, in Jersey cattle the presence of favorable genotypes for cheese manufacturing is widely described. Crosses between Jersey and New Zealand Holstein show a significant highly milk solids yield which could generate a high economic recovery (Delgadillo *et al*., 2016). This better performance results

from heterosis and the favorable input from Jersey biotype (Buitenhuis *et al*., 2014).

In this sense, the dairy sector including the government should initiate a national or regional breeding program including a selection index considering dairy genotypes oriented to milk solids. However, it is important to consider that the association of genetic variants to milk production and milk solids is polygenic. This is, multiples genomic regions or QTLs are involved in these traits (Ibeagha-Awemu *et al*., 2016). For example, a genome-wide study by Buitenhuis *et al*. (2014) reveals that *DGAT1* can explain up to 8-23% of milk fat content in Holstein and Jersey breeds. Therefore, other molecular markers could be included in a prediction scheme of genetic merit, or better, use animals for breeding with validated genomic information for dairy production systems based in pastures.

CONCLUSIONS

This study shows that between dairy breeds used in southern Chile Jersey biotype display the highest frequency of *DGAT1*-KK and *CSN3*-B variants which favor a major content of milk solids and the potential to cheese manufacturing.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

The obtention of blood samples were according to the Institutional Committee of Animal Care and Use from the Instituto de Investigaciones Agropecuarias.

AUTHOR CONTRIBUTIONS

A.M.C. planned the research; D.L. and A.M.C performed the analysis and write the manuscript; A.M.C. and R.dlB. made the discussion of the data and its implications.

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