Genome-wide association studies (GWAS) and post-GWAS analyses for technological traits in Assaf and Churra dairy breeds

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ABSTRACT

This study aimed to perform a GWAS to identify genomic regions associated with milk and cheese-making traits in Assaf and Churra dairy sheep breeds; second, it aimed to identify possible positional and functional candidate genes and their interactions through post-GWAS studies. For 2,020 dairy ewes from 2 breeds (1,039 Spanish Assaf and 981 Churra), milk samples were collected and analyzed to determine 6 milk production and composition traits and 6 traits related to milk coagulation properties and cheese yield. The genetic profiles of the ewes were obtained using a genotyping chip array that included 50,934 SNP markers. For both milk and cheese-making traits, separate single-breed GWAS were performed using GCTA software. The set of positional candidate genes identified via GWAS was subjected to guilt-by-association-based prioritization analysis with ToppGene software. Totals of 84 and 139 chromosome-wise significant associations for the 6 milk traits and the 6 cheese-making traits were identified in this study. No significant SNPs were found in common between the 2 studied breeds, possibly due to their genetic heterogeneity of the phenotypes under study. Additionally, 63 and 176 positional candidate genes were located in the genomic intervals defined as confidence regions in relation to the significant SNPs identified for the analyzed traits for Assaf and Churra breeds. After the functional prioritization analysis, 71 genes were identified as promising positional and functional candidate genes and proposed as targets of future research to identify putative causative variants in relation to the traits under examination. In addition, this multitrait study allowed us to identify variants that have a pleiotropic effect on both milk production and cheese-related traits. The incorporation of variants among the proposed functional and positional candidate genes into genomic selection strategies represent

an interesting approach for achieving rapid genetic gains, specifically for those traits difficult to measure, such as cheese-making traits.

Key words: dairy sheep, genome-wide association studies, cheese-making traits, candidate genes

INTRODUCTION

Improving important production traits in livestock may gain an advantage of the in-depth understanding of the genetic architecture that underlies the phenotype of interest. From this standpoint, animal genotyping using medium- or high-density SNP panels followed by GWAS has been presented as a powerful approach to reconnect phenotypes of interest back to their underlying genetics in livestock species (Korte and Farlow, 2013). To complement GWAS, post-GWAS analyses, such as gene prioritization analyses, gene set enrichment, or pathway analyses, have been increasingly used over the past few years to better understand the molecular mechanisms involved in the different traits of interest (Otto et al., 2020). Such new approaches help to solve GWAS limitations, such as by taking into account that genes work together in networks across the different biological pathways, thus generating the complex control of quantitative traits (Dadousis et al., 2017).

In the dairy industry, milk yield and milk solids, referred to here as “milk traits,” are major goals for selection. Accordingly, a large number of studies have implemented QTL mapping and GWAS to identify QTL with effects on milk traits in dairy cattle (Li et al., 2020b; Otto et al., 2020), goats (Mucha et al., 2018; Guan et al., 2020), and sheep (García-Gómez et al., 2012a; Li et al., 2020a). Moreover, in recent years, an increasing number of studies have successfully applied post-GWAS analyses to determine those genes potentially involved in controlling milk traits (Sanchez et al., 2019; Otto et al., 2020).

Spain is ranked fourth in the world for ewe milk production (FAOSTAT, 2019). The fact that sheep's milk in Spain is mainly intended to produce cheeses means that the study of cheese-making traits is becoming

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ing increasingly important. Some of the most studied indicators related to cheese-making ability are traits related to milk coagulation properties (**MCP**; McMahon and Brown, 1982; Bynum and Olson, 1982) and different measurements of cheese yield (Othmane et al., 2002a,c). Toward a better understanding of the genetic basis of cheese-making traits in dairy sheep, our research group has recently investigated the genetic parameters for 2 groups of traits characterizing the technological behavior of milk (**MCP** and cheese yield) in 2 of the main dairy sheep breeds in Spain, namely, the Assaf and Churra breeds (Sánchez-Mayor et al., 2019; Pelayo et al., 2021). However, the lack of a straightforward methodology to routinely measure cheese yield-related traits at farms has hampered the integration of these traits into classical breeding programs.

In this sense, identifying genetic markers associated with these difficult-to-measure cheese-making traits may be highly relevant to the sheep dairy industry (Marina et al., 2020b). In the literature, approaches to performing GWAS in dairy sheep have mainly focused on milk traits (García-Gómez et al., 2012a; Di Gerlando et al., 2019), with few methods found in relation to cheese-making traits (Marina et al., 2020b). Moreover, to our knowledge, there are no studies that combine analyses of GWAS for milk and cheese-making traits in 2 different dairy breeds. Different breeds show different behaviors concerning MCP (Pazzola et al., 2014a; Pelayo et al., 2021). In particular, concerning Assaf and Churra sheep, previous studies performed by our research group have identified clear differences between both breeds regarding MCP. Milk from Churra shows shorter coagulation times than milk from Assaf (Sánchez-Mayor et al., 2019; Pelayo et al., 2021). Thus, identifying unique and common genomic regions among breeds associated with cheese-related traits could provide insights into the genetic basis of the milk coagulation process in sheep.

Based on these factors and the interests of the sheep dairy industry in increasing the cheese-making ability of milk, the present study reports the analysis results of GWAS for milk traits and cheese-making traits in Assaf and Churra dairy sheep breeds. In addition, we aimed to identify potential positional and functional candidate genes and their interactions through post-GWAS analyses.

MATERIALS AND METHODS

Sampling, Genotyping, and Quality Filtering

The phenotypic data used in this study were previously described by Sánchez-Mayor et al. (2019) for the

Assaf breed and by Pelayo et al. (2021) for the Churra breed. A total of 2,020 ewes belonging to the Assaf ($n = 1,039$, number of flocks = 4) and Churra breeds ($n = 981$, number of flocks = 2) were analyzed. Following the procedure described in detail by Sánchez-Mayor et al. (2019), a sample of 50 mL of milk was collected from each ewe from the morning milking. Each milk sample was analyzed separately to determine 6 milk traits and 6 cheese-making traits. On the one hand, the milk traits included 5 milk production and composition traits, including milk yield (**MY**, kg), fat percentage (**FP**, %), fat yield (**FY**, kg), protein percentage (**PP**, %), and protein yield (**PY**, kg), and milk SCC (cells/mL) as a functional trait considered a good indicator trait of mastitis resistance. On the other hand, the 6 cheese-making-related traits included MCP and individual cheese yield traits. The studied MCP traits were the rennet clotting time (**RCT**, min), which is the time between rennet addition and the formation of the curd; the time necessary for the curd to reach 20 mm or curd-firming time (**K₂₀**, min); and the curd firmness at 30 and 60 min after rennet addition (**A30** and **A60**, mm). These MCP traits were measured in both breeds at 32°C with a Formagraph viscometer (FRM; Foss Electric A/S) using commercial liquid rennet extract [1:15.000; >70% chymosin, <30% bovine pepsin; 185 international milk clotting units (IMCU)/mL] diluted to 4% in distilled water for 60 min. Full details about the analysis of these traits have been described in a previous study of our research group (Sánchez-Mayor et al., 2019). Concerning the cheese yield, the individual laboratory cheese yield (**ILCY**, g/10 mL of milk) and the individual laboratory dried curd yield (**ILD CY**, g/10 mL) were estimated following Othmane et al. (2002a,c). The genetic parameters estimated for all the traits presented in this work (both milk and cheese-making traits) were previously analyzed by Sánchez-Mayor et al. (2019) for the Assaf breed and by Pelayo et al. (2021) for the Churra breed. Note that in these 2 studies, after the initial assessment of the normality of the variable distributions for each trait, logarithmic base 10 transformations of the **K₂₀** and SCC traits were considered for the different analyses (**logK₂₀**; **logSCC**). Basic descriptive statistics of the phenotype data considered in the present study for both dairy sheep breeds are given in Table 1.

The genetic profiles of the 2,020 ewes were obtained through the same custom SNP chip with 50,934 mapped markers. Quality control (**QC**) of the raw genotypes was carried out in both breeds simultaneously using PLINK version 1.90 (Purcell et al., 2007). Samples with more than 10% missing genotypes and SNPs with call rates under 90% and minor allele frequency (**MAF**)

Table 1. Descriptive statistics for the milk and cheese-making phenotypes measured in the Assaf and Churra breeds analyzed in the present study

Breed	Trait ¹	No. of records	NA ²	NA%	Minimum	Maximum	Mean	SD	CV (%)
Assaf	MY	1,039	0	0.00	0.45	6.53	2.89	1.07	37.13
	FP	1,039	0	0.00	3.04	10.34	5.56	1.05	18.96
	FY	1,039	0	0.00	3.07	54.60	16.01	6.63	41.39
	PP	1,039	0	0.00	3.57	7.68	5.05	0.46	9.17
	PY	1,039	0	0.00	2.22	34.16	14.45	5.09	35.23
	SCC	1,039	0	0.00	11.00	27,139.00	701.89	2,426.99	345.78
	logSCC	1,039	0	0.00	1.04	4.43	2.19	0.61	27.76
	RCT	1,039	131	12.61	8.00	58.45	29.15	10.52	36.08
	K20	1,039	173	16.65	1.30	20.45	4.27	2.48	57.95
	logK20	1,039	173	16.65	0.11	1.31	0.57	0.22	38.96
	A30	1,039	507	48.80	1.04	58.36	29.76	13.96	46.91
	A60	1,039	131	12.61	2.12	64.00	40.96	11.29	27.56
	ILCY	1,039	131	12.61	1.36	4.35	2.49	0.41	16.58
	ILCDY	1,039	131	12.61	0.13	1.76	0.97	0.18	18.07
Churra	MY	981	3	0.31	0.20	4.06	1.71	0.65	38.25
	PP	981	1	0.10	3.78	10.10	5.42	0.64	11.87
	PY	981	4	0.41	1.20	32.80	9.12	3.30	36.16
	FP	981	1	0.10	1.68	11.66	6.27	1.53	24.33
	FY	981	4	0.41	1.79	24.23	10.28	3.66	35.57
	SCC	981	8	0.82	11.00	29,322.00	1,143.46	3,307.96	289.29
	logSCC	981	8	0.82	1.04	4.47	2.33	0.68	29.06
	RCT	981	37	3.77	8.15	56.00	17.47	6.93	39.65
	K20	981	36	3.67	1.30	60.00	3.38	6.15	182.15
	logK20	981	36	3.67	0.11	1.78	0.40	0.25	64.07
	A30	981	36	3.67	1.00	59.96	36.98	13.42	36.28
	A60	981	36	3.67	5.06	64.00	39.99	13.07	32.68
	ILCY	981	36	3.67	1.44	4.30	2.64	0.47	17.80
	ILCDY	981	36	3.67	0.30	2.45	1.10	0.21	18.68

¹MY = milk yield; FP = fat percentage; FY = fat yield; PP = protein percentage; PY = protein yield; logSCC: SCC expressed in log₁₀; RCT = rennet coagulation time; K₂₀ = curd-firming time; logK₂₀ = curd-firming time expressed in log₁₀; A30 and A60 = curd firmness at 30 and 60 min, respectively, after rennet addition; ILCY = individual laboratory cheese yield; ILDCY = individual laboratory dried curd yield.

²NA = number of missing values.

lower than 1% were excluded from the data set. In addition, SNPs mapped on the X chromosome were also removed from the analysis.

Population Structure and Linkage Disequilibrium

First, population stratification was evaluated through a principal component analysis (**PCA**) based on the variance-standardized relationship matrix using the remaining SNPs after QC. In addition, linkage disequilibrium (**LD**) was estimated through the squared correlation coefficient (**r**²) and the absolute value of **D'** (**|D'|**) using HAPLOVIEW v4.2 (Barrett et al., 2005). Finally, to observe the LD decay pattern for the 2 breeds, the average for each parameter was calculated based on the SNP pairwise distance in 1-Mb intervals (Miller et al., 2011). The SNP pairs were stacked according to their physical distance into 14 intervals following García-Gómez et al. (2012b): <10 kb, 10 to 20 kb, 20 to 40 kb, 40 to 60 kb, 60 to 100 kb, 100 to 200 kb, 200 to 500 kb, 0.5 to 1 Mb, 1 to 2 Mb, 2 to 5 Mb, 5 to 10 Mb, 10 to 20 Mb, 20 to 50 Mb, or >50 Mb.

The genetic distance for the estimation of LD decay was calculated as the distance at which both **r**² and **|D'|** reached values lower than 0.1 and 0.6, respectively (Fonseca et al., 2016).

Genome-Wide Association Studies

For each of the milk and cheese-making traits considered in this study, individual GWAS analyses were performed using GCTA software (Yang et al., 2011) applying the following general mixed linear model in both breeds:

$$\mathbf{Y} = \mathbf{X}\mathbf{b} + \mathbf{Z}\mathbf{u} + \mathbf{e},$$

where **Y** is the vector of phenotypes, **X** is the incidence matrix of fixed effects, **b** is the vector of fixed effects, and it included the following factors: DIM as a covariate; age at parturition, with 5 and 7 levels for the Assaf and Churra breeds, respectively; flock test day, with 12 and 10 levels for Assaf and Churra, respectively; and the number of lambs born, with 2 levels for both breeds.

The individual's genotype information (given by the P number of SNPs) is represented in the vector of random effects $\mathbf{u}$ ($P \times 1$), $\mathbf{Z}$ is the incidence matrix of the random effect ($N \times P$), and $\mathbf{e}$ is the vector of residual effects, which is assumed to be normally distributed, with a mean of zero and variance of σ_e^2 . Simultaneously, GCTA estimates $\mathbf{Z}$ by centering and scaling the data matrix $\mathbf{X}$ using Hardy–Weinberg assumptions, including that $\mathbf{u}$ and $\mathbf{e}$ are normally distributed with a mean of zero and variance of $\sigma_u^2\mathbf{I}$ and $\sigma_e^2\mathbf{I}$, respectively (Krishna Kumar et al., 2016). Finally, the association analysis between the SNPs and the different phenotypes was based on the leave one chromosome out strategy, which creates a genetic relationship matrix that includes all autosomal SNPs except those on the one chromosome tested in the GWAS (Lin et al., 2017).

Significance thresholds for the GWAS are usually estimated using a Bonferroni multiple-test correction. However, because the Bonferroni approach, which considers all SNPs included in the GWAS as independent variables, is known to be very conservative, we applied the methodology proposed by Gao et al. (2008) for each chromosome as others authors previously reported (García-Gómez et al., 2012a; Atlija et al., 2016). Thus, we first calculated the effective number of independent tests based on the LD for each chromosome (M_{eff_C}). Through this approach, a total of 22,339 markers across the genome were considered independent markers. Then, we applied the Bonferroni correction formula to calculate the adjusted significance level for each chromosome as follows:

$$\alpha_C = \alpha_e / M_{eff_C},$$

where M_{eff_C} is the number of independently analyzed markers per chromosome, α_e is the chromosome-wise type I error rate considered (0.05), and α_C is the adjusted chromosome-wise significance level. Genome-wise significance thresholds were based on the chromosome-wise significance thresholds by correcting for the total number of independent markers analyzed across the genome. SNPs with a significant association P -value lower than the chromosome-wise threshold (α_C) were considered in the subsequent analyses.

QTL Regions and Gene Annotation

The list of SNPs associated with all traits under study was used for gene and QTL annotation using the GALLO R package (Fonseca et al., 2020). The confidence region considered for extraction of positional candidate genes and assessment of concordance with

previously described QTL was the genomic distance where the LD decays at the level of $r^2 < 0.1$ and $|D'| < 0.6$ concurrently in both populations.

For gene annotation within the QTL confidence regions, we used the annotation file for the Oar_v.3.1 ovine reference genome available from Ensembl (<http://www.ensembl.org/biomart>). For the positional candidate genes annotated within the significant SNP confidence regions identified, we also extracted transcription factors (TF) and co-transcription factors (CF) from the AnimalTFDB 3.0 database (Hu et al., 2019). In addition, the coordinates of QTL previously identified through association analyses reported in sheep in relation to milk production or composition, SCS and cheese-making traits were downloaded from SheepQTLdb (<http://www.animalgenome.org/cgi-bin/QTLdb/OA/search>; Hu et al., 2016).

Gene Prioritization and Networks

The set of positional candidate genes identified through the individual GWAS analyses was submitted to guilt-by-association-based prioritization analyses using ToppGene software (Chen et al., 2009). This tool uses functional information from Gene Ontology (GO), human and mouse phenotypes, metabolic pathways, PubMed publications, coexpression patterns, and diseases from a list of training genes to calculate the functional similarities between the training list and the list of considered positional candidate genes using a fuzzy-based similarity measure. The similarity scores from each functional database were combined using a statistical meta-analysis, and the P -value was determined for each test gene. The training list of genes, which was composed of 1,160 genes in our case, was carefully selected from the literature through different studies related to milk and cheese-making traits. Briefly, the 6 studies were as follows: 1 study aimed at unifying a database of candidate genes involved in mammary gland development and function in dairy cattle (Ogorevc et al., 2009); 1 study focused on the identification of potential causal mutations from a list of candidate genes for milk composition traits using whole-genome resequencing data sets (Marina et al., 2020a); 2 transcriptomic studies focused on candidate genes, with one on cheese-related traits in dairy sheep (Suárez-Vega et al., 2016a) and the other on the citrate content in cow milk, (Cánovas et al., 2013); and 2 studies focused on the identification of co-associated gene networks with milk and cheese-making properties in dairy sheep (Marina et al., 2020b) and dairy cattle (Sanchez et al., 2019). Finally, considering those genes with a statistical meta-analysis P -value lower than 0.05,

a gene network analysis was performed using STRING software v11.5 (Szklarczyk et al., 2019).

RESULTS AND DISCUSSION

Phenotype Basic Statistics and Genotype Quality Filtering

The milk and cheese-making traits analyzed in this study are difficult to measure routinely on farms, especially following the processing of individual milk samples, which is crucial to achieve accurate individualized values that are useful for breeding programs. Individual cheese yield based on micro-cheese manufacturing (ILCY) was first described by Othmane et al. (2002a) as a useful predictor of actual cheese yield. Individual cheese yield can also be predicted by spectroscopy, as Othmane (2000) and Cellesi et al. (2019) showed. However, the laboratory simulation process of individual cheese manufacturing followed in this study is not completely comparable to that of the dairy industry. As other authors have suggested, micro-cheese factoring can determine an overestimation of the actual cheese yield due to the limited amount of milk used (Puledda et al., 2017; Othmane et al., 2002b; Jaramillo et al., 2008). In any case, ILCY is the most commonly used parameter for the calculation of the individual laboratory cheese yield, and many different studies have used it as a good proxy trait to assess the cheese-making ability of dairy species (Othmane et al., 2002b,c; Cellesi et al., 2019). Because genetic selection relies on individual phenotype values, the individual proxy phenotypes here analyzed for MCP and cheese yield traits may be of great value for those breeding programs of dairy species that define the improvement of milk technological properties selection objectives.

The phenotypic data analyzed here for Assaf and Churra sheep breeds, whose basic statistics are given in Table 1, have been previously analyzed in detail by Sánchez-Mayor et al. (2019) and Pelayo et al. (2021), respectively. Briefly, of the 1,039 and 981 milk samples from Assaf and Churra ewes, respectively, included in the study, 131 samples in Assaf and 36 in Churra (13% and 3.6%, respectively) did not coagulate within 60 min after the addition of the clotting enzyme (Sánchez-Mayor et al., 2019; Pelayo et al., 2021). These samples had no values for the cheese-making traits (MCP and cheese yield traits), and therefore were declared missing values for the subsequent analyses. This percentage of noncoagulating samples for the Assaf breed was much higher than the values reported in other dairy sheep breeds, such as 0.44% in Sarda sheep by Pazzola et al. (2014a), 3.8% in Alpine sheep by Bittante et al. (2014),

and 3.7% in Churra sheep by Pelayo et al. (2021). In general, the observed average values shown in Table 1 suggest that milk from Churra sheep shows intermediate coagulation properties between the milk from the Assaf breed (considered a slow coagulation breed) and from other fast coagulation dairy sheep breeds, such as Manchega or Sarda (Pelayo et al., 2021).

Regarding the QC performed on the raw genotypes, none of the individuals included in this study was discarded due to a low call rate. Call rates lower than 95% were detected for 2,139 SNPs, and a MAF lower than 0.05 was found for 5,910 SNPs, which were filtered out. Finally, a total of 43,784 SNPs, distributed on the 26 ovine autosomes remained after the QC filtering steps in both breeds, were included in further analyses.

Population Structure and Linkage Disequilibrium

The PCA revealed that Assaf and Churra breeds are subdivided into 2 highly differentiated groups by the first principal component, which explained 49.50% of the genetic variability in the analysis (Supplemental Figure S1, <https://data.mendeley.com/datasets/64jsbg5n9s/2>, Marina et al., 2021). This finding is consistent with the phylogenetic analysis reported on whole-genome data sets by Marina et al. (2020b), where Assaf and Churra breeds had an evolutionary divergence estimate of 0.20 and the 43 different domestic sheep breeds included in the study had average estimates of 0.16. The second component, which captured 7.22% of the variance, represented the intrabreed genetic diversity. The Churra breed showed higher genetic variability than the Assaf breed, although Churra sampling involved fewer flocks than Assaf (2 flocks in Churra vs. 4 flocks in Assaf).

The LD is a key genetic parameter in GWAS and population genetic diversity analyses. In this study, the LD decay pattern estimated for both r^2 and $|D'|$ at different physical distances is represented in Figure 1. For the Assaf and Churra breeds, the LD decay pattern was very similar, suggesting that the r^2 and $|D'|$ parameters reached very low values ($r^2 < 0.1$ and $|D'| < 0.5$) at distances greater than 100 kb, which was the distance used to define the region of confidence on both sides of the significant SNPs identified in the subsequent analyses (Figure 1 and Supplemental Table S1, <https://data.mendeley.com/datasets/64jsbg5n9s/2>, Marina et al., 2021). The LD pattern described here for Churra agrees with previous studies in different populations of this sheep breed (García-Gómez et al., 2012b; Chitneedi et al., 2017). A similar LD decay distance has been reported in others sheep breeds (Kijas et al., 2014; Cesarani et al., 2019).

Significant Regions Identified by the GWAS

The GWAS analysis results in this work are represented as individual Manhattan plots per breed for the milk traits in Figure 2 and the cheese-making traits in Figure 3.

Their corresponding quantile-quantile plots are represented in Supplemental Figures S2 and S3 (<https://data.mendeley.com/datasets/64jsbg5n9s/2>, Marina et al., 2021) for milk and cheese-making traits, respectively. No significant SNPs were observed at genome-wide significance level. However, a total of 84 chromosome-wise significant associations were identified for the 6 milk traits [MY (11), FP (14), FY (19), PP (15), PY (15), and logSCC (10)], whereas 139 for the 6 cheese-making

traits [RCT (10), logK₂₀ (11), A30 (10), A60 (10), ILCY (67) and ILCDY (31)]. Further studies should be performed to confirm the total of 223 chromosome-wise significant associations found in this analysis. In addition, no significant SNPs were found in common between the 2 studied breeds. A possible explanation for this could be the difference in the frequency alleles between the studied breeds. For example, Marina et al. (2020a) found notable differences in the *LALBA* gene for the missense deleterious mutation *LALBA_OAR3*: 137390760T>C, where the T allele was close to fixation (0.92) in the Assaf breed. In contrast, this allele showed a low-moderate frequency (0.26) in the Churra breed. Another explanation for the lack of common significant SNPs between the 2 studied breeds could be

Figure 1. Average linkage disequilibrium (LD) measured through r^2 and D' parameters as a function of the 19 genomic distances considered between markers across the genome of Spanish Assaf and Churra breeds.

Figure 2. Manhattan plots showing the genome-wide association study results for the 6 milk traits considered in this work. The Assaf breed results are represented on the left-hand side (pink and black chromosomes), and the results for the Churra breed are represented on the right-hand side (blue and black chromosomes). The $\log(1/P\text{-value})$ is depicted here for all 43,784 SNPs used in the GWAS analyses for each of the 6 milk traits under study. The chromosome-wise significance thresholds are depicted as horizontal blue lines above each chromosome. MY = milk yield; FP = fat percentage; FY = fat yield; PP = protein percentage; PY = protein yield; and logSCC = the logarithm of SCC.

Figure 3. Manhattan plots showing the genome-wide association study results for the 6 cheese-making traits considered in this work. The Assaf breed results are represented on the left-hand side (pink/black chromosomes), and the results for the Churra breed are represented on the right-hand side (blue/black chromosomes). The $\log(1/P\text{-value})$ is depicted here for all 43,784 SNPs used in the GWAS analyses for each of the 6 cheese-making traits under study. The chromosome-wise significance thresholds are depicted as horizontal blue lines above each chromosome. RCT = rennet clotting time; logK20 = the logarithm of curd-firming time; A30 and A60 = curd firmness at 30 and 60 min, respectively; ILCY = individual laboratory cheese yield; and ILCDY = individual laboratory dried curd yield.

a possible breed-specific epistatic effect underlying the studied traits (de Camargo et al., 2015). Evidence of pleiotropic effects in the dairy sheep and goat genomes has been previously detailed (Rupp et al., 2015; Martin et al., 2018). These points suggest the difficulties of selecting a specific low-density SNP panel to be used in genomic selection for both breeds due to their genetic heterogeneity.

Only 11 markers in Assaf and 3 markers in Churra showed significant effects, at the chromosome-wise level, on more than 1 trait. No coincident genomic locations for these potential pleiotropic markers were observed among the 2 breeds. As summarized in Supplemental Table S2 (<https://data.mendeley.com/datasets/64jsbg5n9s/2>, Marina et al., 2021), most of these pleiotropic effects influence yield traits (MY, PY, and FY), although some other markers influenced cheese-making traits (e.g., OAR13_18633656, rs413217716, and rs415695399) showed effects on the ILCY and ILDCY in the Assaf breed; and the OAR6_85117333 simultaneously influence the PP and A60 traits in the Churra breed. Most of the potential pleiotropic SNPs listed in Supplemental Table S2 influenced the different traits in the same direction. In contrast, only 1 marker in the Assaf breed (rs427009325) and 2 in the Churra breed (rs417819324 and rs407891698) showed opposite pleiotropic effects on the RCT-A60 and RCT-A30 trait pairs (Supplemental Table S2).

The high genetic correlations estimated by Marina et al. (2020b) in the Assaf breed between the pairs of traits MY-PP (−0.52), MY-FP (−0.42), PP-FP (0.65), ILCY-ILDCY (0.87), and RCT-A60 (−0.79), and the high genetic correlation reported by Pelayo et al. (2021) in the Churra breed between the RCT-A30 trait pair (−0.77), suggest that several genes and mechanisms underlying those traits are partially common (Rupp et al., 2015). Epistatic and pleiotropic background mechanisms for dairy traits have been previously reported in dairy cattle (Sanchez et al., 2017), dairy buffalo (de Camargo et al., 2015), dairy goats (Martin et al., 2018), and dairy sheep (Marina et al., 2020b; García-Gómez et al., 2012a; Rupp et al., 2015; Banos et al., 2019).

QTL Regions and Gene Annotation

Considering the concordance between the regions of confidence, obtained through the LD decay pattern (100 kb), around each of the significant SNPs identified by the GWAS, and the regions of the QTL previously reported in sheep for milk and cheese-making traits (SheepQTL database, <http://www.animalgenome.org/cgi-bin/QTLdb/OA/index>), we identified a total of 16 and 44 QTL overlapping the confidence regions of the

significant SNPs identified here for Assaf and Churra breeds, respectively (Supplemental Table S3, <https://data.mendeley.com/datasets/64jsbg5n9s/2>, Marina et al., 2021). The significant confidence regions identified for the Assaf breed showed concordance with 1 QTL for milk PY and 14 QTL related to milk fatty acid content (Crisà et al., 2010; Li et al., 2020a). For the significant regions defined here for the Churra breed, we found 3 QTL associated with MY, 9 QTL associated with milk protein content, 18 QTL associated with milk fatty acid content, 3 QTL associated with lactose content, 2 QTL associated with rennet coagulation time, and 9 QTL associated with curd firmness (Crisà et al., 2010; García-Gómez et al., 2012a, 2013; Noce et al., 2016; Li et al., 2020a). None of the QTL previously identified in the confidence regions by association analyses were related to the SCS trait.

As mentioned above, the GWAS results did not identify significant SNPs in common between the 2 breeds studied in this paper. However, the 11 QTL previously described by Crisà et al. (2010) on OAR24 (OAR24:26228200–38615161 bp) related to milk fat content traits overlapped with the confidence regions identified here for both Assaf (in relation to FP and ILCY) and Churra breeds (regarding the ILCY and ILCDY traits).

A total of 63 and 176 different genes were annotated in the reference genome within the confidence regions of the significant SNPs related to milk and cheese-making traits in the Assaf and Churra breeds, respectively (Supplemental Table S4, <https://data.mendeley.com/datasets/64jsbg5n9s/2>, Marina et al., 2021). Of the total number of genes located within the confidence region defined around the significant SNPs, 28 genes showed significant results for more than 1 trait and only 4 genes (*5S_rRNA*, *U6*, *HFM1*, and *FBXL18*) were located within confidence regions defined for the 2 analyzed breeds. Interestingly, the noncoding RNAs *5S_rRNA* and *U6* genes were previously found by Taye et al. (2017) in QTL regions related to milk traits in dairy cattle. The *HFM1* gene was previously associated with an increase in the PP in dairy cattle (Pimentel et al., 2011). Finally, the *FBXL18* gene mediates polyubiquitylation and proteasomal degradation of the FBXL7 protein, which was previously associated with clinical mastitis in dairy cattle (Nayeri et al., 2019). Although no significant common SNPs were identified between the 2 studied breeds, we found 2 significant SNPs for both breeds in the surroundings of the *HFM1* and *FBXL18* genes, as shown in Supplemental Table S4. These results could highlight the candidate regions to be targeted by a low-density SNP chip to use in different dairy sheep breeds.

Gene Prioritization and Networks

After the functional prioritization analysis of the positional candidate genes, 60 genes were prioritized based on their functional similarity with the training gene list for milk and cheese-making traits (Supplemental Table S4, <https://data.mendeley.com/datasets/64jsbg5n9s/2>, Marina et al., 2021). Moreover, 11 genes in the confidence regions of the significant SNPs identified in the GWAS matched the genes from the training gene list used for the functional prioritization analyses. These 71 positional and functional candidate (PFC) genes were used to construct a protein-protein interaction network, where only the 44 interconnected genes are represented (Figure 4). These connections showed 8 gene interaction groups, of which the largest group of linked genes was composed of 26 of the identified PFC genes and 9 CF genes. The interaction enrichment analysis performed on the gene network revealed the high connectivity of the network (P -value: $3.79\text{e-}06$).

The PFC genes were colored according to the molecular functions (Ashburner et al., 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (<http://www.genome.ad.jp/kegg/>; Ogata et al., 1999), and network clusters identified in the functional enrichment analysis of the gene network (false discovery rate <0.05). In the gene network, 4 molecular functions have been highlighted: calcium ion binding (GO:0005509; depicted in green color) and ion transmembrane transporter (activity GO:0015075, blue), both of which play a relevant role in the lactation process (Neville, 2006; Pegolo et al., 2018); MAP kinase activity (GO:0004708, purple), which has been related to milk production mediated through insulin hormone signaling (Janjanam et al., 2014); and transcription factor binding (GO:0008134, red), which connects the 9 CF included in the gene network (8 identified in relation to the significant results for Churra breed and 1 with regard to the significant results for Assaf breed). Moreover, 3 KEGG pathways highly related to the lactation process were depicted in the gene network: insulin signaling pathway (gold), oxytocin signaling pathway (light blue), and prolactin signaling pathway (pink). Finally, the casein α/β network cluster (CL:35901, yellow) was depicted, highlighting the relevance of the gene network for the studied traits. All the functionally molecular functions, enriched KEGG pathways, and network clusters in the gene network are summarized in Supplemental Table S5 (<https://data.mendeley.com/datasets/64jsbg5n9s/2>, Marina et al., 2021).

Although no single gene in the network is common to the 2 studied breeds, the fact that significant genes are involved in the different metabolic pathways may

highlight the importance of the actual biological process of complex traits and could help to elucidate the genetic background differences between the Assaf and Churra breeds. In the resulting gene network, the metabolic pathways related to the studied phenotypes could be mediated by different PFC genes. The overlapping genes among the PFC genes located within the confidence region of the significant SNPs and the training gene list carefully selected from the literature are summarized in Table 2. The SNPs located in the surroundings of these genes are associated with several traits in Assaf (FP and ILCY) and Churra (PP, logK20, A60, ILCY, and ILCDY) sheep. This finding might contribute to designing a low-density multibreed SNP chip that contains variants in different genes implicated in the same biological pathways, thus solving their genetic heterogeneity.

The following is a brief description of the most relevant prioritized genes (Table 2 and 3) identified in relation to the significant results reported here for each breed (Figure 4). On the one hand, in the Assaf breed, the solute carrier family 2, member 2 (encoded by *SLC2A2* on chromosomes 1) is related to the biological process of carbohydrate metabolic processes and their transmembrane transport and insulin secretion regulation. In the literature, other solute carrier genes, such as the *SLC27A6* and *SLC37A1* genes, have been associated with milk traits such as the fatty acid composition and the mineral content of bovine milk (Bionaz and Loo, 2008; Nafikov et al., 2013; Sanchez et al., 2019). As shown in Figure 4, this gene is highly connected with 2 genes (*GYS2* and *PCK1*) related to the insulin signaling pathway. Phosphoenolpyruvate carboxykinase (encoded by the *PCK1* gene) has been significant in relation to citrate content in cow milk (Cánovas et al., 2013). This gene related to the insulin signaling pathway was associated with the milk and cheese-making traits together with the *NRP1* and *SCUBE2* genes, also highlighted by the prioritization approach. The miRNA encoded by the *NRP1* gene has been related to 5 milk production- and composition-related traits (MY, SCC, PP, FP, and lactose content) in dairy cattle (Bai et al., 2016; Do et al., 2017b). Finally, the *SCUBE2* gene is related to the molecular function of calcium ion binding, as previously described by Yates et al. (2020). Moreover, the *MYRIP* and *GPM6A* genes (positioned on sheep chromosomes 19 and 26, respectively) were identified within the confidence regions of significant SNPs associated with ILCDY and logK20 traits, respectively. The *MYRIP* and *GPM6A* genes, related to the biological function of positive regulation of insulin secretion and calcium ion transmembrane transport, respectively, have also been included in a co-association

Figure 4. Gene network displaying the connections between the 44 interconnected functional and positional candidate genes related to milk and cheese-making traits in the 2 studied sheep breeds (Assaf and Churra). The genes are represented as nodes, and the edges linking the nodes represent the interactions between the genes. The name of the genes is represented in pink for the Assaf breed and in blue for Churra. The nodes have been colored according to the molecular function: calcium ion binding (GO:0005509; green), ion transmembrane transporter (activity GO:0015075, blue), MAP kinase activity (GO:0004708, purple) and, transcription factor binding (GO:0008134, red); Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways: insulin signaling pathway (mmu04910, gold), oxytocin signaling pathway (mmu04921, light blue), and prolactin signaling pathway (mmu04917, pink); molecular function: calcium ion binding (GO:0005509; green), ion transmembrane transporter (activity GO:0015075, blue), MAP kinase activity (GO:0004708, purple) and, transcription factor binding (GO:0008134, red); and the network cluster: Casein, alpha/beta (CL:35901, yellow). The gene interactions can be classified as 3 groups: (1) known interactions: from curated databases (light blue) and experimentally determined (pink); (2) predicted interactions: gene neighborhood, fusions, and co-occurrence (green, red, dark blue, respectively); (3) others: text mining (yellow), coexpression (black) and protein homology (violet).

network related to milk and cheese-making traits in the Assaf breed (Marina et al., 2020b).

On the other hand, for the Churra breed, Table 2 also shows 9 of the prioritized genes identified within

the confidence regions of significant SNPs. On chromosome 3, the *LALBA* and *PTHLH* genes were related to the PP and ILCY traits in the results reported here, respectively. α -LG is one of the main whey proteins

Table 2. Genes located within the confidence regions (100 kb) of the significant SNPs identified by the GWAS reported in this work and present in the reference gene set selected from the literature

Breed identified	CHR ¹	Gene symbol	Gene name	Trait Identified ²	Reference
Assaf	1	<i>SLC2A2</i>	Solute carrier family 2, facilitated glucose transporter member 2	FP	Marina et al. (2020b)
Assaf	24	<i>ACTB</i>	Actin, cytoplasmic 1	ILCY	Ogorevc et al. (2009)
Churra	3	<i>LALBA</i>	Alpha-lactalbumin	PP	Marina et al. (2020a); Ogorevc et al. (2009); Suárez-Vega et al. (2016a)
Churra	3	<i>PTH1L</i>	Parathyroid hormone-related protein	ILCY	Ogorevc et al. (2009)
Churra	6	<i>CSN1S1</i>	Alpha-S1-casein	A60 and PP	Marina et al. (2020a); Sanchez et al. (2019); Suárez-Vega et al. (2016a); Ogorevc et al. (2009)
Churra	6	<i>CSN1S2</i>	Alpha-S2-casein-like A	A60 and PP	Marina et al. (2020a); Sanchez et al. (2019); Suárez-Vega et al. (2016a); Ogorevc et al. (2009)
Churra	6	<i>CSN2</i>	Beta-casein	A60 and PP	Marina et al. (2020a); Sanchez et al. (2019); Suárez-Vega et al. (2016a); Ogorevc et al. (2009)
Churra	9	<i>CSMD3</i>	CUB and sushi domain-containing protein 3	ILCY	Sanchez et al. (2019)
Churra	16	<i>HCN1</i>	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1	ILCY	Sanchez et al. (2019)
Churra	19	<i>MYRIP</i>	Rab effector MyRIP	ILCDY	Marina et al. (2020b)
Churra	26	<i>GPM6A</i>	Neuronal membrane glycoprotein M6-A	logK ₂₀	Marina et al. (2020b)

¹CHR = chromosome.²FP = fat percentage; PP = protein percentage; logK₂₀ = curd-firming time expressed in log₁₀; A60 = curd firmness at 60 min after rennet addition; ILCY = individual laboratory cheese yield.**Table 3.** Transcription factors and co-transcription factors located within the confidence regions (100 kb) of the significant SNPs identified by the GWAS reported in this work and present in the reference gene set selected from the literature

Breed identified	CHR ¹	Gene symbol	Gene name	Gene type	Trait identified ²	Reference
Assaf	24	<i>ACTB</i>	Actin, cytoplasmic 1	Co-transcription factor	ILCY	Ogorevc et al. (2009)
Chur0	3	<i>CCNT1</i>	Cyclin-T1	Co-transcription factor	PP	
Churra	4	<i>HDAC9</i>	Histone deacetylase 9	Co-transcription factor	ILCY	
Churra	4	<i>NRF1</i>	Nuclear respiratory factor 1	Transcription factor	ILCDY	
Churra	11	<i>BCAS3</i>	Breast carcinoma-amplified sequence 3 homolog	Co-transcription factor	ILCY	
Churra	11	<i>HDAC5</i>	Histone deacetylase 5;Hdac5;ortholog	Co-transcription factor	ILCY	
Churra	18	<i>SIN3A</i>	Paired amphipathic helix protein Sin3a	Co-transcription factor	ILCY	
Churra	21	<i>BARX2</i>	Homeobox protein BarH-like 2	Transcription factor	logK ₂₀	
Churra	22	<i>ANKRD1</i>	Ankyrin repeat domain-containing protein 1	Co-transcription factor	ILCY	
Churra	25	<i>EGR2</i>	E3 SUMO-protein ligase EGR2	Transcription factor	ILCY	

¹CHR = chromosome.²PP = protein percentage; logK₂₀ = curd-firming time expressed in log₁₀; ILCY = individual laboratory cheese yield; ILCDY = individual laboratory dried curd yield.

encoded by the *LALBA* gene, where a missense variant (p. Val27Ala) was significantly related to the milk protein and fat content in Spanish Churra sheep (García-Gómez et al., 2012a). The valuable role of parathyroid hormone-like hormone (encoded by the *PTH1L* gene) during lactation was highlighted through knockout in mice (Ogorevc et al., 2009). The *PTH1L* gene has also been described as a promising candidate gene for the milk protein and FP in Holstein cows (Cui et al., 2014). This gene is closely related to the Ca^{2+} concentration in milk (Onda et al., 2006) and highly connected with another gene related to the molecular function of calcium ion binding in the gene network, namely, the *MMP13* gene. In addition, this gene is expressed in the mammary gland and appears to be critical for the morphogenesis and angiogenesis of this structure (Cros et al., 2002). We also found that the *CSMD3* and *HCN1* genes, which are located on sheep chromosomes 9 and 16, respectively, were related to ILCY in the Churra breed. These 2 genes have also been identified within the confidence region of QTLs previously detected for milk cheese-making traits in dairy cattle (Sanchez et al., 2019). Specifically, hyperpolarization-activated cyclic nucleotide-gated potassium channel 1 (encoded by the *HCN1* gene) has been identified as significantly related to the lactation persistence trait in dairy cattle (Do et al., 2017a).

Last, TF and CF are related to the transcriptional control of gene expression, which underlies the creation and maintenance of tissue-specific protein synthesis and the response to specific cellular signaling pathways (Latchman, 1997). Therefore, TF and CFs could be considered potential regulators of the pathway highlighted by this gene network. Within the PFC gene prioritized in this work, we found a total of 10 TF and CF factors (Table 3). Figure 4 shows a highly connected gene group composed of 5 CF (*BCAS3*, *CBX5*, *HDAC5*, *SIN3A*, and *HDAC9* genes colored in red) related to the PP and ILCY traits identified in Churra breed, and a single CF (*ACTB* gene) related to the ILCY trait identified in the Assaf breed, all of them related to the transcription factor binding molecular function. As shown in Figure 4, the *ACTB* gene is highly connected with several PFC genes discovered in this work and detected within the confidence regions identified here for the 2 breeds. This particular CF encodes the β -actin protein, located on sheep chromosome 24, which is included in the database of cattle candidate genes for dairy-related traits associated with mastitis resistance reported by Ogorevc et al. (2009). The *ACTB* gene has been identified with a high probability of showing a binding site for a TF that is differentially expressed and linked with several genes related to energy conservation metabolism and

cell proliferation in beef cattle (Fonseca et al., 2018). This gene is also highly expressed in the dairy cattle milk somatic cell transcriptome at the peak and end of lactation (Wickramasinghe et al., 2012). The remaining CF (2) and TF (3), highlighted by the analyses for the Churra breed, were found in the areas surrounding SNPs significantly associated with the ILCY, ILCDY, logK20, and PP traits (Table 3).

Finally, in the Churra breed, 3 genes (*CSN1S1*, *CSN1S2*, and *CSN2*) that compose the casein α/β network cluster are related to milk and cheese-making traits (Yousefi et al., 2013; Giambra et al., 2014; Pazzola et al., 2014b). Within the reference gene list previously reported in the literature, we found 3 casein genes (*CSN1S1*, *CSN1S2*, and *CSN2*) located on chromosome 6 that have been identified as significantly associated with the A60 and PP traits and could present pleiotropic effect, thus highlighting the potential effect of this genomic region in the Churra breed. Several authors in dairy species have previously described the effect of these genes on milk and cheese-making traits. Specifically, the *CSN1S1* and *CSN1S2* genes have been associated with MY, PY, FY, and milk casein content in sheep (Barillet et al., 2005; Giambra et al., 2014) and the curd-firming times and efficient renneting properties in Sarda goats (Pazzola et al., 2014b). The *CSN1S2* gene has also been associated with rennet-induced gelation of skim milk in dairy cattle (Gregersen et al., 2015). Moreover, the *CSN2* gene has been significantly associated with milk and cheese-making traits in dairy cattle (Cecchinato et al., 2015) and dairy goats (Pazzola et al., 2014b) and with curd-firming time in Sarda sheep (Noce et al., 2016). A previous transcriptomic analysis of the sheep mammary gland highlighted the very high expression levels of the *CSN2* gene in dairy sheep during lactation (Suárez-Vega et al., 2016b).

CONCLUSIONS

In summary, the GWAS results together with prioritized genes and gene network analyses presented in this study provide a new set of candidate genes related to milk and cheese-making traits for the studied breeds. Some of the genes resulting from these analyses have been previously associated with milk and cheese-making traits in dairy populations, thus supporting our findings. In addition, several of the significant variants located in the area surrounding the candidate genes showed a pleiotropic effect on milk and cheese-making traits; therefore, they are potential markers influencing various pathways related to the studied traits. To our knowledge, this is the first study to perform GWAS for cheese-making traits in parallel in 2 different dairy

breeds, and the results were directly compared. The functional and positional candidate genes highlighted in this work can be further analyzed for genomic variations. Incorporating those variants in genomic selection strategies could lead to more rapid genetic gains, specifically for traits that are difficult to routinely measure on farms, such as cheese-making traits.

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