



RESEARCH ARTICLE

Genetic Association of Candidate Genes with Milk and Mastitis Resistance Traits using SNP-Chip Array in Holstein Friesian and Pakistani Indigenous Dairy Cattle Breeds

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ABSTRACT

The use of genetic markers to improve milk and udder health traits in dairy cattle are rapidly growing worldwide. This study was designed to evaluate genetic associations of markers from a SNP-Chip including 20 SNPs in eight candidate genes, namely *JAK2*, *TRAPPC9*, *DGAT1*, *LAG3*, *STAT5A*, *STAT5B*, *CD4* and *PTK2*, with milk production and mastitis resistance traits in four dairy cattle breeds. The study included 420 lactating dairy cows (Holstein Friesian and indigenous Achai, Red Sindhi, and Sahiwal) that were genotyped using the Chinese Cow's SNPs Chip-1 (CCSC-1). Out of the 20 SNPs evaluated, 13 SNPs were significantly associated with milk and/or udder health traits. Also, genetic associations varied across breeds, with certain SNPs being significantly associated with one or multiple breeds. In Holstein Friesian the CC genotype (SNP 7 on *TRAPPC9* gene) increased 305 milk yield and reduced the incidence of clinical mastitis. Also in Red Sindhi, the CC genotype (SNP 14 on *DGAT1* gene) increased 305 milk yield, protein (%), and lactose (%), and reduced the level of somatic cell score. Candidate genes such as *JAK2* in Achai, *JAK2* and *LAG3* in Holstein Friesian, and *CD4* in Sahiwal were significantly associated with the presence of *Staphylococcus aureus* isolated from raw milk. These results infer that genetic markers located on candidate genes present in CCSC-1 could be potentially used in genetic selection to improve milk production and mastitis resistance in dairy cattle breeds of Pakistan.

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INTRODUCTION

Globally, 81% of milk production comes from cattle. Milk is a crucial part of the human diet providing fat, protein, and minerals. India is the world's largest producer, followed by USA, Pakistan, China, and Brazil (FAO, 2021). In Pakistan the livestock farming represents 60.85% of the agricultural sector and contributes with a 14.63% to the total gross domestic product (Pakistan Economic Survey, 2024). However, one of the leading disease and management problem of the global dairy industry is the mastitis disease. Worldwide the dairy industry incurs a loss of US\$ 40 billion per year due to this disease (Neculai-Valeanu and Ariton, 2022), and South Asia (e.g., India, Pakistan) is not the exception where the prevalence of sub-clinical mastitis (SCM)

ranges from 17% to 81% (Ali *et al.*, 2011; Rafiullah *et al.*, 2017; Ali *et al.*, 2021a; Shahzad *et al.*, 2024). Indeed, SCM and clinical mastitis (CM) incidence severely impacts the development of dairy farming in the region, where the tropical and subtropical environmental conditions benefit to the growth of mastitis pathogens (Cheng and Han, 2020).

The mastitis disease produces an increase in somatic cell count (SCC), due to the white blood cells flooding into the quarter to tackle the infection, reducing milk yield and quality (Ali *et al.*, 2021b). Note that mastitis is positively correlated with SCC, which means lower SCC in dairy cattle could be used as an indicator trait for mastitis resistance (Shook and Schutz, 1994). The main pathogens causing mammary gland infection (i.e., mastitis) in dairy cows are *Staphylococcus aureus* (*S. aureus*), *Streptococcus*,

and *Escherichia coli* (Godden *et al.*, 2002). *S. aureus* is not only the most important cause of mastitis, but also a zoonotic bacterium and methicillin-resistant *S. aureus* is difficult to treat with antibiotics (Wang *et al.*, 2014). To effectively reduce and control mastitis, the incidence of *S. aureus* and the effects caused by antibiotic treatments in dairy cows, the dairy sector could focus on the use of genetic selection targeting genetic regions associated with the disease and the main pathogen.

It is well-known that mastitis affects all breeds. However, most of the research is performed on Holstein Friesian, the most famous dairy breed in the world. On the other hand, Pakistan is enriched with local dairy cattle breeds, such as Achai, Red Sindhi, and Sahiwal, making it necessary to develop genetic tools to reduce mastitis while maintaining or increasing milk quality and milk yield. To address this challenge, our Chinese collaborator developed a Chinese Cow's SNPs Chip-1 (CCSC-1) that included relevant genes associated with mastitis resistance and milk production traits (Khan *et al.*, 2019; Khan *et al.*, 2022). This study aimed to validate in Holstein Friesian and three Pakistani indigenous dairy cattle breeds (Achai, Red Sindhi, and Sahiwal), the association of genes and SNP markers included in CCSC-1 with milk production traits, including 305 milk yield, protein (%), fat (%), lactose (%), and udder health traits, including log(SCC), CM incidence, and presence of *S. aureus*.

MATERIALS AND METHODS

Animals: The study included a total of 420 lactating dairy cows from four breeds: Holstein Friesian (n=100), and three Pakistani local dairy cattle breeds, namely Achai (n=116), Red Sindhi (n=104), and Sahiwal (n=100). We collected performance data, including production, reproduction, and health records from eight farms in Pakistan. Furthermore, we collected milk samples from all cows. The samples (30mL) were collected in sterile bottles and immediately transported in ice to the Animal Breeding and Genetics laboratory (Abdul Wali Khan University Mardan, Pakistan) for further analysis. We analyzed milk composition (protein, fat, lactose and SNF contents expressed in %) from each milk sample with a Lactoscan SA standard 1060 milk analyzer (Milkotronic LTD, Nova Zagora, Bulgaria) (Javed *et al.*, 2024). Also, we determined the SCC using direct microscopy as proposed by Usman *et al.* (2017). Animal study was approved (Dir/A&R/AWKUM/2022/9676) by the Advanced Studies and Research Board of the Faculty of Chemical and Life Sciences, Abdul Wali Khan University Mardan (Mardan, Pakistan).

***Staphylococcus aureus* isolation and molecular identification:** *S. aureus* was isolated and identified as described by Haq *et al.* (2024), using mannitol salt agar plates and confirmed by biochemical assays (gram staining, catalase, and coagulase tests) using a quality-control strain of *S. aureus* ATCC25923. The molecular identification employed the *nuc* gene through Polymerase Chain Reaction (PCR) with the following primers: Forward: 5'- GCGATTGATGGTGATACGGTT-3' and Reverse: 3'-CGAAATCAAGCAGTTCCGAACCGA-5' (Lubna *et al.*, 2023).

Genotyping: For DNA extraction, 5 ml blood from the caudal vein of each cattle was collected in EDTA tube and stored at -20°C till further processing. The DNA was extracted by using EZ-10 Column Blood Genomic DNA Miniprep Kit (Bio Basic Canada INC). After confirming the quality and quantity with a NanoDrop Spectrophotometer by measuring the A260/A280 ratio, all DNA samples were sent to Capital Bio Technology Co., Ltd, Beijing, China, for genotyping using the CCSC-1 Chip. The CCSC-1 was co-designed using microfluidic array techniques by our Chinese collaborator from China Agricultural University, Beijing, China and Capital Bio Technology Co., Ltd (Beijing, China). The CCSC-1 Chip includes 20 SNPs located in eight candidate genes, namely *JAK2*, *TRAPPC9*, *DGAT1*, *LAG3*, *STAT5A*, *STAT5B*, *CD4* and *PTK2* (Table 1).

Table 1: The SNPs information in the eight genes included in Chinese Cow's SNPs Chip-1

| SNP # | Gene | SNP location | Variable type | Consequence type |
|--------|----------------|--------------------|---------------|------------------------------------|
| SNP 1 | <i>JAK2</i> | Exon | T/C | Missense variant |
| SNP 2 | <i>JAK2</i> | Exon | T/C | Synonymous Variant |
| SNP 3 | <i>JAK2</i> | 3' Flanking region | T/C | downstream variant |
| SNP 4 | <i>JAK2</i> | 3' Flanking region | G/A | downstream variant |
| SNP 5 | <i>JAK2</i> | 3' Flanking region | T/C | downstream variant |
| SNP 6 | <i>TRAPPC9</i> | Exon | T/C | Non coding transcript exon variant |
| SNP 7 | <i>TRAPPC9</i> | Exon | T/C | Missense variant |
| SNP 8 | <i>TRAPPC9</i> | Intron | T/G | Intron variant |
| SNP 9 | <i>TRAPPC9</i> | Intron | G/A | Intron variant |
| SNP 10 | <i>TRAPPC9</i> | Intron | G/A | Intron variant |
| SNP 11 | <i>TRAPPC9</i> | Intron | G/A | Intron variant |
| SNP 12 | <i>DGAT1</i> | Intron | G/A | Intron |
| SNP 13 | <i>DGAT1</i> | Intron | G/A | Intron |
| SNP 14 | <i>DGAT1</i> | Exon | T/C | Non coding transcript exon variant |
| SNP 15 | <i>LAG3</i> | 3' Flanking region | G/A | downstream variant |
| SNP 16 | <i>LAG3</i> | 3' Flanking region | A/C | downstream variant |
| SNP 17 | <i>STAT5A</i> | Intron | A/C | Intron variant |
| SNP 18 | <i>STAT5B</i> | Exon | T/C | Exon variant |
| SNP 19 | <i>CD4</i> | 5' Flanking region | T/C | Upstream variant |
| SNP 20 | <i>PTK2</i> | Intron | G/A | Intron variant |

Statistical analysis: The association analysis between the 20 SNPs in CCSC-1 Chip and all milk production and udder health traits were performed using the following linear predictor:

$$\eta = G_i + Farm_j + Parity_k + DIM_l$$

where η is a function of the expected value of the trait, G_i is the i^{th} genotype, $Herd_j$ is j^{th} herd (8 levels), $Parity_k$ is the k^{th} parity (5 levels), and DIM_l is the l^{th} days in milk (DIM) (3 levels).

For 305 milk yield, protein (%), fat (%), lactose (%), SNF (%), and log (SCC), we used the following linear regression model:

$$y = \eta + e$$

where y is the observed value of the trait (305 milk yield, fat, protein, lactose, SNF, or log(SCC)), η is the linear predictor described above, and e is the error term. Furthermore, for incidence of clinical mastitis and the presence of *S. aureus*, we used the following logistic regression model:

$$\log\left(\frac{p}{1-p}\right) = \eta$$

where p is the probability of the binary outcome (0 or 1), and η is the linear predictor.

The association between each SNP marker and milk production and health traits were evaluated using a partial F-test. The F-test was implemented by comparing the full model with the reduced model without the SNP marker effect. All models were performed using the functions *lm* and *glm* implemented in the R software (R Core Team 2023).

RESULTS

Overall, two SNPs (SNP 2 and 9) were excluded from analysis due to bad genotyping quality. Monomorphic SNP markers were found, two were monomorphic across all four breeds (SNP 4 and 6) and 10 monomorphic SNPs in different breeds: in Holstein Friesian (SNP 16 and 18), in Achai (SNP 16), in Red Sindhi (SNP 7 and 18), and in Sahiwal (SNP 3, 5, 7, 12, 13, 15, 16, 17, 18, and 20). Note that is relevant because those genomic markers could be potentially fixed in our populations.

SNP markers associations with milk and udder health traits: Seven SNPs were found to be significantly

associated with milk traits in Holstein Friesian cows (Fig. 1). Note that SNP 1, 7, 8 and 10 were significantly associated with 305 milk yield, SNP 5 was associated with fat (%), SNP 3, 5 and 13 were associated with protein (%), SNP 3 and 5 were associated with lactose (%), and SNP 3, 5, and 7 with SNF (%) (Table 2). Moreover, for Achai cows, five SNPs were significantly associated with milk traits. SNP 17 was associated with 305 milk yield, SNP 18 associated with fat (%), SNP 8 and 19 associated with protein (%), and SNP 7 associated with both lactose (%) and SNF (%) (Table 3). For Red Sindhi cows, five SNPs were significantly associated with the production traits as it is also shown in Fig. 1. SNP 1, 14, 16, and 17 were associated with 305 milk yield, SNP 19 associated with fat (%), and SNP 1, 14, and 16 associated with both protein (%) and lactose (%) (Table 4). Lastly, for Sahiwal cows, only SNP 10 was significantly associated with protein (%) (Table 5).

For our three udder health traits, we found genetic associations in all four breeds (Fig. 2). In Holstein Friesian cows three SNPs were significantly associated with udder health traits. SNP 7 was found to be associated

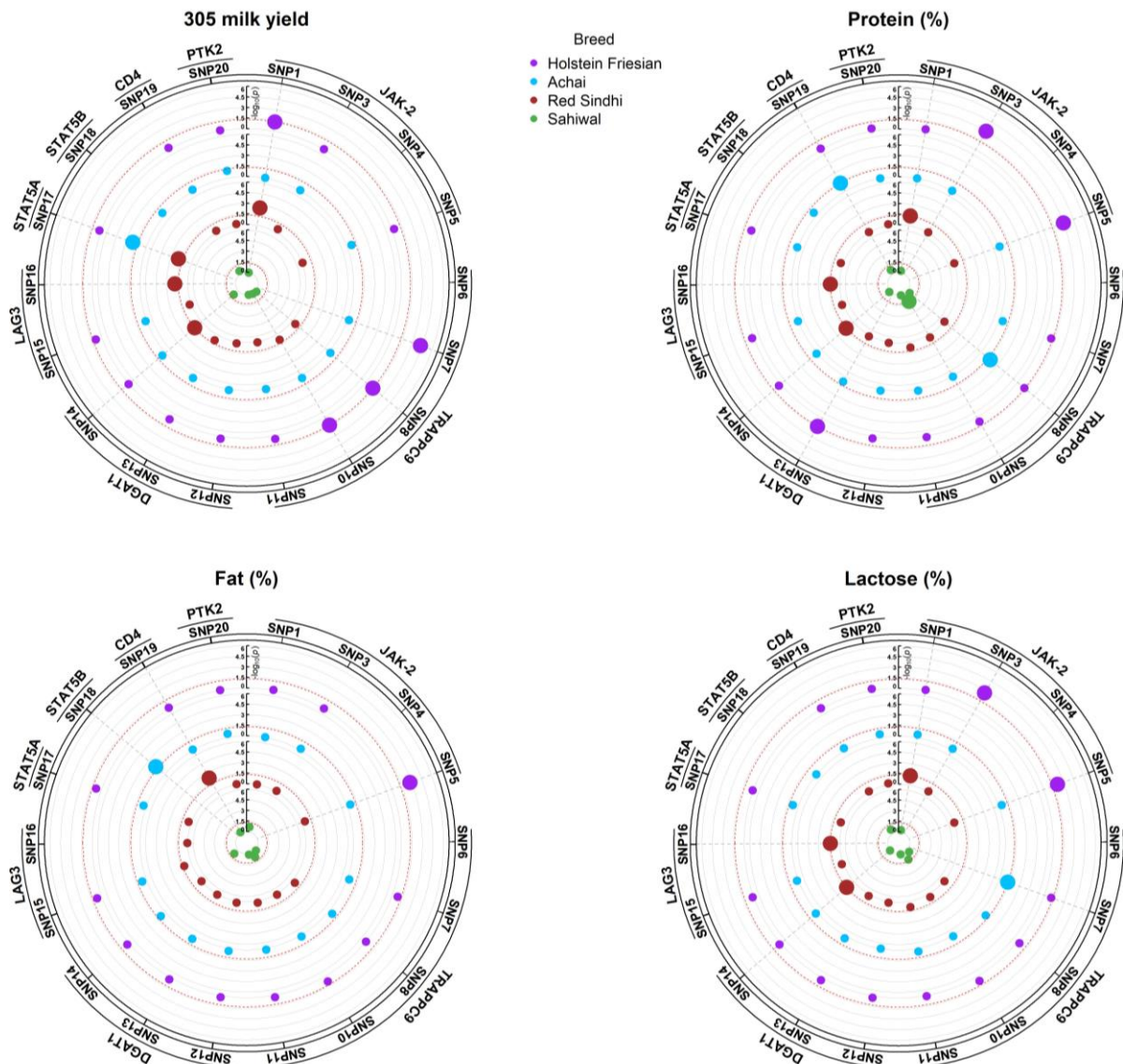


Fig. 1: Circular Manhattan plots showing the association between candidate SNP/genes and production traits: 305 milk yield, fat (%), protein (%), and lactose (%). Larger point sizes indicate that the SNP was significantly associated with the trait (P -value < 0.05). The red dashes lines represent the threshold of P -value $= 0.05$.

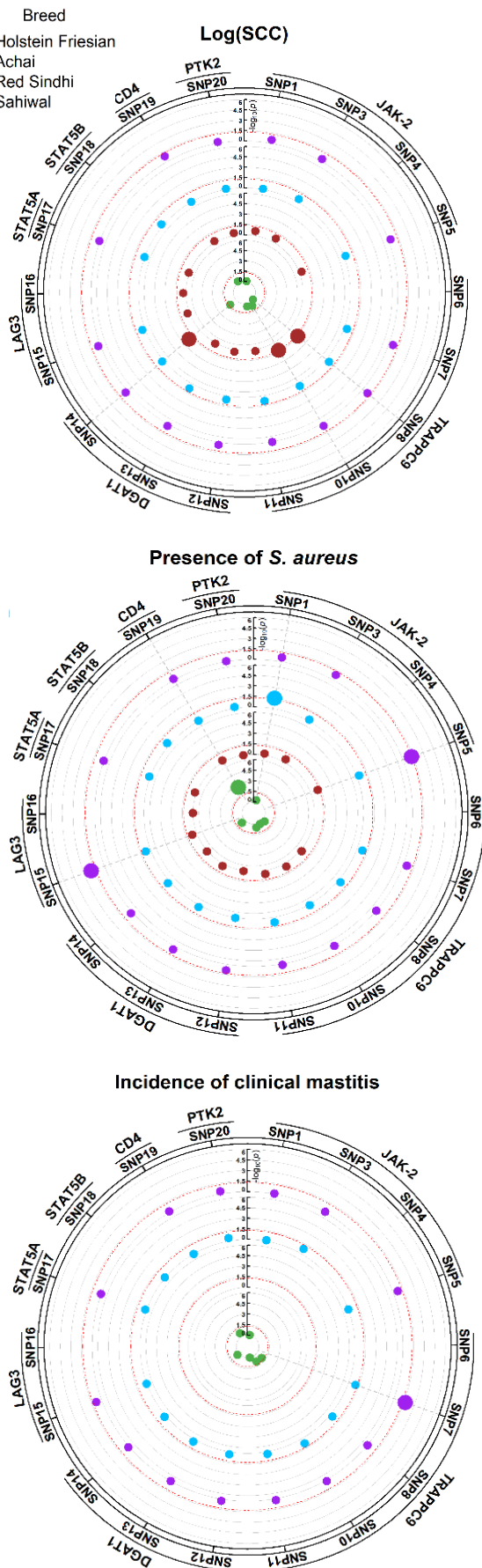


Fig. 2: Circular Manhattan plots showing the association between candidate SNP/genes and udder health traits: log (SCC), incidence of clinical mastitis, and presence of *S. aureus*. Larger point sizes indicate that the SNP was significantly associated with the trait ($P < 0.05$). The red dashes lines represent the threshold of P -value = 0.05.

with CM incidence, and SNP 5 and 15 associated with the presence of *S. aureus* (Table 2). Furthermore, for Achai and Sahiwal cows, SNP 1 and 19 were found to be significantly associated with the presence of *S. aureus*, respectively (Table 3 and 5), and for Red Sindhi cows, three SNPs (SNP 8, 10, and 14) were significantly associated with log (SCC) (Table 4).

Genotype association with milk and udder health traits: We evaluated genotype associations with milk and udder health traits. For Holstein cows, the CC genotype (SNP 7 on *TRAPPC9* gene) was significantly associated with 305 milk yield and with the incidence of clinical mastitis. Note that genotype CC increase milk yield (5056.78 ± 275.12) comparing with TC (4036.68 ± 397.69 ; P -value < 0.05) and TT (2715.12 ± 553.49 ; P -value < 0.01) and reduce the incidence of CM (CC: 0.35 ± 0.09) comparing with TT (0.76 ± 0.17 ; P -value < 0.05) (Table 2). Furthermore, for Red Sindhi cows, the CC genotype (SNP 14 on *DGATI* gene) was significantly associated with 305 milk yield, protein (%), lactose (%) and with log(SCC). Note that CC genotype increase 305 milk yield (1790.12 ± 105.55) comparing to TC (1549.34 ± 100.18 ; P -value < 0.05) and TT (1518.33 ± 176.6 ; P -value < 0.05), increased protein (%) (CC: 3.23 ± 0.07) comparing with TC (3.04 ± 0.07 ; P -value < 0.05), increases lactose (%) (CC: 4.85 ± 0.1) comparing with TC (4.58 ± 0.1 ; P -value < 0.05), while reduce the log(SCC) (CC: 11.85 ± 0.23) comparing with TT (13.12 ± 0.39 ; P -value < 0.05) (Table 4).

DISCUSSION

In dairy cattle, the use of genomic selection to improve milk and health traits is one of the most powerful tools, because it produces permanent and cumulative improvements in the populations. This study targets genetic markers located in eight candidate genes for the traits of interest, namely *JAK2*, *TRAPPC9*, *DGATI*, *LAG3*, *STAT5A*, *STAT5B*, *CD4* and *PTK2*. All these genes have been associated with production and health traits mostly in Holstein Friesian (Usman *et al.*, 2014, 2015, 2017; Jiang *et al.*, 2019; Khan *et al.*, 2019; Yang *et al.*, 2021; Taherkhani *et al.*, 2022; Khan *et al.*, 2022), but not in indigenous breeds like those present in Pakistan. To address this, genetic association between SNP markers and milk and udder health traits were evaluated in Holstein Friesian, Achai, Red Sindhi and Sahiwal breeds, using the CCSC-1 SNP-Chip.

Interestingly, out of the 20 SNPs evaluated, 13 of them were associated either with milk production traits, udder health traits or both. Also, the genetic associations varied across breeds, with certain genetic polymorphisms being significantly associated with one, two or multiple breeds. Note that for genes, such as *JAK2*, *LAG3* and *CD4*, there is limited knowledge about their association with the main pathogen (*S. aureus*) that produce mastitis in dairy cows. In this regard, our findings showed that *JAK2* in Achai cows, *JAK2* and *LAG3* in Holstein Friesian, and *CD4* in Sahiwal cows were significantly associated with the presence of *S. aureus*. These results

Table 2: Association of SNPs in studied genes with milk and udder health traits in Holstein Friesian breed

| Gene | SNP # | Genotype | 305 milk yield | Fats (%) | Protein (%) | Lactose (%) | SNF (%) | Log (SCC) | Incidence of Clinical Mastitis | Presence of <i>S. aureus</i> |
|---------|-----------|----------------|------------------------------|------------------------|-------------------------|------------------------|-------------------------|------------|--------------------------------|------------------------------|
| JAK2 | SNP 1 | TT (0.67) | 4420.45±290.78 ^a | 3.94±0.37 | 2.98±0.06 | 4.44±0.11 | 8.04±0.17 | 11.59±0.78 | 0.45±0.09 | 0.46±0.11 |
| | | TC (0.33) | 5101.59±375.64 ^b | 4.00±0.47 | 3.03±0.08 | 4.4±0.14 | 8.07±0.22 | 12.57±1.01 | 0.47±0.11 | 0.63±0.14 |
| | SNP 3 | TT (0.77) | 4587.26±294.25 | 3.98±0.36 | 2.93±0.06 ^A | 4.33±0.1 ^A | 7.87±0.16 ^A | 11.57±0.77 | 0.44±0.08 | 0.47±0.1 |
| | | CT (0.23) | 4553.79±408.07 | 3.93±0.5 | 3.19±0.08 ^B | 4.73±0.14 ^B | 8.59±0.22 ^B | 12.4±1.07 | 0.42±0.12 | 0.78±0.19 |
| | SNP 5 | TT (0.21) | 4994.14±429.45 | 3.3±0.5 ^A | 3.17±0.09 ^A | 4.72±0.15 ^A | 8.53±0.23 ^A | 11.49±1.13 | 0.31±0.12 | 0.81±0.15 ^a |
| | | TC (0.48) | 4472.47±306.67 | 4.52±0.35 ^B | 3.00±0.06 ^A | 4.41±0.1 ^{AB} | 8.03±0.17 ^{AB} | 12.14±0.8 | 0.51±0.09 | 0.48±0.11 ^b |
| TRAPPC9 | SNP 7 | CC (0.31) | 4481.74±398.55 | 2.96±0.46 ^A | 2.83±0.08 ^B | 4.22±0.14 ^B | 7.65±0.22 ^B | 10.96±1.05 | 0.36±0.11 | 0.4±0.13 ^b |
| | | TT (0.08) | 2715.12±553.49 ^A | 3.18±0.74 | 3.01±0.13 | 4.3±0.21 | 7.77±0.34 ^{AB} | 13.84±1.59 | 0.76±0.17 ^a | 0.34±0.22 |
| | | TC (0.17) | 4036.68±397.69 ^A | 4.7±0.53 | 2.84±0.09 | 4.21±0.15 | 7.55±0.24 ^A | 12.23±1.14 | 0.55±0.12 ^{ab} | 0.57±0.10 |
| | | CC (0.75) | 5056.78±275.12 ^B | 3.85±0.37 | 3.05±0.06 | 4.53±0.11 | 8.26±0.17 ^B | 11.3±0.79 | 0.35±0.09 ^b | 0.43±0.23 |
| | SNP 8 | TT (0.39) | 4039.66±362.86 ^a | 3.89±0.46 | 2.88±0.08 | 4.33±0.13 | 7.74±0.21 | 12.49±0.96 | 0.45±0.11 | 0.53±0.15 |
| | | TG (0.51) | 4728.68±298.7 ^{ab} | 4.02±0.38 | 3.05±0.06 | 4.48±0.11 | 8.19±0.17 | 11.92±0.79 | 0.43±0.09 | 0.54±0.1 |
| | | GG (0.10) | 4994.53±547.68 ^b | 3.92±0.69 | 2.98±0.12 | 4.42±0.2 | 8.05±0.32 | 9.43±1.44 | 0.44±0.16 | 0.5±0.22 |
| | | GG (0.05) | 5597.95±763.13 ^a | 5.7±0.94 | 3.01±0.16 | 4.83±0.27 | 8.24±0.44 | 10.62±2.02 | 0.41±0.23 | 0.32±0.33 |
| DGATI | SNP 10 | AG (0.43) | 4685.74±309.45 ^{ab} | 4.01±0.38 | 3.04±0.07 | 4.43±0.11 | 8.13±0.18 | 11.17±0.82 | 0.42±0.09 | 0.54±0.1 |
| | | AA (0.52) | 4189.77±344.8 ^b | 3.9±0.42 | 2.91±0.07 | 4.35±0.12 | 7.81±0.2 | 12.31±0.91 | 0.5±0.1 | 0.54±0.14 |
| SNP 13 | GG (0.19) | 4444.01±439.67 | 4.48±0.54 | 2.88±0.09 ^a | 4.34±0.16 | 7.81±0.25 | 11.25±1.15 | 0.48±0.13 | 0.36±0.17 | |
| | | AG (0.43) | 4445.91±337.9 | 3.87±0.41 | 2.97±0.07 ^{ab} | 4.38±0.12 | 7.96±0.19 | 12.33±0.88 | 0.43±0.1 | 0.5±0.12 |
| LAG3 | SNP 15 | AA (0.38) | 4775.1±348.82 | 3.85±0.43 | 3.08±0.07 ^b | 4.53±0.12 | 8.26±0.2 | 11.42±0.91 | 0.43±0.1 | 0.67±0.13 |
| | | GG (0.60) | 4622.99±311.7 | 4.26±0.38 | 3.01±0.07 | 4.48±0.11 | 8.1±0.18 | 11.79±0.83 | 0.35±0.09 | 0.63±0.1 ^{AB} |
| | | GA (0.32) | 4780.19±352.46 | 3.48±0.43 | 3±0.08 | 4.4±0.13 | 8.05±0.21 | 11.33±0.94 | 0.55±0.1 | 0.33±0.12 ^A |
| | | AA (0.08) | 3497.45±600.87 | 4.17±0.74 | 2.88±0.13 | 4.31±0.22 | 7.73±0.35 | 13.44±1.6 | 0.51±0.17 | 0.99±0.24 ^B |

Note: Data is displayed as LS means±SE. The values in parenthesis represent the genotype frequency. Different superscripts of the uppercase letters within the same column in a SNP represent that $P<0.01$; lower case letter represent $P<0.05$.

Table 3: Association of SNPs in studied genes with milk and udder health traits in Achai breed

| Gene | SNP # | Genotype | 305 milk yield | Fats (%) | Protein (%) | Lactose (%) | SNF (%) | Log (SCC) | Incidence of Clinical Mastitis | Presence of <i>S. aureus</i> |
|---------|--------|-----------|------------------------------|------------------------|-------------------------|-------------------------|-------------------------|------------|--------------------------------|------------------------------|
| JAK2 | SNP 1 | TT (0.92) | 964.34±87 | 5.1±0.6 | 3.64±0.17 | 5.2±0.15 | 9.33±0.26 | 9.73±1.32 | 0.21±0.08 | 0.17±0.04 ^a |
| | | TC (0.08) | 977.86±127.31 | 5.03±0.88 | 3.63±0.25 | 5.37±0.22 | 9.27±0.39 | 10.1±1.93 | 0.17±0.12 | 0.4±0.12 ^b |
| TRAPPC9 | SNP 7 | TT (0.07) | 880.68±139.26 | 4.26±0.96 | 3.33±0.27 | 4.82±0.23 ^a | 8.62±0.42 ^a | 11.69±2.08 | 0.25±0.13 | 0.16±0.12 |
| | | CC (0.07) | 1034.31±162.27 | 4.6±1.12 | 3.4±0.31 | 5.03±0.27 ^{ab} | 9.09±0.48 ^{ab} | 11.9±2.42 | 0.49±0.15 | 0.51±0.13 |
| | | TC (0.86) | 982.82±89.18 | 5.06±0.62 | 3.61±0.17 | 5.2±0.15 ^b | 9.3±0.27 ^b | 10.06±1.33 | 0.25±0.08 | 0.16±0.04 |
| | | TT (0.78) | 977.62±88.78 | 5.26±0.61 | 3.54±0.17 ^a | 5.16±0.15 | 9.26±0.27 | 9.44±1.34 | 0.22±0.08 | 0.18±0.04 |
| STAT5A | SNP 8 | TG (0.22) | 938.45±98.7 | 4.71±0.68 | 3.86±0.19 ^b | 5.34±0.17 | 9.47±0.3 | 10.52±1.49 | 0.17±0.09 | 0.2±0.08 |
| | | AA (0.05) | 1302.28±142.07 ^A | 3.97±1.02 | 3.52±0.29 | 5.17±0.25 | 9.25±0.45 | 10.58±2.24 | 0.15±0.14 | 0.13±0.14 |
| | | AC (0.07) | 1146.09±152.01 ^{AB} | 5.05±1.09 | 3.45±0.31 | 4.79±0.27 | 9.19±0.48 | 9.89±2.4 | 0.09±0.15 | 0.27±0.15 |
| | | CC (0.88) | 969.17±84.92 ^B | 5.16±0.61 | 3.62±0.17 | 5.16±0.15 | 9.31±0.27 | 9.73±1.34 | 0.19±0.08 | 0.19±0.04 |
| STAT5B | SNP 18 | TT (0.87) | 976.93±87.26 | 4.9±0.59 ^a | 3.66±0.17 | 5.22±0.15 | 9.34±0.26 | 9.89±1.32 | 0.21±0.08 | 0.19±0.04 |
| | | TC (0.13) | 895.83±114.44 | 6.27±0.77 ^b | 3.48±0.22 | 5.2±0.2 | 9.17±0.35 | 9.01±1.74 | 0.19±0.11 | 0.2±0.1 |
| CD4 | SNP 19 | TT (0.31) | 997.4±100.4 | 4.87±0.69 | 3.54±0.19 ^a | 5.11±0.17 | 9.14±0.3 | 9.71±1.53 | 0.2±0.1 | 0.15±0.06 |
| | | CT (0.56) | 966.28±88.05 | 5.11±0.61 | 3.62±0.17 ^{ab} | 5.23±0.15 | 9.34±0.27 | 9.73±1.34 | 0.21±0.08 | 0.21±0.05 |
| | | CC (0.13) | 909.17±115.01 | 5.35±0.79 | 3.96±0.22 ^b | 5.28±0.2 | 9.44±0.35 | 10.15±1.75 | 0.21±0.11 | 0.2±0.09 |

Note: Data is displayed as LS means±SE. The values in parenthesis represent the genotype frequency. Different superscripts of the uppercase letters within the same column in a SNP represent that $P<0.01$; lower case letter represent $P<0.05$.

Table 4: Association of SNPs in studied genes with milk and udder health traits in Red Sindhi breed

| Gene | SNP # | Genotype | 305 milk yield | Fats (%) | Protein (%) | Lactose (%) | SNF (%) | Log(SCC) | Presence of <i>S. aureus</i> |
|---------|--------|-----------|-----------------------------|------------------------|-------------------------|-------------------------|-----------|--------------------------|------------------------------|
| JAK2 | SNP 1 | TT (0.91) | 1689.99±79.84 ^A | 2.26±0.31 | 3.17±0.05 ^a | 4.76±0.08 ^a | 8.29±0.2 | 12.23±0.19 | 0.22±0.08 |
| | | TC (0.09) | 1179.01±170.04 ^B | 2.17±0.65 | 2.93±0.11 ^b | 4.42±0.17 ^b | 7.91±0.43 | 12.72±0.4 | 0.32±0.17 |
| TRAPPC9 | SNP 8 | TT (0.70) | 1580.63±90.16 | 2.37±0.33 | 3.13±0.06 | 4.71±0.09 | 8.17±0.22 | 12.44±0.2 ^a | 0.25±0.09 |
| | | TG (0.30) | 1708.5±119.59 | 1.95±0.44 | 3.14±0.08 | 4.73±0.12 | 8.37±0.29 | 11.87±0.27 ^b | 0.2±0.11 |
| | SNP 10 | GG (0.05) | 1930.28±255.24 | 2.64±0.96 | 3.37±0.17 | 5.08±0.25 | 8.87±0.63 | 11.32±0.57 ^a | 0.06±0.24 |
| | | AG (0.20) | 1822.99±135.48 | 2.02±0.51 | 3.17±0.09 | 4.77±0.13 | 8.35±0.34 | 11.85±0.3 ^{ab} | 0.35±0.13 |
| DGATI | SNP 14 | AA (0.75) | 1592.18±82.84 | 2.29±0.31 | 3.13±0.06 | 4.71±0.08 | 8.23±0.21 | 12.37±0.19 ^b | 0.21±0.08 |
| | | TT (0.08) | 1518.33±176.6 ^a | 1.99±0.66 | 3.21±0.12 ^{ab} | 4.81±0.17 ^{ab} | 8.65±0.43 | 13.12±0.39 ^A | 0.26±0.17 |
| | | TC (0.31) | 1549.34±100.18 ^a | 2.28±0.38 | 3.04±0.07 ^a | 4.58±0.1 ^a | 7.96±0.24 | 12.34±0.22 ^{AB} | 0.26±0.1 |
| | | CC (0.62) | 1790.12±105.55 ^b | 2.33±0.4 | 3.23±0.07 ^b | 4.85±0.1 ^b | 8.43±0.26 | 11.85±0.23 ^B | 0.19±0.1 |
| LAG3 | SNP 16 | CA (0.13) | 1318.99±149.56 ^A | 2.16±0.57 | 2.97±0.1 ^a | 4.47±0.15 ^a | 7.82±0.37 | 12.52±0.35 | 0.23±0.15 |
| | | AA (0.87) | 1673.52±80.34 ^B | 2.26±0.3 | 3.16±0.05 ^b | 4.75±0.08 ^b | 8.3±0.2 | 12.26±0.19 | 0.23±0.08 |
| STAT5A | SNP 17 | AC (0.08) | 1202.74±185.52 ^A | 2.77±0.7 | 3.23±0.13 | 4.85±0.19 | 8.53±0.46 | 12.37±0.43 | 0.15±0.18 |
| | | CC (0.92) | 1665.43±79.54 ^B | 2.21±0.3 | 3.13±0.05 | 4.71±0.08 | 8.23±0.2 | 12.28±0.19 | 0.24±0.08 |
| CD4 | SNP 19 | TT (0.32) | 1568.57±107.81 | 1.92±0.38 ^A | 3.15±0.07 | 4.74±0.11 | 8.12±0.26 | 12.27±0.24 | 0.31±0.1 |
| | | CT (0.44) | 1691.63±96.13 | 2.11±0.34 ^A | 3.15±0.06 | 4.73±0.09 | 8.42±0.23 | 12.37±0.22 | 0.18±0.09 |
| | | CC (0.24) | 1600.74±126.12 | 3.35±0.44 ^B | 3.1±0.08 | 4.66±0.12 | 8.03±0.31 | 12.07±0.28 | 0.24±0.12 |

Note: Data is displayed as LS means±SE. The values in parenthesis represent the genotype frequency. Different superscripts of the uppercase letters within the same column in a SNP represent that $P<0.01$; lower case letter represent $P<0.05$.

suggest that SNPs located in these three genes could be useful to reduce the incidence of *S. aureus* infection.

Furthermore, SNPs in *JAK2* and *TRAPPC9* genes were found to be associated with milk production traits. Three SNP markers (SNP 7, 8, and 10) were associated

with 305 milk yield, protein (%), and/or log(SCC) in Holstein Friesian, Red Sindhi and Sahiwal cows. Similar results were obtained by authors in Chinese Holstein (Khan *et al.*, 2022) and US Holstein (Cole *et al.*, 2011), and even more, the SNP 10 was reported in the top 50

Table 5: Association of SNPs in studied genes with milk and udder health traits in Sahiwal breed

| Gene | SNP # | Genotypes | 305 milk yield | Fats (%) | Protein (%) | Lactose (%) | SNF (%) | Log (SCC) | Incidence of Clinical Mastitis | Presence of <i>S. aureus</i> |
|---------|--------|-----------|----------------|-----------|------------------------|-------------|-----------|------------|--------------------------------|------------------------------|
| JAK2 | SNP 1 | TT (0.89) | 2052.03±60.54 | 4.05±0.28 | 3.33±0.05 | 5.01±0.07 | 9.1±0.13 | 13.31±0.17 | 0.03±0.03 | 0.35±0.06 |
| | | TC (0.11) | 2051.55±156.13 | 3.07±0.73 | 3.25±0.12 | 4.89±0.18 | 8.9±0.33 | 13.41±0.45 | 0.01±0.08 | 0.26±0.16 |
| TRAPPC9 | SNP 8 | TT (0.90) | 2056.37±58.86 | 3.94±0.28 | 3.33±0.05 | 5.01±0.07 | 9.09±0.12 | 13.32±0.17 | 0.03±0.03 | 0.33±0.06 |
| | | TG (0.10) | 1983.84±158.68 | 4.12±0.75 | 3.22±0.12 | 4.88±0.18 | 8.85±0.33 | 13.33±0.45 | -0.1±0.08 | 0.46±0.16 |
| | SNP 10 | AG (0.08) | 2016.36±175.32 | 5±0.82 | 3.07±0.14 ^a | 4.67±0.2 | 8.47±0.36 | 13.89±0.5 | -0.1±0.09 | 0.26±0.18 |
| | | AA (0.92) | 2053.88±58.8 | 3.89±0.28 | 3.34±0.05 ^b | 5.02±0.07 | 9.11±0.12 | 13.29±0.17 | 0.03±0.03 | 0.35±0.06 |
| | | GG (0.88) | 2054.68±60.7 | 3.95±0.28 | 3.33±0.05 | 5.01±0.07 | 9.09±0.13 | 13.36±0.17 | 0.03±0.03 | 0.36±0.06 |
| | SNP 11 | GA (0.12) | 2046.87±139.73 | 4.13±0.65 | 3.3±0.11 | 5±0.16 | 9.09±0.29 | 12.99±0.4 | 0.03±0.07 | 0.21±0.14 |
| | | TC (0.18) | 1906.91±116.59 | 4.59±0.55 | 3.36±0.09 | 5±0.13 | 9.09±0.25 | 13.83±0.33 | 0.06±0.06 | 0.23±0.12 |
| DGATI | SNP 14 | CC (0.82) | 2079.42±60.64 | 3.82±0.29 | 3.32±0.05 | 5±0.07 | 9.08±0.13 | 13.23±0.17 | 0.02±0.03 | 0.36±0.06 |
| CD4 | SNP 19 | TT (0.26) | 2064.63±99.59 | 3.63±0.47 | 3.42±0.08 | 5.15±0.11 | 9.37±0.21 | 13.06±0.29 | -0.04±0.05 | 0.32±0.1 ^A |
| | | CT (0.57) | 2084.85±69.8 | 4.1±0.33 | 3.27±0.05 | 4.93±0.08 | 8.94±0.15 | 13.4±0.2 | 0.05±0.03 | 0.26±0.07 ^A |
| | | CC (0.17) | 1902.45±117.37 | 3.81±0.55 | 3.34±0.09 | 5.03±0.13 | 9.13±0.24 | 13.4±0.34 | 0.02±0.06 | 0.69±0.12 ^B |

Note: Data is displayed as LS means±SE. The values in parenthesis represent the genotype frequency. Different superscripts of the uppercase letters within the same column in a SNP represent that P<0.01; lower case letter represent P<0.05.

markers associated with milk yield (Taherkhani *et al.*, 2022). However, only SNPs in *TRAPPC9* gene were associated with CM in Holstein Friesian cows. That result was unexpected based on previous studies, where authors found associations of *JAK2* with mastitis resistance (Usman *et al.*, 2014; Khan *et al.*, 2019). That could be explained by the limited number of animals used in this study, mostly for local breeds, but also by the potential polymorphisms in the *JAK2* gene across populations. Note that the genotype frequency revealed that SNP 4 (genotype GA) on *JAK2* region was monomorphic across all four breeds. A previous study showed that the GA genotype was associated with higher SCC in Chinese Holsteins (Usman *et al.*, 2014), suggesting a potential higher mastitis susceptibility for the studied Holstein Friesian population.

It is well known that the *DGATI* gene is associated with milk traits in dairy cows (Spelman *et al.*, 2002; Li *et al.*, 2021). Remarkably, some SNP markers, such as SNP 13, was found to be significantly associated with protein (%) in our Holstein Friesian population, while others, such as SNP12 was not associated with any trait across the four breeds evaluated in our study. However, previous studies reported SNP 12 associated with fat (%) and mastitis resistance traits, including SCS (Khan *et al.*, 2019). The association observed for the SNP 13, were also reported in different studies (Jiang *et al.*, 2019; Bakhshalizadeh *et al.*, 2021; Yang *et al.*, 2021; Taherkhani *et al.*, 2022). For the local breeds, SNP 14 was found to be significantly associated with milk production and mastitis resistant traits in the Red Sindhi breed. Note that CC genotype increased milk yield, protein (%), lactose (%), and reduce log(SCC), which increases dairy cow resistance to mastitis with higher milk production.

Several genetic markers were found that have not been previously reported to be associated, such as the SNP 17 and SNP 18 on *STAT5A* and *STAT5B* gene regions, respectively. The SNP 17 was associated with 305 milk yield in two breeds (Achai and Red Sindhi), and the SNP 18 with fat (%). These results suggest that we could be identifying novel markers associated with milk traits for local breeds in Pakistan.

Overall, our study identified genetic markers on relevant genes that could be used to reduce the incidence of mastitis in the Pakistani cattle population. Also, the significant associations obtained in our study showed the

potential to improve milk yield, enhance mastitis resistance and reduce *S. aureus* infection. We demonstrated the relevance for exploring genetic diversity in local breed by the use of a SNP-Chip (CCSC-1). Future studies should continue to explore these associations in larger populations, integrating advanced genomic techniques to refine our understanding of these genetic markers and their role in dairy cattle productivity and health.

Conclusions: This study validated the CCSC-1 SNP-Chip as an effective tool for identifying genetic markers in Holstein Friesian and indigenous Pakistani dairy cattle breeds (Achai, Red Sindhi, and Sahiwal). The results verify that SNPs located on selected genes (*JAK2*, *TRAPPC9*, *DGATI*, *LAG3*, *STAT5A*, *STAT5B*, and *CD4*) could be considered as powerful genetic markers associated with milk yield, composition, and udder health traits. These markers could contribute to breeding programs to select cattle with enhanced resistance to mastitis, reducing *S. aureus* infection and improved productivity.

Authors contribution: Conceptualization: TU and YU Methodology: MK, YU and FP Software: MK, GM and PF Investigation: MK Resources: TU and NR Data curation: MK Writing—original draft preparation: MK Writing—review and editing: GM, FP and TU Supervision: NR, FP and TU Project administration: TU, NR and YU Funding acquisition: TU and YU All authors have read and agreed to the published version of the manuscript.

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