SINGLE NUCLEOTIDE POLYMORPHISM OF INTERFERON GAMMA (IFN-γ) GENE AND ITS ASSOCIATION TO GASTROINTESTINAL PARASITE BURDEN OF CROSSBRED ANGLO-NUBIAN GOATS

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ABSTRACT

Gastrointestinal parasite (GIP) infection is a significant health issue in goats. Selecting genetically resistant animals offers a sustainable solution. Thus, this study investigated GIP burden, species, and polymorphisms in the interferon-gamma (IFN-γ) gene and its association with worm burden of crossbred Anglo-Nubian goats. Bucks that had not been dewormed for 30 days were subjected to fecal analysis to assess worm burden based on eggs per gram (EPG) and to identify parasite species. Genomic DNA was extracted from goat hair follicles, and the exon 2-3 of the IFN-γ gene was amplified and sequenced to determine polymorphism. The study found moderate GIP infections in most goats, with mixed infections of *Haemonchus* spp., *Trichostrongylus* spp., *Strongyloides* spp., and *Moniezia* spp. Two single nucleotide polymorphisms (SNPs), IFNG1359C/T and IFNG1469C/T, which conformed to Hardy-Weinberg equilibrium were identified. Notably, IFNG1469C/T exhibited a moderate polymorphic information content (PIC) of 0.500, and was significantly associated with worm burden (p = 0.022). These results highlight the need for further research with larger sample sizes, different breeds, and varied farm conditions to better understand how specific alleles affect parasite burden. Additionally, associating GIP prevalence with identified polymorphisms could aid in identifying genetic markers for resistance to specific parasites.

Keywords: cytokine, endoparasites, polymorphism, ruminants, worm burden

INTRODUCTION

Polymorphisms in genes are known to have potential as markers for economically important traits in livestock. One important trait is resistance to gastrointestinal parasites (GIP), which is heritable, making breeding resistant animals a proposed sustainable alternative for controlling parasite infections (Khobra *et al.*, 2012; Mpofu *et al.*, 2022; Brahma *et al.*, 2023). Key genes studied in association with resistance to GIP are immune-related genes, such as the pro-inflammatory cytokine interferon-gamma (IFN-γ) gene (Benavides *et al.*, 2016). The IFN-γ molecule is essential for immune system communication; it activates

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macrophages to enhance their response and functions by binding to specific receptors on the cell surface, inducing conformational changes that trigger downstream signaling pathways (Kak *et al.*, 2018; Deng *et al.*, 2023). Hence, any genetic variation that alters the expression or receptor-binding affinity of IFN-γ could potentially affect resistance against pathogens (Benavides *et al.*, 2016). Additionally, among indicator traits, fecal egg count (FEC) is the most reliable, practical, and common method to determine resistance or tolerance to GIP; it is a repeatable and heritable trait among animal breeds (Saddiqi *et al.*, 2012). Eggs per gram (EPG) is typically used for FEC (Valilou *et al.*, 2015; Mpofu *et al.*, 2022), and studies investigating the polymorphism of IFN-γ in association with parasite resistance utilized EPG counts (Coltman *et al.*, 2001; Sayers *et al.*, 2005; Bressani *et al.*, 2014; Omar *et al.*, 2019; Brahma *et al.*, 2023).

In the Philippines, the goat industry supports the livelihoods of millions of Filipinos and is considered a sunrise industry (Rupa and Portugaliza, 2016). As of September 2023, the goat population in the Philippines stood at 3.86 million heads (PSA, 2023a). The majority of the goat industry is composed of backyard raisers with Northern Mindanao ranking among the top goat-producing regions, together with other regions in Mindanao that contribute to 38.44% of the Annual Volume of Production of goats in the country (PSA, 2023b). However, GIPs are a significant constraint on goat husbandry in the Philippines, hindering the optimization of the industry's full potential (Rupa and Portugaliza, 2016). The impact of GIPs is more severe in tropical countries due to favorable environmental conditions, resulting in clinical diseases, productivity losses in meat and milk, increased mortality, and various other issues (Mpofu *et al.*, 2022).

Several studies have examined the association of IFN-γ polymorphism with GIP resistance in various sheep breeds. Coltman *et al.* (2001) described that the 126 allele (126/126 genotype) of IFN-γ was significantly associated with reduced fecal egg count (FEC) and a trend was also observed in the titer of IgA, as it is positively associated with 126 allele in Soay sheep breed. Sayers *et al.* (2005) discovered four haplotypes of IFN-γ gene in the Suffolk breed and two previously identified haplotypes in the Texel breed; and only one haplotype (haplotype B) in the Texel breed was associated with resistance to nematode infection. Patra *et al.* (2016) determined that one single nucleotide polymorphism (SNP) in exon 2 of IFN-γ is significantly associated with resistance in Gerole sheep. Brahma *et al.* (2023) identified 7 SNPs in Gerole sheep, four exclusive to the resistant sheep. On the other hand, very few studies have been conducted on goats. Bressani *et al.* (2014) showed a significant association between the polymorphism in exon 3 of IFN-γ and the FEC distribution in crossbred Saanen and Anglo-Nubian goats. Omar *et al.* (2019) determined that the expression levels of IFN-γ in the abomasal tissues of goats are higher in resistant Yichang white goats.

With these, this study was conducted to determine the gastrointestinal parasite burden, parasite species, polymorphism of the IFN-γ gene and its association with worm burden in crossbred Anglo-Nubian goats housed on a farm in Sultan Naga Dimaporo, Lanao del Norte, Philippines. The result of this study provides a preliminary record of the worm burden affecting goats at the specified farm, offering essential data on the parasite species infecting the animals and seeks to enhance marker-assisted breeding practices in the country by providing data on potential markers for selecting goats to improve livestock quality and survivability.

MATERIALS AND METHODS

Ethical Approval

This study was conducted with the approval of the Research Integrity and Compliance Office, Institutional Animal Care and Use Committee of the Mindanao State University Iligan Institute of Technology with the IACUC Protocol Approval No. 2024A02.

Animals and Fecal Sample Collection

Crossbred Anglo-Nubian x Native goats reared at Mindanao State University – Lanao del Norte Agricultural College in Sultan Naga Dimaporo, Lanao del Norte, Philippines were used in this study. The dry season in the area starts in January and ends in June, while the rainy season begins in late June and lasts until early January. The initial male-to-female ratio of animals that started the population was 3:40 (male: female). The goats were raised in a cut-and-carry system with water *ad libitum*. Goats were dewormed with Fenbendazole and Ivermectin every 60 days alternatively. Supplements were also given, such as Vitamin B complex for goats below one-year-old and Vitamin ADE for goats above one-year-old. During the sample collection, there were 117 goats, of which 34 were males. Only 30 goats were included in the sample, as those younger than four months were excluded from the study (Valilou *et al.*, 2015; Yadav *et al.*, 2016). This age threshold ensures that the animals possess a mature immune response (Bishop, 2012). Additionally, does were also excluded from the study due to the possibility of pregnancy during the sample collection period (Pratap *et al.*, 2024) to avoid putting the animals at risk of stress.

The method for collecting fecal samples was adopted from the study of Valilou *et al.* (2015) with some modifications. Fecal samples were collected from thirty bucks that had not been given any anthelmintic treatments for the last 30 days (Mohammedsalih *et al.*, 2020) allowing any remaining parasite egg to reinfect the animals (Taylor *et al.*, 2016). The collection was performed thrice, each with a week interval. An estimated 5g of fecal sample was collected directly from the goat's rectum using sterile disposable gloves and placed inside a clean plastic zip-lock pack and then labeled. All samples were chilled in a cooler box with ice packs then transported to the laboratory and refrigerated at 4°C. Fecal analysis was performed immediately within 48 hours after the collection (Matsepe *et al.*, 2021).

Fecal Analysis

Fecal analysis was conducted using the modified McMaster egg counting technique described by Zajac and Conboy (2012). Two grams of fecal matter were weighed and placed in a plastic container and mixed with 28ml saturated sodium chloride (NaCl) solution. This solution was prepared by dissolving 180g of laboratory-grade NaCl in 500ml of distilled water. The fecal material was manually homogenized using a scoopula, and the mixture was filtered through a mesh sieve and the filtrate was collected in a separate container. A sufficient volume of the suspension was pipetted into the chambers of a McMaster slide (Eggzamin® McMaster Microscope Slides), filling each chamber individually. The slides were left to stand for at least 5 minutes to allow the parasite eggs to float. A compound microscope was used to observe and count the GIP eggs under low magnification (10x). The counting process was completed within 60 minutes to prevent the formation of crystals in the chamber. EPG counting involves only eggs of GIP with measurements greater than 60-80µm (Zajac and Conboy, 2012; Taylor *et al.*, 2016). EPG of feces was calculated using the

formula: (Chamber 1 + Chamber 2) X 50.

GIP eggs were identified in the fecal samples through direct microscopic examination (Minnatt, 2014; Ghimire and Bhattarai, 2019; Sharma *et al.*, 2020), and micrographs were taken. The species of GIP eggs were identified according to several works of literature by Christensen (2015), Aiello and Moses (2016), Taylor *et al.* (2016), Ghimire and Bhattarai (2019), Sabatini *et al.* (2023), and Sharma *et al.* (2020).

DNA Extraction, Amplification, and Sequence Analysis of IFN-γ Gene

Genomic DNA (gDNA) was extracted from the hair follicles of goats using the QIAGEN DNEasy kit with minor modifications. The 487bp fragment in Exons 2-3 of the IFN-γ gene was amplified using the primers described by Bressani *et al.* (2014): F-5'-AAATAGTGCCAGCATCCAAGTT-3' and R- 5'-TGCAATGATACCAAAGAAAGCA-3'. Thirty µL PCR reaction mixture composed of 1x buffer, 1µM for each forward and reverse primers, 0.2mM dNTPs, 0.5U Taq Polymerase, at least 10ng DNA sample, and dH2O was optimized under the following PCR conditions: initial denaturation at 95°C for 5mins, followed by 35 cycles of denaturation at 94°C for 45secs, annealing at 57.7°C for 45secs, extension at 72°C for 1min, and a final extension at 60°C for 4mins.

Amplicons were sent to Biofact Co., Ltd in South Korea for PCR purification and sequencing. The obtained sequences in fasta format were aligned using MEGA11 (Tamura *et al.*, 2021) with reference sequence from NCBI: NC 030812.1 (Lowe and Eddy, 1997). The genotypes of each SNP identified were determined by viewing and aligning the chromatograms (Sayers *et al.*, 2005) using Geneious Prime.

Data Analysis

The data obtained for the EPG counts of goats were tabulated. The mean EPG of goats (Mean+SD) was distributed to three different worm burden categories (WBC) used by Khobra *et al.* (2012) and Mohammedsalih *et al.* (2020), which are low (<500), moderate (500-2000), and high (2000>). Frequency and percentage are used to present the number of animals per WBC and the presence of GIP species in the samples.

The identified single nucleotide polymorphisms (SNPs) were analyzed for their allele and genotype frequencies, deviation from the Hardy-Weinberg equilibrium (HWE), and polymorphism information content (PIC) using R studio (Posit, 2024). The association of the IFN-γ SNP with the worm burden categories was determined through the Chi-square test (Steel and Torrie, 1980) using Jamovi Software (The Jamovi Project, 2024; R Core Team, 2023) at a 95% confidence interval.

RESULTS AND DISCUSSION

Worm Burden and GIP Species in Crossbred Anglo-Nubian Goats

Poor sanitation and hot, humid weather allow GIP infection among animals to be successful making parasitism one of the top 3 challenges in livestock farming in the Philippines (Rupa and Portugaliza, 2016). However, reports on the detection and prevalence of gastrointestinal parasites in goats in the Philippines are only limited to some parts of the country. Thus, this study presents one of the few records assessing GIP in goats from a smallholder farm, specifically one located in Lanao del Norte, Mindanao, Philippines.

As shown in Table 1, the worm burden levels of the goats were determined by distributing the mean EPG count of animals across the categories used by Khobra *et al*. (2012) and Mohammedsalih *et al.* (2020). Notably, the majority of the goats investigated were experiencing moderate GIP infection.

Table 1. Distribution of the mean EPG of crossbred Anglo-Nubian goats based on worm burdens.

WORM BURDEN CATEGORY	n	%	MEAN EPG±SD
Low Infection (EPG <500)	4	13.3	356.8±48.3
Moderate Infection (500-2000)	18	60.0	998.1±396.7
High Infection (2000>)	8	26.7	3470.8±978.3

n: number of goats
%: percentage of goats (n/30 × 100)

Table 2. Prevalence of gastrointestinal parasite species in crossbred Anglo-Nubian goats.

GASTROINTESTINAL PARASITE SPECIES	NE	NP	%
Nematodes Strongyle eggs/ *Strongyloides* spp.	30	30	100%
Cestodes *Moniezia* spp,	30	13	43.33%
Nematodes + Cestodes Strongyle eggs/ *Strongyloides* spp., *Moniezia* spp	30	13	43.33%

NE: Number of goats examined
NP: Number of goats positive
%: percentage prevalence

The gastrointestinal parasites found in the fecal samples of the animals belong to nematodes (roundworms) from the family *Strongylidae* and cestodes (tapeworms) of the genus *Moniezia* (Table 2) which were simultaneously present in 43.33% of the goats whereas 100% of the goats were infected with nematodes. This variation in parasite prevalence may suggest that nematodes thrive better than cestodes in this goat population, possibly due to factors such as the presence of an intermediate host, egg production rates, environmental conditions, and parasite susceptibility to anthelmintics used on the farm (Taylor *et al.*, 2016). For instance, *Moniezia* requires an intermediate host like mites, whereas roundworms have direct life cycles that do not involve an intermediate host (Aiello & Moses, 2016).

The mixed infections observed among the animals are illustrated in Figure 1. Letters

were used to label the specimens to distinguish between GIP species and pseudoparasites. The occurrence of mixed infections was similarly reported in studies conducted by Rupa and Portugaliza (2016) in the Philippines, and other researchers internationally (Minnat, 2014; Azrul *et al*., 2017; Abdi-Soojeede, 2018).

Subsequently, the species of parasites were determined based on the morphological characteristics of the parasite eggs. However, it is important to note that distinguishing between different species of strongyle eggs can be highly challenging; hence, nematodes with such eggs are generally categorized within the superfamilies *Trichostrongyloidea* and *Strongyloidea* (Sabatini *et al.*, 2023). Most of the parasites identified belong to phylum *Nematoda*, characterized by strongyle-type eggs (Figure 2A, 2B, 2C, 2D, 2E, 2F, 2G, 2H) with smooth surfaces, ellipsoidal shells, and visible morulae (Taylor *et al.*, 2016; Ghimire and Bhattarai, 2019; Sabatini *et al.*, 2023). The strongyle eggs identified were *Haemonchus* spp. (Figure 2A, 2B, 2D), which are distinguished by their round shape and visible blastomeres, and *Trichostrongylus* spp. (Figure 2C, 2E), which are often kidney-bean-shaped, with one side rounder than the other, and a visible space surrounding the morula (Christensen, 2015; Taylor *et al.*, 2016; Sharma *et al.*, 2020). Particularly, *Haemonchus* spp., also known as the barber pole worm, is the most common gastrointestinal parasite in small ruminants globally

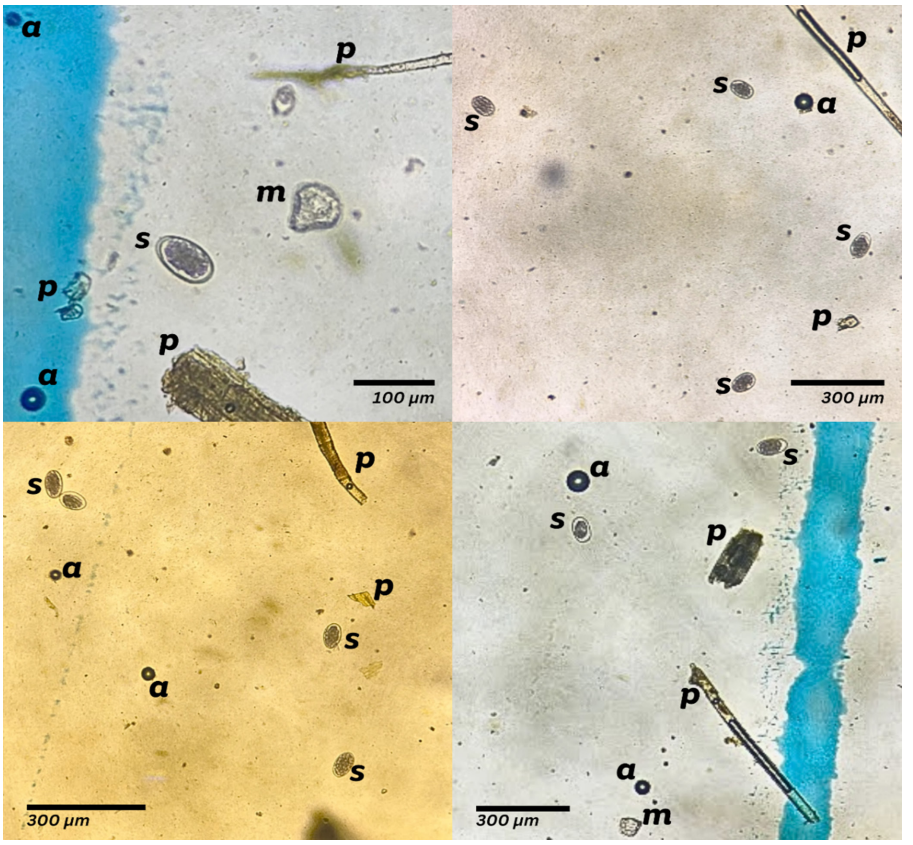

Figure 1. Mixed infection of gastrointestinal parasite observed in the fecal samples of crossbreed Anglo-Nubian goats. s: Nematodes, m: Cestodes, p: plant debris/ psuedoparasites, a: air bubbles.

that causes acute hemorrhagic anemia in its host (Taylor *et al.*, 2016; Mpofu *et al.*, 2022). Another nematode belonging to the genus *Strongyloides* (Figure 2K, 2L), identified by its smaller size and the presence of L1 larvae, was also observed (Aiello & Moses, 2016; Taylor *et al.*, 2016). Additionally, *Moniezia* spp. (Figure 2I, 2J) - a tapeworm - was easily recognized by its irregular/quadrangular shape containing pear-shaped structures (Aiello & Moses, 2016).

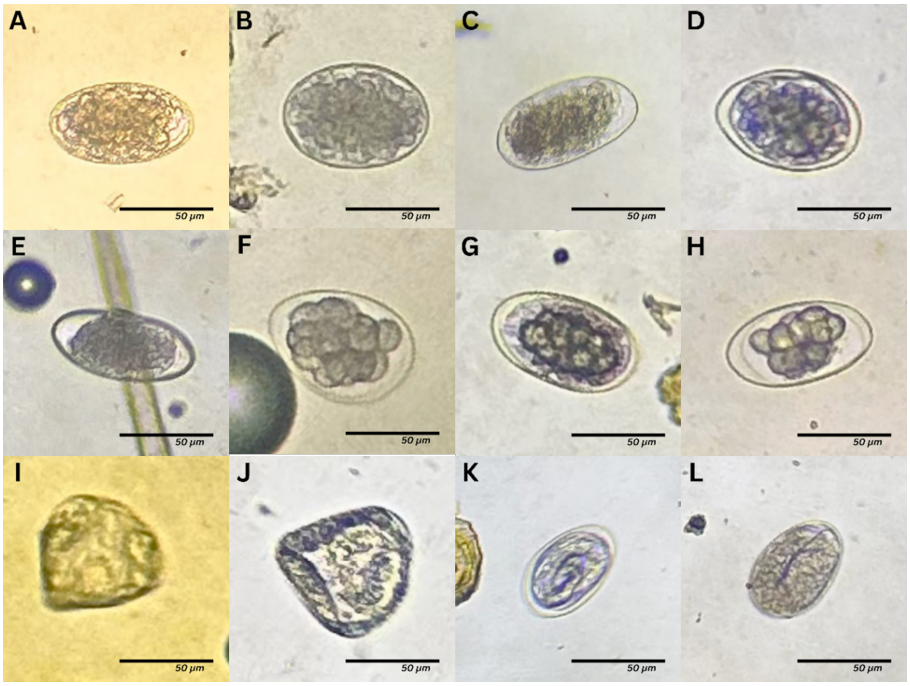

Figure 2. Gastrointestinal parasites species found in the fecal samples of crossbreed Anglo-Nubian goats. A, B, C, E, F, G, H: Strongyle-type eggs; I, J: *Moniezia* spp; and K, L: *Strongyloides* spp.. Bar is equal to 50 um.

On the other hand, Rupa and Portugaliza (2016) identified additional parasite species, besides those found in the present study, including *Oesophagostomum* spp., *Trichuris* spp., *Chabertia* spp., and *Cooperia* spp., in goats from Baybay City, Leyte. Their study reported that 82.89%, 15.56%, and 1.56% of the goat population exhibited mild, moderate, and heavy infections with nematodes, respectively. These results differ from those of the present study, where a significant number of animals experienced moderate to high GIP burdens. This may indicate that there is a need to reassess the farm's GIP control strategy, as it may no longer be effective.

IFN-γ Gene Polymorphism and its Association to Worm Burden of Crossbred Anglo-Nubian Goats

IFN-γ is a key cytokine that plays a crucial role in immune regulation. Specifically, it binds to receptors on target cells, such as macrophages and dendritic cells, triggering

a signaling cascade that enhances immune responses, including antigen processing and presentation via the upregulation of class II MHC. This activation, in turn, promotes autophagy to clear intracellular pathogens and stimulates the secretion of pro-inflammatory cytokines. Furthermore, IFN-γ activates NK cells and regulates B cell antibody production, thereby facilitating pathogen clearance through mechanisms such as phagocytosis, inflammation, and lymphocyte recruitment (Kak *et al.*, 2018; Deng *et al.*, 2023).

Table 3. Diversity indices of SNP in IFN-γ gene of crossbred Anglo-Nubian goats.

SNP ID[1]	Type of Mutation	Allele	Allele Frequency	Genotype	Genotype Frequency	PIC	Deviation form HWE (p value)
IFNG1359C/T	Synonymous	C	0.250	CC	0.067	0.375	0.993
		T	0.750	CT	0.367		
				TT	0.567		
IFNG1469C/T	Synonymous	C	0.500	CC	0.233	0.5	0.936
		T	0.500	CT	0.533		
				TT	0.233		

[1]Site locations were determined based on NCBI reference: NC 030812.1.

Table 4. Association of the SNPs in the IFN-γ gene with worm burden of crossbred Anglo-Nubian goats using chi-square test.

SNP ID	Genotype	Worm burden category			x^2	df	p-value
		Low N (mean EPG)	Moderate N (mean EPG)	High N (mean EPG)			
IFNG1359C/T	CC	1 (367.00)	0 (0.00)	1 (4017.00)	4.45	4	0.348
	CT	1 (417.00)	8 (3154.71)	2 (2816.50)			
	TT	2 (325.00)	10 (956.60)	5 (3623.20)			
IFNG1469C/T	CC	2 (392.00)	2 (1292.00)	3 (4127.67)	11.5	4	0.022*
	CT	0 (0.00)	14 (2085.64)	2 (2075.00)			
	TT	2 (325.00)	2 (808.50)	3 (3744.33)			

N: Number of animals with the corresponding genotype and WBC.
*Significant at $p<0.05$

The sequence analysis of the 487bp fragment of the IFN-γ gene (exons 2-3) is shown in Table 3. The researchers identified two SNPs: IFNG1359C/T in intron 2 and IFNG1469C/T in exon 3 (Figure 3 and Figure 4). The alignment of the translated sequences

from these coding regions revealed that the SNP mutations were synonymous, resulting in no changes to the amino acid sequences. The Hardy-Weinberg equilibrium analysis revealed that the samples did not significantly deviate from HWE ($p > 0.05$) with moderate PIC values of 0.375 and 0.500, respectively. Markers with PIC > 0.5 are highly informative, $0.25 < PIC \leq 0.5$ are moderately informative, and PIC < 0.25 are less informative; high PIC values are ideal for association studies (Serrote *et al.*, 2020).

Subsequently, the association analysis (Table 4) demonstrated that only the polymorphism in exon 3 (IFNG1469C/T) was significantly associated with worm burden categories ($p = 0.022$). The mean EPGs for each worm burden and genotype are also provided in the table. Although the association analysis showed statistical significance, the frequency distribution of the genotypes for IFNG1469C/T across various worm burden levels (Figure 5) shows an inconclusive distribution for the CC and TT genotypes. In contrast, the CT genotype was more frequently observed in the moderate worm burden category, suggesting that individuals with the CT genotype are more likely to experience moderate worm burdens compared to those with either the C or T alleles alone. However, the specific allele linked to high or low worm burdens was not distinctly identified in the current study.

In comparison to previous research, the results of this study conform to the findings of Bressani *et al.* (2014) in Saanen crossbred Anglo-Nubian goats, where similar polymorphisms in intron 2 and exon 3 of the IFN-γ gene were reported. Their study also found an association between the second SNP and fecal distribution categories—extreme, resistant, and susceptible—though it did not specify which allele corresponded to each category. On the other hand, another study on goats investigated the expression levels of

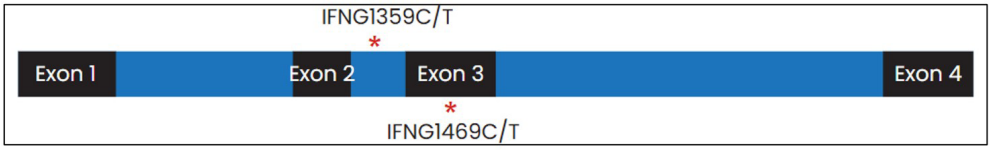

Figure 3. Illustration of the IFN-γ gene showing the locations of the single nucleotide polymorphisms.

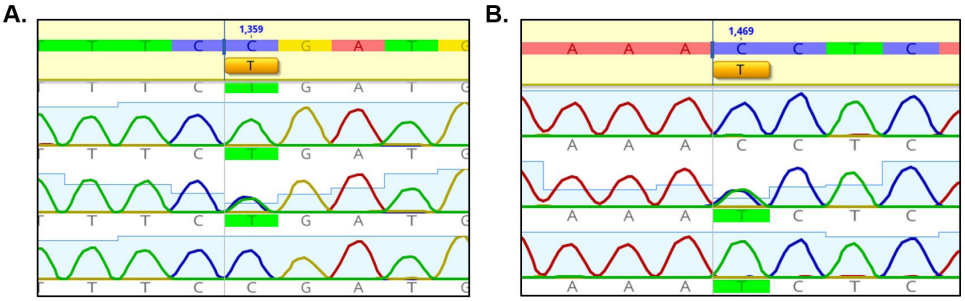

Figure 4. A section of the IFN-γ gene chromatograms of the Anglo-Nubian goats aligned with the reference sequence (NC 030812.1) in Geneious. A: IFNG1359C/T in Intron 2, B: IFNG1469C/T in exon 3.

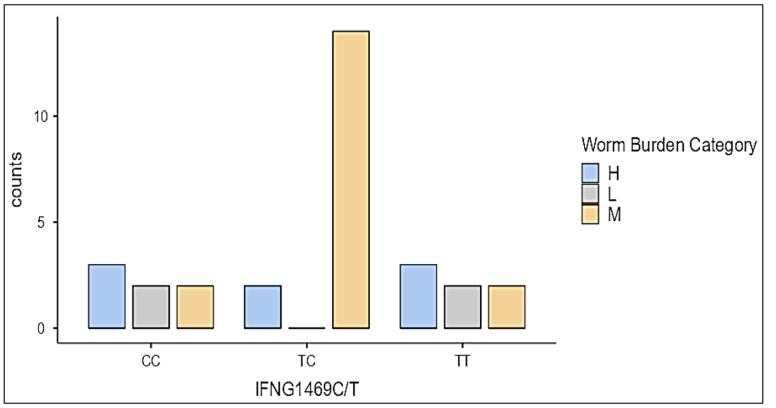

Figure 5. Frequency distribution of IFNG1469C/T genotypes among the three worm burden categories of crossbred Anglo-Nubian goats. Low (L): <500, Moderate (M): 500-200, High (H): 2000 above.

IFN-γ in the abomasal tissues; Omar *et al.* (2019) found an association between the high expression of IFN-γ and resistant Yichang White goats based on FEC.

Meanwhile, the majority of published studies investigating the IFN-γ gene were conducted in different sheep breeds. Coltman *et al.* (2001) reported that the 126 allele (126/126 genotype) of IFN-γ was associated with a significant decrease in FEC in Soay sheep and higher levels of IgA. Sayers *et al.* (2005) investigated intron 1 of the IFN-γ gene and discovered four new haplotypes (A, B, C, and D) in the Suffolk breed and two previously identified haplotypes (A and B) in the Texel breed; however, only haplotype B in the Texel breed was associated with low FEC for nematode infection. Similar to the current study, Patra *et al.* (2016) found a polymorphism in exon 3 of three resistant Gerole sheep. The SNP was also observed to have a C/T substitution and was a synonymous mutation, causing no amino acid changes. Although there is no definitive evidence that the IFN-γ gene in sheep and goats are identical, the authors have successfully aligned the IFN-γ (exon 2-3) gene sequence of *Ovis aries* (sheep) retrieved from the NCBI (NC_056056.1) with the current study's sequences using MEGA11, and all sequences matched. Thus, the polymorphism in exon 3 identified by Patra *et al.* (2016) might be similar to the current study's IFNG1469C/T. Additionally, Brahma *et al.* (2023) identified 4 SNPs (Promoter_A/T, 1001Exon1_A/G, 1033Exon1_A/G, and Intron1_A/G) exclusive to the resistant (low EPG) Gerole sheep; the expression of the IFN-γ gene was significantly higher in infected resistant sheep compared to susceptible sheep, suggesting that resistant sheep exhibit an enhanced immune response compared to susceptible goats.

CONCLUSION

This research is one of the few studies, if not the sole, to illustrate the association of IFN-γ gene polymorphisms, particularly IFNG1469C/T in exon 3, and worm burdens in crossbred Anglo-Nubian goats in the Philippines. Results revealed that a higher number of

goats with moderate worm burdens possessed the CT genotype; however, it remains unclear whether the distribution of high or low worm burden is specifically linked to allele C or allele T. Nevertheless, this result presents a critical step in identifying genetic markers that could enhance selective breeding strategies to improve goat resistance to parasitic infections. Thus, further research is recommended to replicate these findings to distinctively identify specific alleles associated with GIP resistance using a larger sample size, different goat breeds, and different farm environments. Future researchers may also explore the association of specific GIP species prevalence and other economically important traits with the identified polymorphisms or investigate other loci within the IFN-γ gene.

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