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PRELIMINARY SURVEY VISIT

AREA V
RESEARCH

D. PUBLICATION AND DISSEMINATION

D.3. Copies of Published Articles

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Effectiveness of Drug-Based Interventions and Integrated Community Strategies for Soil-Transmitted Helminth Control in Southeast Asia: A Systematic Review

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Abstract

Soil-transmitted helminth (STH) infections continue to pose a significant health threat in Southeast Asia (SEA), where a third of global STH infections are estimated to occur. Preventive chemotherapy (PC) through mass treatments has been recommended by the WHO to combat STH infections and their dire consequences in susceptible populations. This review aims to update PC efforts for STH infection control in SEA. We systematically searched MEDLINE via PubMed, ScienceDirect, Scopus, EBSCO Host and ProQuest using specific terms for papers published from 2011 through 2025. From the 4294 initial search hits, 27 studies were included in this research. All SEA countries, except Brunei Darussalam and Singapore, were represented. Benzimidazole (BZ) drugs, namely, albendazole (400 mg) and mebendazole (500 mg), were the most commonly used drug interventions in PC efforts. BZ treatment effectively reduced the prevalence and intensity of STH infections, reinforcing its role in control programs. However, recent research has shown that BZ treatments fail to reduce infection prevalence and have diminished efficacy. Combination therapies, such as ivermectin (200 μg/kg) + albendazole (400 mg), enhanced treatment outcomes across STH species. Combination therapy integrated with health education and water, sanitation and hygiene improvement can accelerate control and elimination. Our review provides valuable insights for strengthening STH control programs in SEA, advocating for a multi-faceted approach informed by the current evidence.

Keywords: mass drug administration; neglected tropical disease; benzimidazoles; public health programs

Introduction

Soil-transmitted helminth (STH) infections remain a significant threat to public health in endemic areas with tropical and subtropical climates.[1] The WHO estimated that approximately 1.5 billion people are affected, accounting for approximately a quarter of the global population.[2] Recent data from the 2021 Global Burden of Disease study indicate that there are approximately 642 million cases worldwide, which contribute to 1.38 million disability-adjusted life-years.[3] The age-standardized prevalence is 8429.89 per 100,000 population, representing a 70% decrease from the 1990 data.[3] These infections are caused by *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm species like *Necator americanus* and *Ancylostoma duodenale*, and *Strongyloides stercoralis*.[4] *Ancylostoma ceylanicum* is a zoonotic hookworm that affects companion animals and humans in the Asia-Pacific region.[5] Southeast Asia (SEA) is a hotspot for STH infections, accounting for one-third of all reported cases globally.[6] A 2008 WHO report documented that the prevalence of STH infections exceeded 50% in some areas of Cambodia, Indonesia, Myanmar, the Philippines and Vietnam.[7] A 2018 systematic review revealed that the prevalence of *A. lumbricoides* exceeded 20% in Myanmar, the Philippines, Malaysia and Indonesia, while hookworm infections surpassed this threshold in these countries and Cambodia.[8] In addition, a recent analysis indicates that *T. trichiura* affects over 685 million people in SEA, with a pooled prevalence of 20.95%.[9] The latest Philippine national survey revealed that the prevalence of STH infections was 8% higher than the 20% national target, and the prevalence of moderate-to-heavy infections more than 30% higher than the 2% target.[10,11] Recent studies in Myanmar and Vietnam using qPCR and Kato-Katz smears, respectively, revealed alarmingly high rates of STH infections among schoolchildren, with 78.8% of this population infected in Myanmar (predominantly *A. lumbricoides* and *T. trichiura*) and 76.1% in Vietnam.[12,13] In Malaysia and Thailand, persistently high STH infections in indigent and tribal communities remain a significant health problem, especially among children living in these remote and rural areas.[14,15] STH infections have had a shared history with countries in SEA. Hence, it is not surprising that various efforts to control those parasitic health threats have been implemented across the region.

STH infections pose a significant health threat to vulnerable segments of the population within endemic regions, particularly schoolchildren, women of reproductive age and immunocompromised individuals.[1-4] Among children, the pathologic consequences of infection include iron-deficiency anemia, stunting, malnutrition, cognitive development issues and motor deficits.[4,16,17] The severe effects of high-burden infections among schoolchildren have led to efforts to control, eliminate and prevent STH infections in this vulnerable population.[18] The cornerstone of these efforts has been preventive chemotherapy (PC) with benzimidazole (BZ) drugs through mass drug administration (MDA) programs.[18,19] Control and elimination efforts against STHs aim to reduce infection prevalence, lower the pathologic consequences of infections and prevent reinfection.[18,20,21] According to 2023 WHO data, nearly 900 million preschool and school-aged children around the world require PC against STH infections.[22] The 2018 WHO data showed that several endemic SEA countries had varying levels of PC coverage: Indonesia did not reach 75%, the Philippines and Vietnam achieved higher than 75%, but for less than five years, and only Cambodia, Lao PDR and Myanmar sustained a coverage above 75% for more than five years.[21] Among preschool and school-aged children, the 2023 WHO data revealed that only Indonesia, Myanmar, Cambodia and Vietnam achieved the target 75% benchmark for PC coverage, while the Philippines and Lao PDR lagged behind, at 29.73% and 57.05%, respectively.[22] SEA nations have demonstrated a sustained commitment to combating STH infections through various control programs and MDA initiatives. Significant disparities in coverage and long-term success highlight the ongoing challenges of achieving consistent and equitable elimination across the region.

The fight against STH infections remains a difficult and ongoing process in endemic SEA countries. Trends and developments in these control and elimination efforts need to be assessed to forge the most appropriate path towards achieving national and global elimination targets.[2,11,23] Hence, the objective of this comprehensive review is to provide an update on drug-based control and elimination efforts for the control and elimination of STH infections in SEA by summarizing and analyzing findings from peer-reviewed research. Here, we analyzed research published between 2011 and 2025 to document trends in PC using mono- and combination drug administration, present the value of integrated control efforts that include health education and water, sanitation and hygiene (WASH), and highlight research indicating diminished efficacy of MDA efforts from many countries in the region.

Results

Characteristics of the studies included

Five databases were searched, and a total of 4294 hits were retrieved (Figure 1). Of these, 67% were removed due to duplication. The initial screening of 1244 articles resulted in a 97% removal rate, with exclusions based on publication type (reviews and abstracts), study location (outside SEA), pathogen focus, publication date (outside January 2011 to January 2025), incomplete metadata (no authors/abstract), and language (non-English). Eligibility screening was conducted on the full text of the remaining 41 papers, among which 34% were excluded due to an incorrect study design or outcome. A total of 27 papers were included in the study (Table S1, http://links.lww.com/IMD/A110), and all of them passed the risk of bias assessment (Tables S2-4, http://links.lww.com/IMD/A110).

The included studies span all the countries of SEA, except Brunei Darussalam and Singapore, where PC is not required.[22] Lao PDR had the greatest number of studies (six), followed by Myanmar, Indonesia and the Philippines (four). Three studies were conducted in Timor-Leste, while two studies each were performed in Thailand, Cambodia and Vietnam. Malaysia was represented by one study. Among these countries, Thailand and Malaysia do not require PC.[22] Studies were performed in children (ie, preschool and school-aged) and community folks. Among the STHs studied, the most commonly represented was *T. trichiura*, followed by *A. lumbricoides* and hookworms. Most of the studies included in this review were analytical cross-sectional studies, followed by randomized controlled trials, with quasi-experimental studies being the least represented. Key information regarding the included studies is summarized in Tables 1–3.

BZ drugs, particularly albendazole (ABZ, 400 mg) and mebendazole (MBZ, 500 mg), were the most frequently employed interventions for MDA in the included studies. Combination therapy, in which drugs from different classes of anthelmintic are combined to expand the range of activity and increase efficacy, was also an emerging trend in the included studies. BZ drugs were combined with macrocyclic lactones such as ivermectin (IVM)[10] and pyrimidine derivatives such as pyrantel pamoate (PYR).[23] Similarly, a combination of drugs that integrate the control of numerous parasitic helminths of public health importance was also reported. For instance, the combination of ABZ, IVM and diethylcarbamazine (DEC) against lymphatic filariasis, scabies and STHs has been tested for its ability to reduce infection prevalence.[50] Comprehensive control efforts that integrate drug-based interventions with health education, the provision of clean water, and the improvement of sanitation and hygiene standards have also been reported.[26,33]

BZs as the primary intervention

Several cross-sectional studies included in this review assessed the effects of BZ monotherapy on infection prevalence. For instance, Sanza et al.[37] reported a significant decrease in the prevalence of STH infections in several provinces in the central Philippines where several rounds of MBZ monotherapy have been administered. Similarly, a preliminary study by Staal et al.[41] reported a decrease in *A. lumbricoides*, *T. trichiura* and hookworm infection prevalence in Indonesia at follow-up after ABZ treatment. Studies on monotherapy and combination therapy have also reported the cure rate (CR) and egg reduction rate (ERR) as efficacy parameters. An early trial by Soukhathammavong et al. observed that ABZ and MBZ had subpar CR (less than 95%) against *A. lumbricoides*, but had superior ERR (almost 100%), while the efficacy against hookworms and *T. trichiura* was suboptimal, characterized by low CRs and ERRs.[24] A similar trend was observed by Levecke et al.[25] in trials conducted in Vietnam and Cambodia, and by Vaz Nery et al.[42] among school-aged children in Timor Leste. A community-wide study by Ash et al. in the Lao PDR revealed that two rounds of triple-dose ABZ significantly reduced the prevalence and resulted in superior ERRs in *A. lumbricoides*, hookworms and *T. trichiura*.[40] In Vietnam, community-wide treatment with ABZ resulted in a more substantial reduction in the prevalence and intensity of infection than treatment involving only schoolchildren, as observed by Dyer et al.[31] The use of BZ drugs for the control and elimination of STH infections relies on their efficacy, together with their safety and ease of administration.[21] The challenge, however, is varied efficacies among different STH species.[31] Hence, its combination with other dewormers from different classes has been explored.

Combination therapy: the new frontier?

Studies assessing the effects of combination therapy against STH infections have also reported varying outcomes. Sapulete et al.[27] assessed the efficacy of the combination of PYR and ABZ in comparison with ABZ alone in school children from Indonesia, which revealed that the addition of PYR increased the CR by 20%.[27] Another trial by Keller et al.[18] revealed that, compared with

ABZ monotherapy, IVM+ABZ combination treatment resulted in superior ERRs at 2 to 3 weeks, 6 months and 1 year after treatment. Utilizing qPCR as their diagnostic tool, Hürlimann et al.[32] reported that, compared with ABZ monotherapy, the ABZ+IVM treatment resulted in higher CRs and ERRs for *A. lumbricoides*, *T. trichiura* and hookworms. The addition of DEC, IVM and ABZ resulted in a reduced infection prevalence of several hookworm species.[50]

PC with health education and WASH initiatives: the ol' reliables

The combination of nonpharmaceutical interventions with drug-based treatments also showed promising effects on STH infections. The "Fit for School" program, which included daily handwashing with soap, daily brushing with fluoride toothpaste and biannual deworming with ABZ, resulted in a reduced prevalence of STH infections of moderate to heavy intensity.[35] Another innovation in health education was the incorporation of Magic Glasses, which is a health education intervention aimed at preventing intestinal worms in schoolchildren that involves using engaging cartoon videos and educational materials.[52] The trial performed by Mationg et al.[29] in the Philippines revealed that the integration of the Magic Glasses program into the biannual ABZ administration program resulted in insignificant changes in the infection prevalence but markedly improved the knowledge and behavior of the schoolchildren receiving the intervention. In addition to health education, the provision of WASH infrastructure with deworming also improved infection control. A preliminary study showed that the provision of latrines and health education, together with ABZ treatment, significantly decreased the prevalence of STH infections among schoolchildren in Indonesia.[34] These results show the value of incorporating health education and WASH initiatives with drug administration for the control and elimination of STH infections. Recently, Mationg et al.[52] conducted a review of studies that assessed the effectiveness of health education packages against STHs and their impacts on infection control. Only three relevant studies were identified, highlighting the neglect of this intervention in the current drug-based paradigm. As the articles included in our review purport, health education plays a crucial role in improving the knowledge and attitudes of the target populations. Likewise, improving WASH facilities enables the elimination of open defecation and improves hygiene practices that prevent STH infection and reinfection.[53] A multipronged approach that includes health education and

WASH facility provision with mass treatment can facilitate faster achievement of control and elimination targets.

Indications of diminished efficacy

Several alarming reports of reduced mass treatment efficacies, which were lower than the threshold in the WHO guidelines,[51] were documented by some of the included studies. The WHO guideline stipulates that an anthelmintic drug's effectiveness is categorized as "satisfactory" if its ERR meets or exceeds the reference value, and as "doubtful" or "reduced" if the ERR falls below the reference value by less or more than 10 percentage points, respectively.[51] The aforementioned study comparing the efficacy of ABZ and MBZ in Lao PDR reported doubtful ERR (ie, less than 10 percentage points lower than the 90% reference value) among hookworms when the former was administered.[24] More recently, Colella et al.[47] used qPCR to assess the efficacy of ABZ treatment and showed a diminished efficacy because ERRs of hookworm infections in Cambodia were reduced (ie, more than 10 percentage points lower than the 90% reference value). A similar trend was also observed in Timor-Leste, where ABZ treatment did not result in ERRs for *N. americanus* that were within satisfactory levels.[42] Persistent prevalence despite a history of repeated treatments was also reported. A history of BZ treatment in schoolchildren and women of reproductive age failed to decrease the prevalence of *A. lumbricoides*, *T. trichiura* and hookworms in northern Lao PDR, where it remained relatively high.[36] In Myanmar, six rounds of ABZ treatment failed to consistently bring down the prevalence of *T. trichiura* infections.[48] The national and global control targets of the prevalence of STH infections have not been met after a decade of MDA with BZ drugs in schoolchildren in the Philippines, where the prevalence of *A. lumbricoides* and *T. trichiura* remained higher than 50%.[38]

Discussion

Our results show that BZ monotherapy remains central to STH control in Southeast Asia. However, some studies have reported that the efficacy of these drugs have been decreasing over time. To address this, combination therapy with ABZ+IVM has been trialed and has shown improved efficacy across different STH species. Moreover, health education regarding hygiene and improving access to clean water and sanitation in conjunction with drug administration have been shown to be effective and essential for the long-term control of STH infections. Based on the current WHO roadmap, the primary goal for STH control is to achieve elimination as a public health problem, defined as reducing the prevalence of moderate-to-heavy intensity infections to below 1% in all endemic districts by 2030.[51] The initial phase of intervention focuses on achieving disease control, which aims to significantly reduce the morbidity caused by these infections through regular MDA.[18]

Among the selected studies in this systematic review, BZ drugs, such as ABZ (400 mg) and MBZ (500 mg), were the most commonly used interventions in mass chemotherapy efforts. Indeed, MDA with BZ monotherapy remains the cornerstone of control and elimination efforts against STH infections.[18,19] This is particularly true in other endemic regions such as South America,[54,55] South Asia[56] and Africa.[57,58] Several SEA nations recognized the serious threat of STH infections and established control programs early: Malaysia in 1974, Indonesia in 1975, Thailand in 1980 and the Philippines in 1999.[8] Similarly, school-based deworming efforts were initiated in Vietnam, Lao PDR and Myanmar with aid from foreign agencies and support from affluent countries.[8] Some of these programs were discontinued due to a lack of funding or reliance on donations, loans and support from foreign agencies for the drugs needed for mass PC.[8] At the turn of the millennium, renewed enthusiasm for MDA against STH infections was seen. For instance, a 95% MDA coverage was reported during a country-wide, school-based deworming program conducted in Lao PDR in 2006.[59] Likewise, in 2006, the high infection prevalence reported previously prompted the Philippine government to implement the Integrated Helminth Control Program, which is still ongoing.[7,11,19,60] Similar efforts were implemented in Indonesia through governmental programs and in Cambodia through foreign support.[61]

Similar to our results, reports from other regions affirm the enhanced efficacy when BZs are combined with IVM. A recent meta-analysis revealed a greater reduction in the prevalence of *T. trichiura* infections when ABZ+IVM was used than when IVM was used alone (89.40% vs. 49.93%).[62] Against strongyloidiasis, however, IVM (84.49%) alone was slightly better in reducing infection prevalence than ABZ+IVM (81.37%).[62] In India, ABZ+IVM was also shown to increase the ERR of *A. lumbricoides* when compared with ABZ monotherapy (93.22% vs. 90.61%).[63] A cross-sectional study in a population receiving concurrent ABZ–IVM mass treatment in Cameroon revealed that the drug combination could have collateral impacts that can hasten the control and elimination of STHs.[64] Moreover, the efficacy of ABZ+IVM in reducing the incidence of STH-associated anemia and infections and their relative safety during pregnancy, were assessed in Uganda.[65] The results of the studies included in our review and those from the literature indicate that adding drugs from different classes to BZs improved the efficacy of treatments across a variety of STH species that affect populations within endemic areas. This point was highlighted by Emerson et al.,[66] which reported that combination therapy with appropriate drugs targeted toward the most susceptible helminth species can hasten the achievement of global control and elimination targets and therefore lessen the risk of resistance development.

The results from the selected papers indicate that mass treatments with BZs may have reduced efficacy depending on the species of the helminth and the drug used. This issue provides an opportunity for combination therapy regimens that can enhance efficacy, as proven by the aforementioned studies. The long-term consequences of combination treatment against STHs have yet to be fully assessed. However, the emergence of multidrug-resistant STHs as a consequence of combination therapy has become a concern.[67-69] Likewise, evidence of multidrug-resistant animal hookworms due to the protracted use of combination therapy in the United States and Canada should also be kept in mind.[70-72] The role of other factors, such as treatment coverage and compliance, needs to be thoroughly evaluated in all endemic countries in SEA. Moreover, the indication of resistance has yet to be fully elucidated by field data in this region. Guidelines on the determination of resistance in STHs have yet to be promulgated.[73] Only guidelines for the assessment of PC efficacy against STH infections and schistosomiasis have been established by the WHO.[51]

Our comprehensive review has several limitations. First, we were not able to incorporate data from governmental databases because we focused only on peer-reviewed literature. Some PC efforts may not be described in peer-reviewed literature. However, through extensive searches and sufficient selection and eligibility criteria, we were able to include studies that were performed in areas where government-implemented MDA was being carried out, such as those in the Philippines[23,38] and Lao PDR.[36] Second, the variability in the sample sizes, control groups used (if necessary), outcomes measured and study designs hamper the integrated analysis of the data from the included studies. Key limitations arise from the necessity to acknowledge the potential

publication and language bias inherent in restricting the search to nonmultilingual and indexed sources. The methodological rigor of the overall synthesis is also limited by the inadequate statistical power of many individual trials due to their small sample sizes. A final constraint is the absence of longitudinal data beyond five years, making it impossible to evaluate long-term effectiveness or the definitive emergence of resistance. Despite these issues, our utilization of appropriate risk-of-bias tools allowed for the assessment of each study's quality, confirming that the included results and their synthesis were of acceptable scientific merit.

Conclusions

This comprehensive review aimed to update the knowledge on mass treatment for STH control in SEA countries by collecting and synthesizing peer-reviewed literature. We searched several databases and included 27 studies that passed our screening and eligibility criteria. These papers had different study designs and utilized various drug interventions. BZ drugs, primarily ABZ and MBZ, remained the most commonly used anthelmintic in mass deworming efforts in the region, with often satisfactory but inconsistent results in terms of efficacy and reducing infection prevalence. An emerging trend that the papers reported on is the combination of anthelmintics from different classes with BZs to enhance efficacy. Moreover, comprehensive efforts that include health education and WASH facility improvement with deworming result in enhanced infection reduction. Several reports have documented diminished efficacies, such as insufficient ERRs and failure to significantly reduce infection prevalence, when BZs are used against hookworms and *T. trichiura* infections. Combination therapy (eg, ABZ+IVM) provides an opportunity to address this issue and enhance efficacy. Therefore, judicious combination therapy with health education packages and improvement of WASH can hasten the achievement of control and elimination targets and abate the threat of resistance.

Materials and methods

Research questions

This systematic review aimed to answer the following research questions:

1. Which SEA countries have implemented drug-based STH control efforts, as reported in peer-reviewed literature published from 2011 through 2025?
2. What drugs have been used as interventions in these control efforts?

3. What were the treatment outcomes of drug administration in controlling infections in these countries?

Search strategy

Five databases were searched in this systematic review, MEDLINE via PubMed (https://pubmed.ncbi.nlm.nih.gov/), Scopus (https://www.scopus.com/sources), Science Direct (https://www.sciencedirect.com/search/entry), EBSCO Host (https://search.ebscohost.com/) and ProQuest (https://www.proquest.com/) were searched for literature (accessed 7 through 11 February 2025). All database searches were restricted to the publication date from January 2011 through January 2025. This time restriction was chosen to provide a follow-up to the review on the same topic by Jex et al.[6] This review is limited to the member countries of the Association of Southeast Asian Nations. For EBSCO Host, the Applied Science and Technology Source Ultimate, CINAHL Complete, MEDLINE and Newswires databases were selected. The specific terms used to search the databases can be found in Table S5, http://links.lww.com/IMD/A110. The .ris and .nbib files of the searches were downloaded.

Study screening, selection and eligibility

The search results were uploaded to Rayyan (https://www.rayyan.ai/), where study selection, screening and eligibility checking were performed (accessed 14 through 21 February 2025). Rayyan is an AI-assisted platform for streamlining study screening, with the ability to import and deduplicate references from various sources.[14] The platform was used for duplicate detection, with 95% text similarity as the resolution limit. Duplicates with <95% similarity were resolved manually. Titles and abstracts were used for the initial screening, and papers lacking these components were excluded. The detailed inclusion and exclusion criteria used for assessment are summarized in Table S6, http://links.lww.com/IMD/A110. The first, second and third authors conducted the study selection, screening and eligibility assessment, and all authors collaboratively resolved disagreements to reach a consensus. An overview of the study selection, screening and eligibility assessment process is provided in Figure 1.

Risk of bias assessment, data extraction and synthesis

The papers finally included in this study were of several types: cross-sectional studies, quasi-experimental studies and randomized controlled trials. Due to the diversity of study types, several

risk-of-bias tools were utilized. The *Joanna Briggs Institute's Critical Appraisal Checklist for Analytical Cross-Sectional Studies*, the *Critical Appraisal Checklist for Quasi-Experimental Studies*, and the *Critical Appraisal Tool for Assessment of Risk of Bias for Randomized Controlled Trials* were used (https://jbi.global/critical-appraisal-tools). The second and third authors independently reviewed the risk of bias assessment. The fourth and fifth authors resolved disagreements. The results of the risk of bias assessment are provided in Tables S1-3, http://links.lww.com/IMD/A110.

Codes were assigned to the included papers (Table S6, http://links.lww.com/IMD/A110). Data extracted from the studies include authors' names, year of publication, title, type of study, specific locale and country of the study, population, STH species studied, drug-based intervention, comparison groups (if any), test used and key outcomes. These were input into a spreadsheet. The study results were described, summarized and synthesized using descriptive statistics, specifically counts and percentages.

Supplementary digital contents

Supplementary materials are available at, http://links.lww.com/IMD/A110

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Figure legends

Figure 1. PRISMA summary of the systematic literature search, screening and selection process of this review. STH, soil-transmitted helminth.

Table 1. Key results from randomized controlled trials included in this review

Study	Country	Location	Population	STH studied	MDA intervention (dose)	Comparison group	Test used	Key outcomes
Soukhathammavong et al. (2012)[24]	Lao PDR	Batieng district, Champasak Province	Children aged 6–12 y	Hookworms, *Ascaris*, *Trichuris*	ABZ (400 mg)	MBZ (500 mg)	Kato-Katz thick smear	Hookworms: ABZ CR, 32.0%; ABZ ERR, 86.7%; MBZ CR, 15%; MBZ ERR, 76.3% *Ascaris*: ABZ CR, 92.9%; ABZ ERR, 100%; MBZ CR, 93.3%; MBZ ERR, 100% *Trichuris*: ABZ CR, 33.3%; ABZ ERR, 67%; MBZ CR, 27.9%; MBZ ERR, 66%

Study	Country	Location	Population	STH studied	MDA intervention (dose)	Comparison group	Test used	Key outcomes
Levecke et al. (2014)[25]	Cambodia and Vietnam	Cambodia and Vietnam	Schoolchildren aged 4–18 y	Hookworms, *Ascaris*, *Trichuris*	MBZ (500 mg)	ABZ (400 mg)	McMaster egg counting method	*Ascaris*: ABZ, 100% FECR in Vietnam; BZ, 93.9% FECR in Vietnam *Trichuris*: ABZ, 92.3% FECR in Vietnam; MBZ, 76.8% FECR in Vietnam Hookworms: ABZ, 97.6% FECR in Cambodia; MBZ, 79.7% FECR in Cambodia; ABZ, 100% FECR in Vietnam; MBZ, 95% FECR in Vietnam
Clarke et al. (2018)[16]	Timor Leste	Aileu and Manufahi municipalities	Community-wide and school children	Hookworms, *Ascaris*, *Trichuris*	Community-wide ABZ (400 mg) MDA and WASH	ABZ (400 mg) MDA and WASH only in schools	Real-time multiplex PCR	Control arm prevalence: *Ascaris*, 48.7%; *Trichuris*, 2.2%; *Necator americanus*, 13.7% Intervention arm prevalence: *Ascaris*, 7.6%; *Trichuris*, 1.9%; *N. americanus*, 15.1%

Study	Country	Location	Population	STH studied	MDA intervention (dose)	Comparison group	Test used	Key outcomes
Sapulete et al. (2020)[27]	Indonesia	Bangli, Bali	Children aged 8–12 y	*Trichuris*	ABZ (400 mg) and PYR (10 mg/kg) for 3 consecutive d	ABZ (400 mg) for 3 consecutive d	Kato-Katz thick smear	Control arm CR, 40%; intervention arm CR, 60% Control arm % satisfactory ERR (≥ 50%), 44.7%; intervention arm % satisfactory ERR (≥ 50%), 55.3%
Keller et al. (2021)[28]	Lao PDR	Nam Bak district in Luang Prabang Province	Community-wide, aged 6–60 y	Hookworms, *Ascaris*, *Trichuris*	ABZ (400 mg) and IVM (200 µg/kg)	ABZ (400 mg)	Kato-Katz thick smear	14- to 21-d ERR: control arm, 68.8%; intervention arm, 99.2% 6-mo ERR: control arm, 79.6%; intervention arm, 99% 12-mo ERR: control arm, 91.3%; intervention arm, 99.6%

Study	Country	Location	Population	STH studied	MDA intervention (dose)	Comparison group	Test used	Key outcomes
Mationg et al. (2021)[29]	Philippines	Laguna Province	Schoolchildren aged 9–10 y	Total STHs	ABZ (400 mg) MDA with Magic Glasses	ABZ (400 mg) MDA with DOH/DepEd's standard health education	Kato-Katz thick smear	Control arm: baseline prevalence, 20.1%; 1st FU, 16.4%; 2nd FU,18.8% Intervention arm: baseline prevalence ,25.8%; 1st FU, 19.7%; 2nd FU, 21.3%
Hürlimann et al. (2022)[30]	Lao PDR	Nam Bak district in Luang Prabang Province	Community-wide, aged 6–60 y	Hookworms, *Ascaris* *Trichuris*	ABZ (400 mg) and IVM (200 μg/kg)	ABZ (400 mg)	qPCR	Control arm: *Trichuris* ERR, 69%; *Trichuris* CR, 8%; *Ascaris* ERR, 100%: *Ascaris* CR, 100%; hookworms ERR, 99%; hookworms CR, 56% Intervention arm: *Trichuris* ERR, 99%; *Trichuris* CR, 66%; *Ascaris* ERR, 100%; *Ascaris* CR, 100%; hookworms ERR, 99%; hookworms CR, 59%

Study	Country	Location	Population	STH studied	MDA intervention (dose)	Comparison group	Test used	Key outcomes
Dyer et al. (2023)[31]	Vietnam	Dak Lak Province	Community-wide and school children	*N. americanus*	ABZ (400 mg) to schoolchildren and the community-wide	ABZ (400 mg) to schoolchildren only	Multiplex qPCR	Control arm: Baseline: prevalence, 16.7%, EPG, 1678 8 12-mo FU: prevalence ,11.7%; EPG, 1622.0 Relative reduction: prevalence, 30.1%; EPG, 3.4% Intervention arm: Baseline: prevalence, 11.7%; EPG, 1699.4 12-mo FU: prevalence, 7.7%; EPG, 747.7 Relative reduction: prevalence, 34.6%%; EPG, 56.0%

Study	Country	Location	Population	STH studied	MDA intervention (dose)	Comparison group	Test used	Key outcomes
Welsche et al. (2024)[12]	Lao PDR	Nambak district, Luang Prabang	Community-wide	*Trichuris*	ABZ (400 mg) and IVM (200 µg/kg)	ABZ (400 mg)	Kato-Katz thick smear	Control arm: arithmetic ERR, 0–64.1%; geometric ERR, 56.2%–80.2% Intervention arm: arithmetic ERR, 91.9%–97.2%; geometric ERR, 98.9%–99.1%

ABZ, albendazole; CR, cure rate; DEC, diethylcarbamazine; EPG, egg per gram; ERR, egg reduction rate; FECR, fecal egg count reduction; FU, follow-up; IVM, ivermectin; MDA, mass drug administration; MBZ, mebendazole; PYR, pyrantel pamoate; STH, soil-transmitted helminth WASH, water, sanitation and hygiene.

Table 2. Results from quasi-experimental studies included in this review

Study	Country	Location	Population	STH studied	MDA intervention (dose)	Comparison group	Test used	Key outcomes
Monse et al. (2013)[23]	Philippines	Municipalities of Mambajao, Catarman, Guinsiliban, Mahinog and Sabay in Camiguin Province, and Gingoog City in Misamis Oriental Province	First-grade students (aged 6–7 y) of public elementary schools	Total STHs	ABZ (400 mg), daily, supervised brushing with a fluoride toothpaste	External concurrent control groups and internal non-concurrent control groups	Kato-Katz thick smear	Intervention arm: 10.7% reduction in the prevalence of moderate-to-heavy STH infections Control arm: 12.4% reduction in the prevalence of moderate-to-heavy STH infections
Park et al. (2016)[24]	Indonesia	Gunungpatin sub-district, Semarang, Central Java	Children aged 3–13 y	Total STHs	ABZ (400 mg). BALatrines	No intervention	Sodium nitrate fecal flotation technique	STH prevalence: control arm, 20.4%, intervention arm, 4%

Study	Country	Location	Population	STH studied	MDA intervention (dose)	Comparison group	Test used	Key outcomes
Nachaiwieng et al. (2024)[35]	Thailand	Omkoi District, Chiang Mai Province	Community-wide	*Ascaris* and *Trichuris*	ABZ (400 mg) with community health education	ABZ (400 mg)	Formalin ethyl acetate concentration method	Control arm: Baseline prevalence: *Ascaris*, 0.6%; *Trichuris*, 1.8% 3-mo FU prevalence: *Ascaris*, 0%; *Trichuris*, 1.3% 6 mos FU prevalence: *Ascaris*, 0%; *Trichuris*, 0.5% Intervention arm: Baseline prevalence: *Ascaris*,0.8%; *Trichuris*, 5.4% 3 mos FU prevalence: *Ascaris*, 0%; *Trichuris*, 4.8% 6 mos FU prevalence: *Ascaris*, 0.9%; *Trichuris*, 6.4%

ABZ, albendazole; CR, cure rate; DEC, diethylcarbamazine; EPG, egg per gram; ERR, egg reduction rate; FU, follow-up; IVM, ivermectin; MDA, mass drug administration; MBZ, mebendazole; PYR, pyrantel pamoate; STH, soil-transmitted helminth WASH, water, sanitation and hygiene

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Table 3. Results from cross-sectional studies included in this review

Study	Country	Location	Population	STH studied	MDA history	Test used	Key outcomes
Conlan et al. (2012)[36]	Lao PDR	Oudomxay, Luangprabang, Houaphan and Xiengkhuang Provinces	Community-wide, 6–11-y-old children, women of childbearing age	*Ascaris*, *Trichuris*, Hookworms, *Strongyloides*	ABZ (400 mg)	Formalin-ether concentration	STH prevalence: total STH, 70.6%; *Ascaris*, 26.1%; *Trichuris*, 41.5%; hookworms, 46.3%; *Strongyloides*, 8.9%
Sanza et al. (2013)[37]	Philippines	Aklan, Antique and Capiz	Third-grade students aged 8-9 y	*Ascaris*, *Trichuris*	MBZ (500 mg)	Kato-Katz thick smear	Prevalence of moderate-to-heavy STH infections: significantly decrease from the baseline Effect on egg counts: decreased within the first follow-up but increased in the last follow-up
Belizario et al. (2016)[38]	Philippines	Municipalities of Aroroy, Cawayan, Milagros and Monreal in Masbate Province	Preschool-age children	Hookworms, *Ascaris*, *Trichuris*	10 years of MDA with ABZ (400 mg) or MBZ (500 mg)	Kato-Katz thick smear	STH prevalence: *Ascaris*, 59.0%; *Trichuris*, 54.0%; hookworms, 2%

Study	Country	Location	Population	STH studied	MDA history	Test used	Key outcomes
Dunn et al. (2017)[39]	Myanmar	Udo village, Taikkyi township, Yangon Region and Kyee Kan Theik village, Nyaung Don township, Ayeyarwady Region	Community-wide	Hookworms, *Ascaris*, *Trichuris*	ABZ (400 mg), DEC (6 mg/kg)	Kato-Katz thick smear	STH prevalence: total STHs, 27.81%; *Ascaris*, 5.34%; *Trichuris*, 18.12%; hookworms, 8.71%
Ash et al. (2017)[40]	Lao PDR	Phongsaly Province	Community-wide	Hookworms, *Ascaris*, *Trichuris*	2 rounds of MDA with triple ABZ (400 mg) 5 mos apart	McMaster egg counting method; PCR species identification	MDA1 STH prevalence reduction: *Ascaris*, 95.6%; *Trichuris*, 69.2%; hookworms, 83.4% STH ERR: *Ascaris*, 99.4%; *Trichuris*, 77.2%; hookworms, 94.4% MDA2 STH prevalence reduction: *Ascaris*, 93.5%; *Trichuris*, 61%; hookworms, 84.5% STH ERR: *Ascaris*, 99.3%; *Trichuris*, 88.5%; hookworms, 97.8%

Study	Country	Location	Population	STH studied	MDA history	Test used	Key outcomes
Staal et al. (2018)[41]	Indonesia	Nangapanda Village, Ende District, Flores Island	School children	Hookworms, *Ascaris*, *Trichuris*	ABZ (400 mg)	Kato-Katz thick smear	Baseline STH prevalence: *Ascaris*, 8.4%; *Trichuris*, 11.2%; hookworms, 4.9% FU STH prevalence: *Ascaris*, 1.0%; *Trichuris*, 3.9%; hookworms, 1.0%
Vaz Nery et al. (2018)[42]	Timor Leste	Manufahi municipality	Community-wide	*Necator*, *Ascaris*	ABZ (400 mg)	Real-time multiplex PCR	*Necator americanus*: CR, 58.3%; infection intensity reduction: 88.9% *Ascaris*: CR, 91.4%; infection intensity reduction, 95.5%
Sungkar et al. (2018)[43]	Indonesia	Perobatang Village, Southwest Sumba District	Children aged 1–15 y	Hookworms, *Ascaris*, *Trichuris*	Triple dose ABZ (400 mg)	Kato-Katz thick smear	Baseline STH prevalence: *Ascaris*, 65.4%; *Trichuris*, 55.8%; hookworms, 15.4% FU STH prevalence: *Ascaris*, 8.3%; *Trichuris*, 12.8%; hookworms, 0%

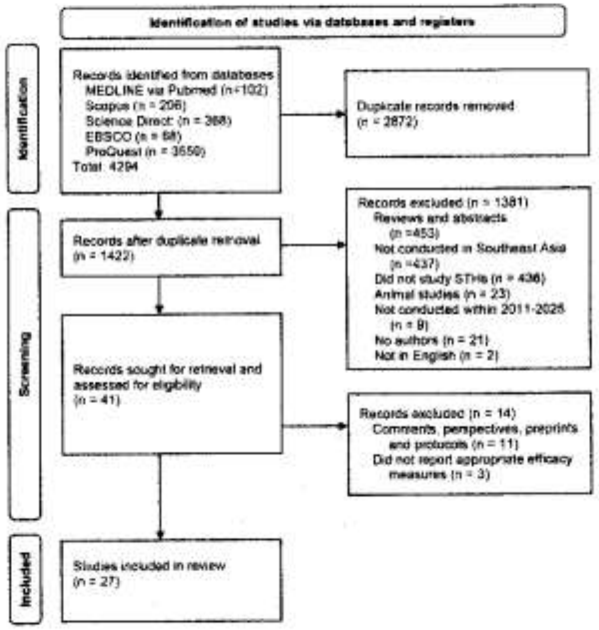
Study	Country	Location	Population	STH studied	MDA history	Test used	Key outcomes
Dunn et al. (2019)[44]	Myanmar	Udo village, Taikkyi township, Yangon Region and Kyee Kan Thaik village, Nyaung Don township, Ayeyarwaddy Region	Community-wide	Hookworms, *Ascaris*, *Trichuris*	Thrice administration of ABZ (400 mg) 4 and 6 mos apart	Kato-Katz thick smear	Baseline STH prevalence: *Ascaris*, 5.5%; *Trichuris*, 17.02%, hookworms, 9.75% 1st FU STH prevalence: *Ascaris*, 1.72%; *Trichuris*, 14.82%; hookworms, 6.69% 2nd FU STH prevalence: *Ascaris*: 2.49%; *Trichuris*: 11.85%; hookworms: 5.54%
Chai et al. (2020)[45]	Myanmar	South Dagon, North Dagon, and Hlaing-thar-yar of Yangon Region	School children	Hookworms, *Ascaris*, *Trichuris*	ABZ (400 mg)	Kato-Katz thick smear	STH prevalence: *Ascaris*, 17.2%; *Trichuris*, 19.4%; hookworms, 0.2%
Stracke et al. (2021)[46]	Thailand	Mae Song subdistrict, Tha Song Yang district, Tak Province	Pre-school and school-aged children aged 2–6 y	*Ancylostoma ceylanicum*, *Ascaris*, *Trichuris*	Annual MBZ (500 mg) administration	multiplexed-tandem qPCR	STH prevalence: *Ascaris*, 39.2%; *Trichuris*, 36.6%; *A. ceylanicum*, 1.1%

Study	Country	Location	Population	STH studied	MDA history	Test used	Key outcomes
Colella et al. (2021)[47]	Cambodia	Chambak Pa'em, Chamlong, Kam-pot, Rovieng Thoung, Sangker Roung, Pal Hal, Anglong Svay, Cham-long, Bos Pey and Sra'er Thom, Rovieng district, Preah Vihear Province	Community-wide and animals	*A. ceylanicum*, *Necator americanus*	ABZ (400 mg)	multiplex qPCR	All hookworms: CR, 46.4%; geometric ERR, 83.2%; arithmetic ERR, 72.5% *N. americanus*: CR, 48.3%; geometric ERR, 84.5%; arithmetic ERR, 77.6% *A. ceylanicum*: CR, 52.6%; geometric ERR, 98.7%; arithmetic ERR, 62.9%

Study	Country	Location	Population	STH studied	MDA history	Test used	Key outcomes
Hong et al. (2021)[47]	Myanmar	Shwe Pyi Thar, Twantay, and Kyauktan, Yangon Region	Primary school-children	Hookworms, *Ascaris*, *Trichuris*	9 rounds of ABZ (400 mg)	Kato-Katz thick smear	1st FU prevalence: *Ascaris*, 23.3%; *Trichuris*, 23.3%; hookworms: 0.1% 2nd FU prevalence: *Ascaris*,17.3%; *Trichuris*, 26.2%; hookworms: 0.8% 3rd FU prevalence: *Ascaris*, 17.3%; *Trichuris* 27.4%; Hookworms, 0.3% 4th FU prevalence: *Ascaris*, 2.9%; *Trichuris*, 21.3%; hookworms, 0% 5th FU prevalence: *Ascaris*, 7.2%; *Trichuris*, 14.5%; hookworms, 0.2% 6th FU prevalence: *Ascaris*, 3.6%; *Trichuris*, 20.2%; hookworms, 0.1%
Tee et al. (2022)[48]	Malaysia	Rasau (Perak state), Sungai Judah, Tanjung Sepat and Bukit Bangkon, Selangor States	Community-wide, aged 4-85 y	Hookworms, *Ascaris*, *Trichuris*	3-d course of ABZ (400mg)	Modified Kato-Katz technique	3-wk FU *Ascaris*: CR, 100%; ERR, 100% *Trichuris*: CR, 64.6%; ERR, 94.2% Hookworms: CR, 100%; ERR, 100% 6 wk FU: *Ascaris*: no infection *Trichuris*: 25.8% prevalence, 250.2 EPG Hookworms: no infection
Le et al. (2023)[49]	Timor Leste	Dili, Ermera, and Manufahi municipalities	Schoolchildren	*Ascaris*, *Trichuris*, *N. americanus*, *A. ceylanicum*, *Acylostoma duodenale*	IVM (200 µg/kg), DEC (6 mg/kg), ABZ (400 mg)	Multiplex qPCR	Baseline prevalance: *Ascaris*, 18.1%; *Trichuris*, 4.8%; *N. americanus*, 7.4%; *A. ceylanicum*, 0.6%; *A. duodenale*, 0.2% 18 mos FU prevalence: *Ascaris*, 13.2%; *Trichuris*, 0.6%; *N. americanus*, 5.9%; *A. ceylanicum*, 3.4%; *A. duodenale*, 0%

ABZ, albendazole; CR, cure rate; DEC, diethylcarbamazine; EPG, egg per gram; ERR, egg reduction rate; FU, follow-up; IVM, ivermectin; MBZ, mebendazole; MDA, mass drug administration; STH, soil-transmitted helminth.

Figure 1



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Unraveling the mechanisms of benzimidazole resistance in hookworms: A molecular docking and dynamics study

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A R T I C L E I N F O

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A B S T R A C T

Background: Benzimidazole resistance is an emerging challenge among parasitic helminths. It is caused by single nucleotide polymorphisms (SNPs) in specific loci in helminths' β-tubulin genes. Field studies and laboratory investigations reported resistance-associated SNPs in 4 codon locations with 7 allelic variations among hookworms. This study aimed to determine the effects of these mutations on the binding efficiency and behavior of the β-tubulin protein in four hookworm species against four benzimidazole drugs.
Methods: β-tubulin gene coding sequences of *Ancylostoma caninum*, *A. duodenale*, *A. ceylanicum*, and *Necator americanus* were retrieved, assessed phylogenetically, and used to construct the 3D structure models of the proteins. The modeled protein structures were verified and edited to contain the reported SNPs: Q134H, F167Y, E198A, E198K, E198V, F200L, and F200Y. Benzimidazole drugs such as albendazole (ABZ), fenbendazole (FBZ), mebendazole (MBZ) and oxfendazole (OBZ) were used as ligands. Molecular docking experiments were performed with the wild-type and mutated proteins. Molecular dynamics simulation assessed the dynamic behavior of the β-tubulin-benzimidazole complex.
Results: *In silico* docking assessments showed that various amino acid substitutions due to resistance-associated SNPs cause alterations in binding affinities and positions. E198K and Q134H in hookworm β-tubulins substantially weakened the binding affinities and altered the binding positions of benzimidazole drugs. Molecular dynamics analysis revealed that these mutations also caused marked reductions in the binding free energies owing to diminished hydrogen bond contacts with the benzimidazole ligands.
Conclusion: The evidence shown herein indicates that mutations at positions 198 and 134 are detrimental to conferring benzimidazole resistance among hookworms. The presence of these mutations may alter the efficacy of pharmacological interventions. Hence, further studies should be conducted to assess their emergence among hookworms in endemic areas with histories of chemotherapy.

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1. Introduction

Hookworm infections continue to pose a significant threat to public and veterinary health worldwide. Human hookworm infections are primarily caused by *Necator americanus* and *Ancylostoma duodenale*, while *A. caninum*, *A. braziliense*, and *A. tubaeforme* are the primary etiological agents of hookworm infections in animals. *A. ceylanicum* is a true zoonotic hookworm widespread in countries within the Asia-Pacific region. Hookworm infections impose a substantial burden on endemic populations, with an estimated loss of 800,000–4 million disability-adjusted life years. Moreover, zoonotic hookworm infections cause approximately 239–500 million cases globally. Among animals, hookworms reportedly infect approximately 41 % of domestic dogs in the Asian continent, with *A. caninum* and *A. ceylanicum* accounting for 27 % and 24 % of these infections, respectively. To abate the health threats caused by hookworm infections, numerous control efforts have been mobilized by the public health and veterinary sectors in many countries. Preventive chemotherapy for humans and routine deworming for animals continue to be the cornerstone of hookworm control programs. The repeated and sustained administration of benzimidazole drugs, such as albendazole, mebendazole, and fenbendazole, has raised concerns about the development of benzimidazole resistance in soil-transmitted helminths, including hookworms. Drug-resistant helminths pose significant challenges to public and veterinary health. These resistant parasites can cause more severe infections and spread more easily. Additionally, the limited availability of effective drugs to treat these infections further exacerbates the problem.

Single nucleotide polymorphisms (SNPs) in the β-tubulin isotype 1 gene result in benzimidazole resistance in helminths. Canonical nucleotide mutations F167Y (TTC,TTT to TAC,TAT), E198A (GAG,GAA to GCG,GCA), and F200Y (TTC,TTT to TAC,TAT) cause amino acid substitutions that change the structure of the β-tubulin protein, thereby adversely affecting its binding efficiency with benzimidazole drug ligands. These mutations have been reported in different hookworm species that affect both humans and animals. Recently, multidrug-resistant *A. caninum* isolates that carry such mutations have emerged in the United States and Canada. Moreover, studies on these multidrug-resistant hookworms have resulted in the discovery of novel SNPs that have not been previously found among members of Ancylostomatidae: Q134H (CAA to CAT), E198K (GAG, GAA to AAG, AAA) and E198V (GAG, GAA to GTA). Laboratory investigations that induced resistance in *A. ceylanicum* have produced inconsistent findings regarding the correlation between these SNPs and benzimidazole resistance. One of these laboratory studies identified F200L (TTC,TTT to TTA) mutation which was linked to the induced resistance. Despite these reports, the exact mechanisms underlying benzimidazole resistance in hookworms remain unclear and require further study.

Recent *in silico* studies determined the key interactions between helminth β-tubulins and benzimidazole drugs. However, these studies focused on the use of albendazole as the sole drug ligand. This seems limiting since albendazole is not frequently used against canine or feline hookworms, where fenbendazole and oxfendazole are used in deworming preparations. Moreover, these studies have not explored the effects of other SNPs reported recently in addition to the canonical mutations. Therefore, to gain novel insights into benzimidazole resistance, this study aimed to determine the effects of all SNPs reported in hookworms on the binding behavior and efficacy of hookworm β-tubulin isotype 1 protein with several benzimidazole drugs. Here, we report the effects of resistance-associated mutations in the hookworm β-tubulin proteins when docked *in silico* with benzimidazole ligands.

2. Materials and methods

2.1. *Hookworm β-tubulin nucleotide and amino acid sequences*

As described previously by Jones et al., four isotypes of β-tubulin exist among the members of Ancylostomatidae (Supplementary Table 1). Among hookworms of public and veterinary health concerns, the coding nucleotide and amino acid sequences of these proteins have only been available for *A. caninum*, *A. ceylanicum*, *A. duodenale*, and *N. americanus*. These β-tubulin sequences, both nucleotide and amino acids, were obtained from WormBase ParaSite. Moreover, the amino acid sequences were confirmed by searching UniProt.

2.2. *Phylogenetic analysis*

BioEdit Sequence Alignment Editor version 7.2.5 was used to align both nucleotide and amino acid sequences. The sequences were aligned using ClustalW Multiple Alignment function in BioEdit. The aligned sequences were trimmed using the alignment editor of MEGA version 11. The best model was determined for both the trimmed nucleotide and amino acid sequences, and phylogenetic trees were constructed. Maximum likelihood trees were drawn based on the K2 (Kimura-2 parameter) + G model for the nucleotide sequences and JTT (Jones-Taylor-Thornton) model for amino acid sequences with 1000 bootstraps in MEGA version 11.

2.3. *Homology Modelling and quality assurance checks*

β-tubulin proteins of the four hookworm species were modeled using SWISS-MODEL (https://swissmodel.expasy.org/) [Accessed 27 May 2024]. The amino acid sequences were used for protein structure modeling, with a previously described β-tubulin (PDB ID No. 6fkj) as the template. 6fkj was selected because it contains β-tubulin proteins (chains B and D), whose crystal structures were determined with a ligand bound to the colchicine binding site in a similar manner to benzimidazole drugs. The percentage of sequence identity similarity and GMQE (Global Model Quality Estimate) scores were noted (Supplementary Table 2). Modeled β-tubulins were submitted to various platforms for quality assurance checks. Before downloading the PDB file of the homology-modeled protein, the Ramachandran plot, which gives the percentage of amino acids in their favored conformation in SWISS-MODEL's structure analysis function, was inspected and noted. Moreover, the modeled proteins were submitted to the UCLA-DOE LAB — SAVES v6.0 (https://saves.mbi.ucla.edu/) [Accessed: 27 May 2024], and the results of the PROCHECK Ramachandran plots and VERIFY3D plots were recorded (Supplementary Table 2).

Since mutations occurring in the β-tubulin isotype 1 gene are the cause of benzimidazole resistance, modeled isotype 1 proteins were selected for *in silico* docking assessments with benzimidazole drugs. Known resistance-associated SNPs at positions 134, 167 198, and 200: Q134H, F167Y, E198A, E198K, E198V, F200Y, and F200L were introduced into amino acid sequences and were used to model proteins using SWISS-MODEL. These mutations were identified through a prior systematic review that our team conducted. All mutations that we reported in the systematic review were included in this study. These mutated proteins were also assessed using PROCHECK and VERIFY 3D (Supplementary Table 2). Both wild-type and mutated hookworm β-tubulins were evaluated for their structural similarity to the mammalian template using the PDB Pairwise Structure Alignment (https://www.rcsb.org/alignment) [Accessed: 29 May 2024]. The mammalian β-tubulin monomer (PDB ID No. 1FJJ) was used as the template. RMSD, TM Score, Identity %, Aligned residues, and sequence lengths were documented and assessed.

2.4. *In silico docking*

To obtain the best binding poses and their respective affinity values, in silico docking analyses with the modeled β-tubulin isotype 1 proteins and benzimidazole drugs were performed using AutoDock Vina[12] in the PyRx-Virtual Screening Tool version 0.8 (https://pyrx.sourceforge.io/). Before uploading the proteins to PyRx, polar hydrogens were added to all wild-type and mutated β-tubulins using the BIOVIA Discovery Studio Visualizer. These proteins were then used as docking macromolecules for PyRx. 3D ligand structures of Albendazole (as Albendazole sulfoxide, ABZ), Mebendazole (MBZ), Fenbendazole (FBZ), and Oxfendazole (OBZ) were obtained from PubChem[13] (https://pubchem.ncbi.nlm.nih.gov/) [Accessed 25 May 2024]. ABZ, MBZ, FBZ, and OBZ were used because these drugs are the most commonly used anthelmintics in clinical treatment and control programs.[14] Also, previous studies of similar methodologies and aims have utilized these drugs.[10,11] These structures were imported to PyRx, set as ligands, and energy-minimized.

In silico docking was performed by setting the center of the search grid cube at position 200, with the surface of the cube at 25 Å from the center (coordinates: X = 15.6; Y = 57.5; Z = 33.1). This search grid configuration ensured that all positions where the SNPs occurred were inside the cube. The binding positions with the highest binding affinities (kcal/mol) and the lowest RMSD scores (lower and upper bounds, preferably 0) were considered the best and were saved as PDB files. The binding poses of the in silico docking experiments were visualized using the Receptor-Ligand Interaction function in BIOVIA Discovery Studio Visualizer version 21.1.0.20298 (https://discover.3ds.com/discovery-studio-visualizer-download). Both the 3D and 2D representations of the docking results were evaluated. The list of interacting amino acids and their frequencies were summarized and recorded (Supplementary Tables 4 and 5).

2.5. *Molecular dynamics simulations and trajectory analysis*

Docked complexes of the top two resistance-associated mutations that caused the highest reduction in binding affinity were analyzed using molecular dynamics (MD) to determine the binding behavior and binding free energies. MD simulations were performed using GROMACS 2023.2. The topology of the benzimidazole ligands (Albendazole and Fenbendazole) was assigned using the Chemistry at Harvard Macromolecular Mechanics (CHARMM) General Force Field (CGenFF) server (https://app.cgenff.com/login). The protein topology of the Ancylostoma ceylanicum β-tubulin receptor was generated using the CHARMM36 force field (July 2022 version), which was incorporated into GROMACS. The protein complex was simulated inside a cubic box with a 10 Å buffer from the box edge to avoid interactions with the periodic image from the adjacent unit cell. The box was filled with TIP3P water molecules and neutralized with Na and Cl ions. Then, 0.15 M NaCl was added. The system was energy minimized using steepest descent for 50,000 steps to remove steric clashes. It was then equilibrated to 300 K and 1 bar using NVT and NPT ensembles, respectively. Both equilibration methods used 50,000 steps or 100 ps of simulation time. Temperature was regulated using the V-rescale thermostat, while pressure was controlled using the Berendsen barostat. The MD run was conducted for 50,000,000 steps or 100 ns, saving snapshots every 5,000 steps, resulting in a trajectory file composed of 1,000 frames. Hydrogen bonds (H-bonds) were identified based on two criteria: a donor-acceptor distance of no more than 3.5 Å and a donor-hydrogen-acceptor angle of at least 120 degrees. RMSD (Root Mean Square Deviation), RMSF (Root Mean Square Fluctuation), SASA (Solvent Accessible Surface Area) plots, and H-bond plots were constructed to assess the behavior of the β-tubulin-benzimidazole ligand complexes. The equilibration point was determined to be beyond the 80 ns mark of the simulation.

The absolute binding free energies, determined through MM-GBSA (Molecular Mechanics-Generalized Born Surface Area) and MM-PBSA (Molecular Mechanics-Poisson-Boltzmann Surface Area) methods, were calculated using the last 20 ns of the simulations. MM-GBSA and MM-PBSA estimates were derived using Equation 1. ΔGGAS represents the sum of electrostatic and van der Waals energies, ΔGSOLV is the sum of polar and nonpolar solvation energy, and −TΔS represents the interaction entropy calculated from the last 25 frames (Supplementary Tables 6 and 7). The MM-GBSA and MM-PBSA methods employed the Lennard-Jones and Coulomb potentials for van der Waals and electrostatic energy calculations, respectively. MM-GBSA employed the Generalized Born model to estimate the polar solvation energy, while MM-PBSA used the Poisson-Boltzmann model. Nonpolar solvation energies were assumed to have a linear dependency on the SASA values. Per-residue binding free energy decomposition was done using the MM-GBSA method. Binding free energies were calculated using the last 20 s of the simulation after the equilibration point was reached.

$$\Delta G_{bind} = \Delta GGAS + \Delta GSOLV - T\Delta S \tag{1}$$

3. Results

3.1. *Hookworm β-tubulins and their phylogeny*

All modeled β-tubulins passed the quality assurance checks based on the constructed Ramachandran Plots in SWISS-MODEL and PROCHECK (Supplementary Table 2). However, the modeled isotype 3 β-tubulins of *A. caninum*, isotype 4 of *N. americanus*, and both isotypes 3 and 4 of *A. duodenale* failed the VERIFY3D check (i.e., the modeled protein scored less than 80 %). All isotype 1 β-tubulins of all hookworm species mutated to contain the reported SNPs passed quality assurance checks (Supplementary Table 3). These results justify the use of only isotype 1 β-tubulins in the in silico docking experiments. In addition, the alignment of both coding nucleotide and amino acid sequences revealed that in all species and isotypes, the positions holding the SNPs related to benzimidazole resistance had very similar codon constitutions, resulting in similar sets of amino acids (Fig. 1).

The cladograms derived from the phylogenetic analysis of nucleotide coding (Fig. 2A) and amino acid sequences (Fig. 2B) revealed that hookworm β-tubulins clustered based on their respective isotypes. Isotypes 1 and 2 in all species were more evolutionarily related, and the same was true for isotypes 3 and 4. As expected, the β-tubulins of *N. americanus* were genetically distant from those of the three *Ancylostoma* species based on the cladogram from the nucleotide sequences. Conversely, based on the tree derived from the amino acid sequences, *A. duodenale* β-tubulin isotype 4 was genetically distant from those of *A. caninum*, *A. ceylanicum*, and *N. americanus*. Moreover, based on the same tree, isotypes 3 and 4 showed greater evolutionary divergence than isotypes 1 and 2. The pairwise structural alignment of the predicted hookworm β-tubulin proteins revealed that all structure predictions had high structural similarity to the defined β-tubulin crystal structure from PDB ID No. 1jff (Supplementary Fig. 1).

3.2. *BZ resistance mutations affect benzimidazole binding affinities*

The in silico docking analysis revealed varying binding affinities between different hookworm species and benzimidazole drugs (Fig. 3). Among the β-tubulin isotype 1 protein across all species, MBZ had the highest binding affinity, while ABZ had the lowest. Moreover, the binding affinities of the different benzimidazole ligands were lowest in *N. americanus*; the decrease was particularly marked for FBZ, OBZ, and ABZ reflecting the results of the phylogenetic analysis. Mutations of the β-tubulins through the introduction of the reported SNPs resulted in marked alterations in binding affinities. Overall, the Q134H and E198K mutations markedly decreased the binding affinities of the hookworm β-tubulins, regardless of the interacting benzimidazole drug. In addition, a similar but less reduction in binding affinity was observed when β-tubulins had the F200L mutation; the opposite was observed in proteins with the F200Y mutation. In contrast, the other mutations caused either slight increases or unchanged docking binding affinities.

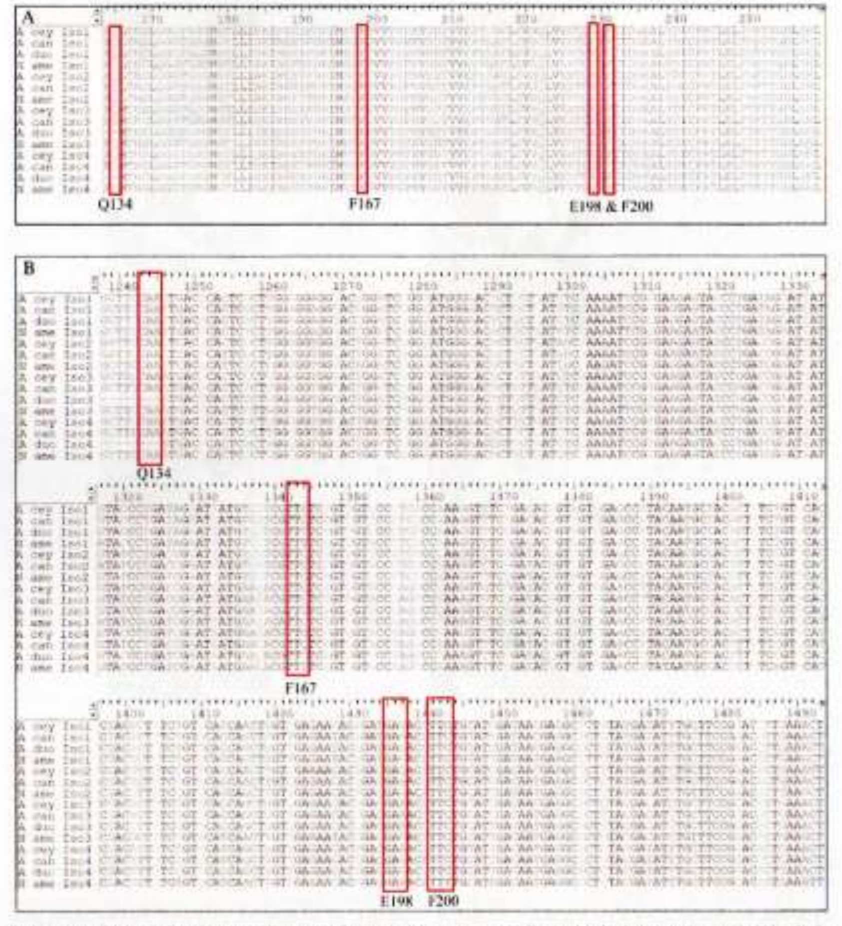

Fig. 1. Sequence alignment of the hookworm β-tubulin amino acids (A) and nucleotide sequences (B). The amino acid and codon positions carrying SNPs related to benzimidazole resistance are highlighted and labeled. Alignments revealed similar sequence profiles across different β-tubulin isotypes and hookworm species. Sequence alignment was performed using ClustalW Multiple Alignment in the BioEdit Sequence Alignment Editor version 7.2.5.

3.3. *BZ resistance mutations impact the binding positions and interacting residues*

In addition to the altered binding affinities, SNPs also affected the binding pose between the β-tubulin proteins and benzimidazole drugs (Fig. 4). The observed reduction in binding affinities of hookworm β-tubulins with E198K and Q134H was supported by alterations in the docking poses of the protein and benzimidazole ligands. The loss of key carbon-hydrogen bonds was observed when glutamine was swapped with histidine at position 134. However, this mutation resulted in the

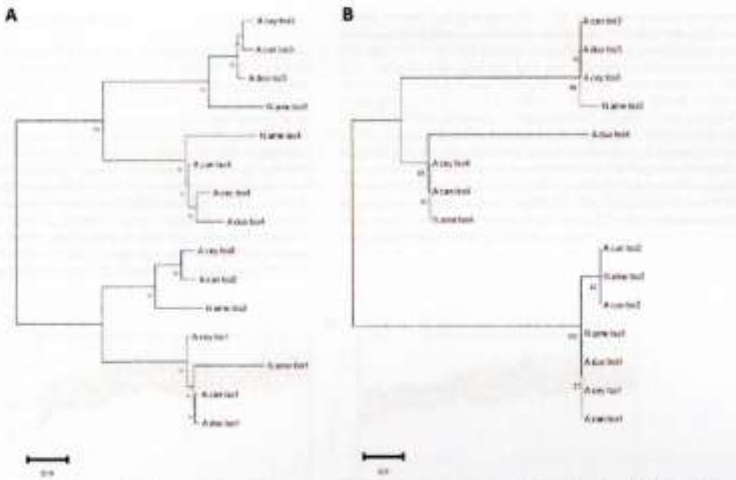

Fig. 2. Phylogenetic trees of β-tubulin nucleotide (A) and amino acid (B) sequences of *Ancylostoma caninum* (A can), *A. ceylanicum* (A cey), *A. duodenale* (A duo), and *Necator americanus* (N ame). All β-tubulin sequences were clustered according to their isotypes. Both cladograms were constructed using MEGA v. 11 (https://www.megasoftware.net/). Maximum likelihood phylogenies were constructed with K2 + G model at 1000 bootstraps for the nucleotide sequences and the JTT model at 1000 bootstraps for the amino acid sequences.

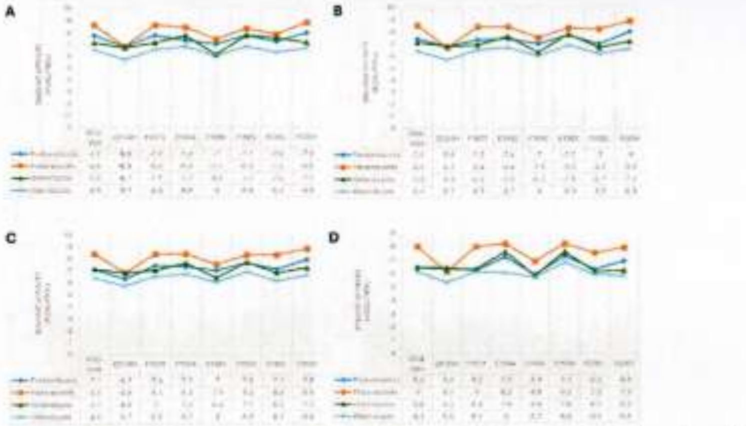

Fig. 3. Binding affinities generated by in silico docking experiments. A: *A. caninum*, B: *A. ceylanicum*, C: *A. duodenale*, D: *N. americanus*. The wild type and mutated (7 proteins with the SNPs) β-tubulin proteins were docked with Fenbendazole (blue diamond), Mebendazole (orange box), Oxfendazole (green triangle) and Albendazole (sky blue X), and the binding affinities of these interactions are shown above. In general, the Q134H and E198K mutations decreased the binding affinity of the β-tubulin-benzimidazole interactions.

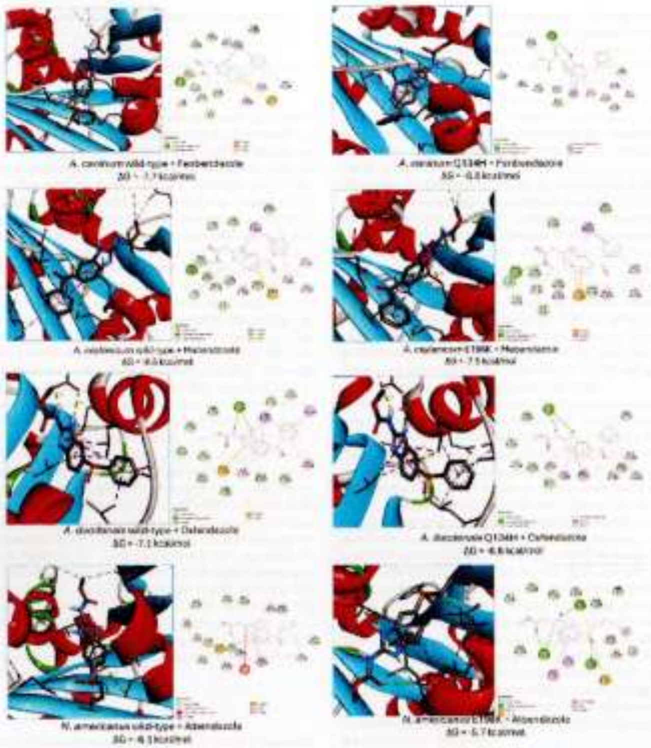
A. caninum wild-type + Fenbendazole
ΔG = -7.7 kcal/mol
A. caninum Q134H + Fenbendazole
ΔG = -6.8 kcal/mol
A. ceylanicum wild-type + Mebendazole
ΔG = -8.6 kcal/mol
A. ceylanicum E198K + Mebendazole
ΔG = -7.5 kcal/mol
A. duodenale wild-type + Oxfendazole
ΔG = -7.1 kcal/mol
A. duodenale Q134H + Oxfendazole
ΔG = -6.6 kcal/mol
N. americanus wild-type + Albendazole
ΔG = -8.1 kcal/mol
N. americanus E198K + Albendazole
ΔG = -5.7 kcal/mol

Fig. 4. 3D and 2D representations of interactions from in silico docking of hookworm β-tubulin isotype 1 wild-type and mutated proteins and benzimidazole drugs. Alterations to binding poses, such as shifting of the ligand-binding position, loss of significant bonds, or changes in bond types, were observed when 3D and 2D representations of interactions with mutated β-tubulins were inspected against that of wild-type proteins. These 2D and 3D interactions were viewed and captured using BIOVIA Discovery Studio Visualizer (https://discover.3ds.com/discovery-studio-visualizer-download).

same interaction as the wild-type counterpart. Taken together, these examples and the rest of the results show that resistance-associated mutations in the β-tubulin isotype 1 protein of different hookworm species caused alterations in binding positions when compared to ligand interactions with wild-type proteins. Moreover, changes in the types of bonds formed were also induced by the mutations. In particular, replacing glutamic acid with lysine at position 198 resulted in the emergence of Pi-cation bonds instead of hydrogen bonds. Lastly, shifts in binding positions due to mutations also affect interactions with other amino acids. For *N. americanus* β-tubulin with the E198K mutation, the mutant lysine formed conventional hydrogen bonds that reduced the drug interaction of phenylalanine at position 200 to noncovalent pi bonds. These changes in binding positions may result in the emergence of unfavorable interactions, changes in the type of bonds, and loss of key interactions thereby similarly affecting binding affinities.

The alteration of binding positions and interactions also resulted in several changes in the amino acids that interacted with benzimidazole drugs (Supplementary Tables 4 and 5). Leucine at position 253, methionine at 257, and alanine at position 314 were the most prevalent amino acid residues observed in all docking experiments with both wild-type and mutated proteins. Both wild-type (i.e., Glutamic Acid) and mutated (i.e., Alanine, Lysine, and Valine) amino acids at positions 198 and 134 were also observed to interact frequently with the drugs. These results revealed that among the positions that contain resistance-associated mutations, only those at positions 198 and 134 showed a significant number of interactions with ABZ, FBZ, MBZ, and OBZ collectively. These observations further strengthen the observed deviations in docking binding affinities, implicating mutations at positions 198 and 134 as markers of benzimidazole resistance.

3.4. *Molecular dynamics simulations*

To further investigate the results of the molecular docking experiments, hookworm β-tubulin with the E198K and Q134H mutations complexed with FBZ and ABZ were analyzed using molecular dynamics simulations. Overall, the hookworm β-tubulin, both wild-type and mutated, and benzimidazole ligand complexes stabilized around 20 to 30 ns and continued beyond the equilibration point at 80 ns (Fig. 5A and B). The wildtype-, E198K- and Q134H-ABZ complexes have comparable RMSD values post-equilibration point (wildtype = 2.42 ± 0.06 Å, E198K = 2.26 ± 0.09 Å, Q134H = 2.51 ± 0.08 Å). A similar trend was observed when the β-tubulins were docked with FBZ (wildtype = 2.21 ± 0.09 Å, E198K = 2.52 ± 0.07 Å, Q134H = 2.26 ± 0.08 Å) over the 100 ns simulation. The RMSF values showed that the complexes shared a similar profile of flexible regions, regardless of the mutations. Among the β-tubulins complexed with ABZ, flexible regions included residues 37–45, 94–99, and 274–284 (Fig. 5C). For those docked with FBZ, increased flexibility was observed at residues 172–180, 244–253, and 275–282 (Fig. 5D). The Solvent-Accessible Surface Area (SASA) plots (Fig. 5E and F) revealed that both the wild-type and mutant proteins maintained similar levels of solvent exposure when bound to ABZ (wildtype = 1.87 ± 0.02 Å^2, E198K = 1.78 ± 0.02 Å^2, Q134H = 1.84 ± 0.02 Å^2) and FBZ (wildtype = 1.81 ± 0.03 Å^2, E198K = 1.81 ± 0.02 Å^2, Q134H = 1.82 ± 0.04 Å^2) after the equilibration point was reached. This suggests that the overall structural conformations of the proteins were not significantly altered by the mutations or the binding of the BZ ligand. The mutations and ligand binding did not induce substantial changes in the protein's overall shape or flexibility. The RMSF, RMSD, and SASA plots reveal that the wild-type and mutated β-tubulin-benzimidazole complexes share similar dynamic behaviors.

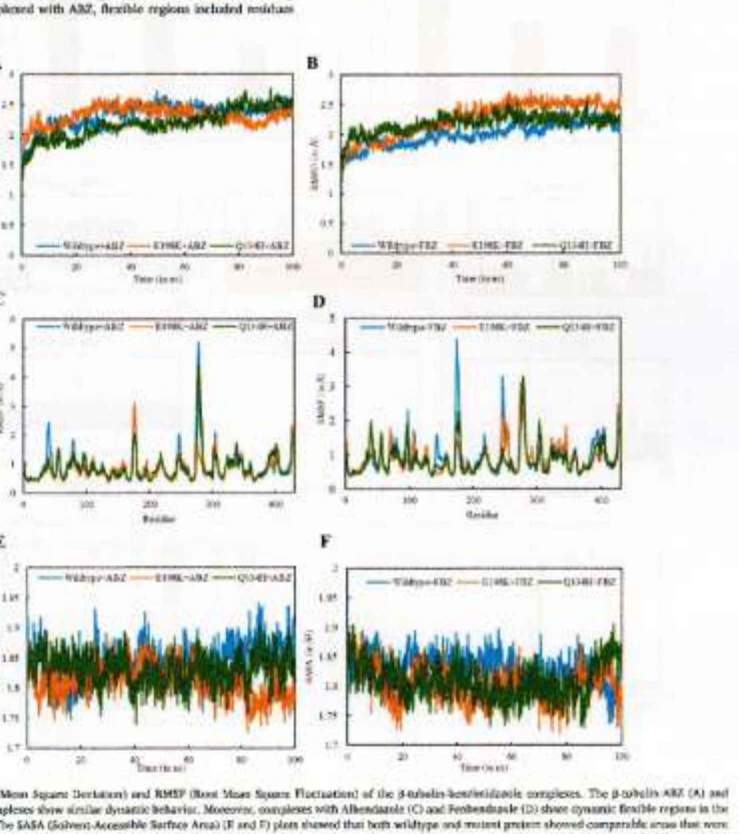

Fig. 5. RMSD (Root Mean Square Deviation) and RMSF (Root Mean Square Fluctuation) of the β-tubulin-benzimidazole complexes. The β-tubulin-ABZ (A) and β-tubulin-FBZ (B) complexes show similar dynamic behavior. Moreover, complexes with Albendazole (C) and Fenbendazole (D) share dynamic flexible regions in the β-tubulin backbone. The SASA (Solvent-Accessible Surface Area) (E and F) plots showed that both wildtype and mutant protein showed comparable areas that were solvent-exposed across the simulation time indicating similar structural conformations regardless of the mutation and BZ ligand used. The RMSF, RMSD, and SASA plots reveal that the wild-type and mutated β-tubulin-benzimidazole complexes share similar dynamic behaviors.

3.5. *E198K and Q134H reduce the binding efficiency of β-tubulin-BZ complex*

The binding free energy is used to estimate the energetic differences between the bound and unbound states of the protein–ligand complexes, which can be used to quantify the stability and favorability of binding. In this study, the MM-PBSA and MM-GBSA methods were used to estimate the binding free energy between the β-tubulins and benzimidazole ligands (Supplementary Tables 6 and 7). The results showed that complexes with E198K and Q134H mutations in β-tubulins showed reduced binding energies compared to those with the wild-type (Fig. 6A). Regardless of the benzimidazole ligand and binding free energy determination method, the Q134H mutation caused significant decreases in binding energies, thereby causing weaker interactions. Hydrogen bonds between the β-tubulin receptors, both wild-type and mutated, and the benzimidazole drug ligands were determined during molecular dynamics simulations (Fig. 6B). Overall, more H-bond contacts were formed between the wild-type and ABZ and FBZ. A decrease in the number of H-bonds was observed when the β-tubulin containing the E198K mutation interacted with ABZ. This decrease was more pronounced when the β-tubulin had the Q134H mutation. Using FBZ as a ligand, marked reductions in the number of H-bonds were observed when E198K and Q134H mutations were present in the β-tubulin receptor. These results indicate that the E198K and Q134H mutations cause weaker binding interactions with benzimidazole drugs indicating their role in benzimidazole resistance among hookworms. The per-residue energy decomposition analysis (Fig. 6C) indicated that amino acids at positions 134, 167, 198, and 200, which were mutation sites, were involved in the binding interaction. Moreover, the strongest interactions were observed in residues 236–248, 251–258, 315–318, 350, and 368. The latter interacting residues correspond to the positions that were frequently interacting with the BZ ligands in the docking experiments. The results depicted in Fig. 6 showed that the reduction in binding free energies among the complexes with the β-tubulin mutations is caused by a diminished number of hydrogen bonds between the protein and the ligand. Molecular dynamics simulations proved that the E198K and Q134H mutations in hookworm β-tubulins confer benzimidazole resistance. Likewise, other interacting amino acid positions may also harbor mutations that tend to undermine the binding efficiency of the β-tubulin-BZ drug complex.

4. Discussion

Benzimidazole resistance is an emerging threat that can potentially undermine efforts to control and eliminate STH, including hookworms. Despite concerns raised recently, the exact mechanism of resistance among these parasites remains unclear. In this study, we used *in silico* docking with open-source platforms to study the potential consequences of SNPs in the β-tubulin isotype 1 gene of several hookworm species. Phylogenetic analysis revealed that the hookworm β-tubulin nucleotide and amino acid sequences clustered within their specific isotypes. The in silico docking analysis revealed that amino acid substitutions brought about by SNPs in 4 codon locations of the hookworm β-tubulin isotype 1 protein resulted in altered binding affinities and binding positions. Among these mutations, E198K and Q134H may be important in conferring benzimidazole resistance. Molecular dynamics analysis revealed that the inclusion of these mutations did not significantly alter the binding dynamics of the β-tubulin-benzimidazole complex. However, the E198K and Q134H mutations caused marked reductions in the H-bonds formed, which resulted in significant reductions in the binding free energy. These results indicated that these mutations confer benzimidazole resistance.

The clustering of various β-tubulin genes based on their isotypes observed in this study was similar to those reported previously. Reconstruction of Ascaridomorpha phylogenies based on β-tubulin genes showed that ascarid β-tubulins were clearly separated into seven known isotypes (isotypes A–G). This clustering is believed to have arisen early in the evolution of various roundworm species, such as *Ascaris lumbricoides*, *Anisakis simplex*, and *Parascaris equorum*, within the *Ascaridomorpha* infraorder. Our results, which indicated that hookworm β-tubulin isotypes 1 and 2 are monophyletic but distant from isotypes 3 and 4, which are also closely related, are also echoed by the results of Jones et al. They observed a similar clustering of strongyle β-tubulins and revealed a similar phylogenetic profile for other important parasitic nematodes, such as *Haemonchus contortus* and *Trichuris trichiura*. The results of our phylogenetic analysis and the aforementioned results from other published studies may have implications for benzimidazole resistance. Since benzimidazole resistance is often linked to SNPs in the β-tubulin isotype 1 gene of nematodes, its close monophyletic relationship with isotype 2 suggests that SNPs occurring in the latter may also confer or assist in the emergence of benzimidazole resistance. This was previously observed by Doyle et al. when they studied resistant *H. contortus*. They observed that increasing Thiabendazole EC$_{50}$ concentrations correlated well with the E198V variant frequency in β-tubulin isotype 2 genes. Moreover, they noted that this mutation in isotype 2 may mediate a higher level of resistance than those conferred by SNPs in isotype 1 (e.g., F200Y). However, it is important to note that there are differences in expression levels of different nematode β-tubulin isotypes based on life stage and cell type.

In silico docking of the wild-type and mutated β-tubulin isotype 1 proteins with various benzimidazole drug ligands in this study revealed that resistance-associated mutations alter binding affinities and positions. Our results showed that E198K and Q134H mutations caused a marked decrease in binding affinity, regardless of the benzimidazole ligand used. The Q134H mutation was recently found to be widespread among *A. caninum* in the United States. The results of our study suggest that the same mutation may also confer resistance in *A. ceylanicum*, *A. duodenale*, and *N. americanus*. The E198K mutation, which caused a marked reduction in binding affinities in all hookworm species studied herein, has only been reported recently in *A. caninum* from Australia. The same SNP was recently reported in *H. contortus* infecting goats from several districts of Uganda. Fungal pathogens of crops in China that were resistant to benzimidazole derivatives also carry these mutations. Currently, there is no information regarding their effect on resistance among parasitic nematodes of public health concern. Hence, for the first time, we provide evidence of marked binding affinity reduction caused by the E198K mutation in hookworm β-tubulin isotype 1 proteins with several benzimidazole drugs. Moreover, we also showed that the Q134H mutation may confer resistance in many hookworm species, as proven previously in *A. caninum*.

In the present study, we observed that mutations in the β-tubulin isotype 1 protein caused a shift in the ligand-binding position, modifications in bonds formed, and loss of important interactions. These alterations have been reported in previous in silico docking studies of other nematode species. Despite the modifications in binding positions in mutated proteins, the profile of interacting amino acids with the benzimidazole drug ligands in this study remained similar to those of the wild-type proteins. Numerous drug interactions with leucine at position 253, methionine at position 257, and alanine at position 314 have also been noted in previous prediction and molecular dynamics studies. Likewise, the frequent interactions with amino acids at 198, both in wild-type and mutated β-tubulins, observed in this study were also confirmed by prior research, thereby implicating that SNPs occurring at this position may be crucial in inducing resistance. Our results, together with those previously reported, indicate that amino acid substitutions at positions 198 and 134 caused by resistance-related SNPs induce modifications in bond formation and other ligand–protein interactions that play roles in benzimidazole resistance in helminths of public and veterinary health significance. This should be explored further in future laboratory and field studies.

The molecular dynamics simulations revealed that hookworm β-tubulins, both wild-type and mutated, bound with benzimidazole ligands,

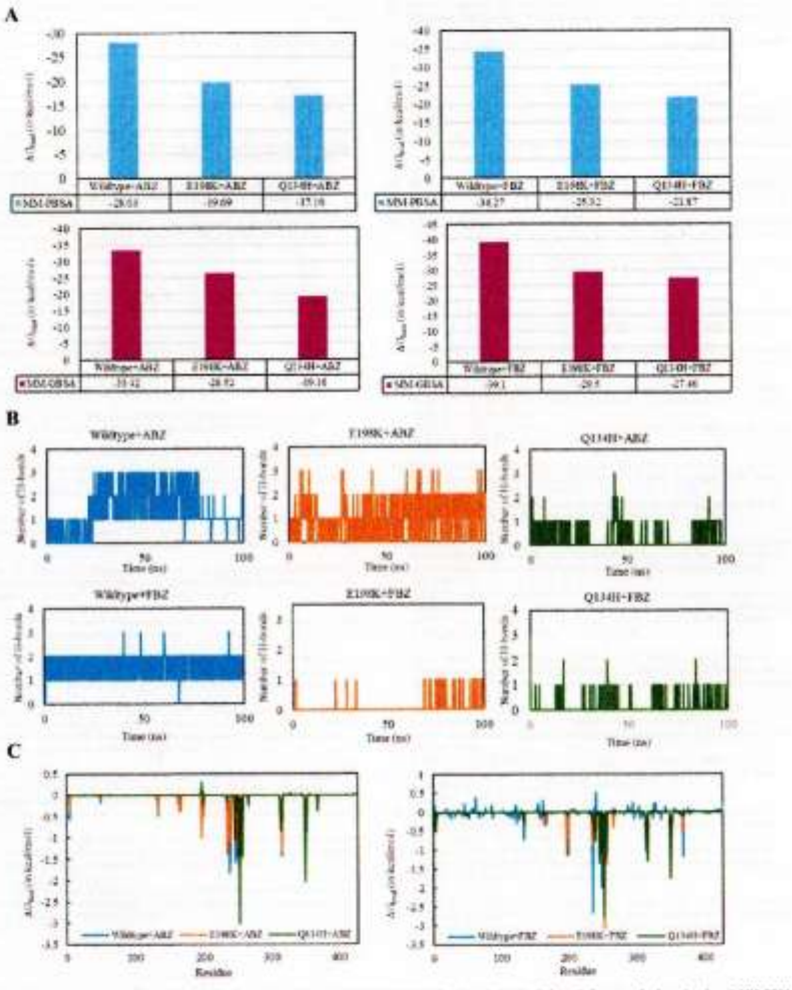

Fig. 6. The interaction of the β-tubulin-BZ complexes. A: The result of the MM-PBSA (Molecular Mechanics-Poisson-Boltzmann Surface Area) and MM-GBSA (Molecular Mechanics-Generalized Born Surface Area) binding free energies of the β-tubulin-BZ complexes from the MD simulations. Overall, complexes with mutated β-tubulins caused a reduction in the binding energies, resulting in weaker interactions. B: The hydrogen bond contact plots during the 100 ns MD simulation. The E198K and Q134H mutations in hookworm β-tubulins caused reductions in the number of hydrogen bonds formed. C: The per-residue energy decomposition of the binding free energy plots. It showed that the interacting amino acids involved those in residues where mutations occurred (positions 134, 167, 198, and 200). Also, the strongest interactions occurred in residues 236 to 248, 251 to 258, 315 to 318, 350, and 356. Molecular dynamics (MD) simulation analysis revealed that BZ resistance mutations reduce the binding free energy of the BZ-β-tubulin interaction. This reduction is caused by a decrease in hydrogen bond contacts. Other residues within the binding pocket that interact with the drug may also be targets of resistance mutations.

share similar behavior in terms of atomic displacements and protein backbone flexibility. This indicated that resistance-associated mutations significantly altered the simulated behavior of the β-tubulin-benzimidazole ligand complex. This observation was also noted by Jones et al.[illegible] in dynamics simulations of *A. lumbricoides* β-tubulin with the canonical mutations (i.e., F167Y, E198A, and F200Y) bound with ABZ. Despite these dynamic similarities, analysis of the H-bonds formed between the receptor and the benzimidazole ligand provided insights into how the E198K and Q134H mutations cause resistance. The two mutations decreased the number of H-bonds between the complexes. Benzimidazole resistance-associated mutations causing reduction in interactions have also been reported in *in silico* studies that assessed *H. contortus* and *Giargia lamblia* β-tubulins, where residues cause intra-protein interactions instead of the ligand.[illegible] The marked differences in absolute binding free energy between complexes with wild-type and mutated β-tubulins also confirm the effects of lowering the number of H-bonds observed in this study. The reduced number of hydrogen bonds resulting in diminished binding free energy is due to the loss of attraction forces and structural stability, as observed in the analysis of different drug candidates docked with the hACE2-S protein.[illegible] Our molecular dynamics results provide an *in silico* background of how these mutations cause phenotypic resistance. Using *C. elegans* that were edited to have the Q134H mutation, which is widespread among *A. caninum* from the US, phenotypic resistance as measured by regressed animal length was proven after exposure to 30 μM of ABZ.[illegible] Moreover, phenotypic resistance was also observed in *C. elegans* edited to contain E198K mutation when challenged with 30 μM of ABZ and FBZ.[illegible] The fitness costs and other untoward effects of these mutations on tubulin dimerization have also been documented previously.[illegible] Altogether, the molecular dynamics analysis of the β-tubulin-benzimidazole ligand complexes proved that the E198K and Q134H confer benzimidazole resistance by negatively affecting binding efficiency by reducing the number of interactions which drives down the absolute binding free energy.

5. Conclusion

Our results provided several insights into benzimidazole resistance in hookworms. Our phylogenetic analysis revealed that the hookworm β-tubulins clustered with respect to their isotypes. Hence, resistance-causing mutations in a particular β-tubulin isotype in one species may potentially cause the same as in other closely related species. Moreover, our *in silico* docking studies found that various amino acid substitutions brought about by resistance-related SNPs caused alterations in binding affinities and positions. In particular, the E198K and Q134H mutations caused marked reductions in binding affinities regardless of the hookworm species and interacting benzimidazole ligand. These mutations also resulted in shifts in the ligand-binding position, loss of interactions, and/or changes in the types of bonds formed. Molecular dynamics (MD) simulation analysis revealed that BZ resistance mutations reduce the binding free energy of the BZ-β-tubulin interaction. This reduction is caused by a decrease in hydrogen bond contacts. Additionally, other residues within the binding pocket that interact with the drug may also be targets of resistance mutations. Our results support the notion that these SNPs occurring in the β-tubulin isotype 1 gene are implicated in causing benzimidazole resistance in hookworms.

6. Ethical standards

This research does not require ethical approval for it did not use animal or human subjects.

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CRediT authorship contribution statement

Jan Clyden B. Tenorio: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Muhammad Fikri Heikal:** Writing – review & editing, Formal analysis. **Alok Kafle:** Writing – review & editing, Formal analysis. **Mark Andrian B. Macalalad:** Writing – review & editing, Formal analysis. **Fredmoore L. Orosco:** Writing – review & editing, Formal analysis. **Prasert Saichua:** Writing – review & editing, Supervision, Conceptualization. **Sutas Suttiprapa:** Writing – review & editing, Supervision, Conceptualization.

7. Data availability

Data generated in this study can be made available upon request from the corresponding author.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgeb.2025.100472.

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Molecular docking and dynamics as a tool to study benzimidazole resistance in helminths: A scoping review

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Systematic Review

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Abstract

Background: Benzimidazole (BZ) resistance remains an emerging grave concern in helminths of public and veterinary health concerns. Resistance against BZ drugs is due to mutations that change the amino acid comprising the β-tubulin protein, which negatively affects its interactions with BZ drug molecules. Several in silico modeling studies have been published to decipher the precise mechanism of BZ resistance, but inconsistencies on the resistance consequence mutations confer and the effect of different BZ ligands have led to further confusion regarding the exact mechanism of resistance. Hence, this scoping review was done to unravel the mechanism of BZ resistance based on published research on molecular docking and dynamics.

Methods: A scoping review was conducted in ScienceDirect, MEDLINE via PubMed and Scopus using the search term "Benzimidazole Resistance AND Beta Tubulin AND Molecular Docking". A total of 37 hits were recovered and from these 6 were included after selection, inclusion, and risk of bias assessment.

Results: The six research papers included in this review studied several helminth species: *Haemonchus contortus, Trichinella spiralis, Ancylostoma duodenale, Ancylostoma caninum, Ancylostoma ceylanicum, Necator americanus, Trichuris trichiura, Trichuris suis, Anisakis simplex, Ascaris suum, Ascaridia galli, Parascaris equorum, Toxocara canis,* and *Fasciola hepatica*. The benzimidazole resistance associated mutations studied included F167Y (TTC, TTT → TAC, TAT), E198A (GAG, GAA → GCG, GCA), and F200Y (TTC, TTT → TAC, TAT). The results show that the E198A can markedly reduce the binding affinity of BZ ligand-β-tubulin interactions. The F167Y and F200Y also showed a similar effect that could vary based on the helminth species. The F200Y mutation can alter the conformation of the β-tubulin active site, negatively affecting drug binding.

Conclusion: While the impact of these mutations can vary depending on the specific helminth species and the BZ drug involved, the overall findings highlight the importance of targeting these residues for the development of novel anthelmintic strategies to address emerging drug resistance.

Introduction

Benzimidazole resistance continues to be an emerging grave concern in helminths of public and veterinary health concern. Benzimidazoles (BZ) are disruptors of microtubule polymerization by binding in the β subunit of the tubulin dimer (Whittaker et al., 2017). The binding of BZ drug molecules in the β-tubulin prevents the polymerization of tubulin subunits into microtubules, disrupting the formation of the cytoskeleton (Furtado et al., 2016). Benzimidazole drugs, like albendazole, mebendazole, and fenbendazole, are used for clinical treatment and preventive chemotherapy in humans and animals (TroCCAP, 2019; World Health Organization, 2011). Resistance against this drug class became a huge concern in the veterinary field as widespread reports of resistant livestock helminths, like *Haemonchus contortus, Teladorsagia circumcincta,* and *Trichostrongylus colubriformis* (Von Samson-Himmelstjerna et al., 2007). Among helminths of public health concern, soil-transmitted helminth infections that do not respond to conventional BZ treatment have been reported in several areas globally (Ng'etich et al., 2023; Schwenkenbecher et al., 2007). Recently, the emergence of BZ-resistant helminth infections among pets (e.g. canine hookworm) in the United States and Canada raises the zoonotic threat these treatment-irresponsive isolates pose (Jimenez Castro et al., 2021; Tenorio et al., 2024; Venkatesan et al., 2023).

The resistance against BZ drugs is due to mutations that change the amino acid comprising the β-tubulin protein expressed by the helminth (Furtado et al., 2016). These amino acid substitutions are brought about by Single Nucleotide Polymorphisms (SNPs) (Von Samson-Himmelstjerna et al., 2007). These mutations include those that occur in amino acid positions 167 (Phenylalanine, F, TTC, TTT → Tyrosine, Y, TAC, TAT), 198 (Glutamic acid, E, GAG, GAA → Alanine, A, GCG, GCA) and 200 (Phenylalanine, F, TTC, TTT → Tyrosine, Y, TAC, TAT) (Furtado et al., 2016; Tenorio, 2023; Tenorio et al., 2024). These mutations alter the amino acid constitution of the expressed protein negatively affecting the binding of BZ drug molecules structurally or biochemically (Lacey and Gill, 1994). These mutations have been reported in a variety of worms that threaten humans and animals globally (Ng'etich et al., 2023). The atomic underpinnings of BZ resistance in helminths remain understudied, hence its precise mechanism has been put into question (Von Samson-Himmelstjerna et al., 2007).

Several *in silico* modeling studies utilizing advances in computational biology have been undertaken to decipher the precise mechanism of BZ resistance. These research have included modeling the wild-type protein's interaction with BZ drug ligands (Aguayo-Ortiz et al., 2013) and predicting the effects of BZ resistance mutations (Jones et al., 2022). However, inconsistency regarding the resistance effects each mutation confers and the consequences of utilizing numerous BZ derivatives as ligands has led to further confusion regarding the exact mechanism of resistance. Hence, this scoping review was done to unravel the mechanism of BZ resistance based on published research that used molecular docking and dynamics. The results show that the E198A can drive down the binding affinity of BZ ligand-β-tubulin interactions regardless of the species and drug used. The F167Y and F200Y also showed a similar effect that could vary based on the helminth species.

Materials and Methods

Research Questions

The scoping review was done based on the guidelines reported by the PRISMA-ScR (PRISMA Extension for Scoping Reviews) (Tricco et al., 2018) (https://www.prisma-statement.org/scoping). Based on published molecular docking and dynamics studies, this research aims to determine the mechanism of benzimidazole. Specifically, this research answers the following questions:

1. What are the *in silico* underpinnings of benzimidazole resistance based on molecular docking and dynamics study?

2. What are the consequences of these mutations on the measurement of binding efficiency of the β-tubulin-benzimidazole drug complex?

3. What are the consequences of these mutations on the interactions between the β-tubulin and the benzimidazole drug ligand/s?

Search Strategy

A systematic search was done in three research databases. Scopus (https://www.scopus.com/search), ScienceDirect (https://www.sciencedirect.com/), and MEDLINE via PubMed (https://pubmed.ncbi.nlm.nih.gov/) were searched using the search term "Benzimidazole Resistance AND Beta Tubulin AND Molecular Docking." The literature search was done on 17 September 2024. The .ris file of the search results was downloaded.

Study Selection, Strategy, and Eligibility

Using the Mendeley citation manager (https://www.mendeley.com), the .ris files were uploaded and utilized for the selection and eligibility assessment. First, duplicates and records with no titles and abstracts (e.g., indexes) were removed. Second, an initial evaluation based on the title and abstract was done. Full-length articles of the studies were accessed for further eligibility appraisal. Figure 1 summarizes the systematic literature search, selection, and eligibility evaluation done.

A study was considered eligible for selection if it fulfilled any of the following inclusion criteria:

1. Studies that utilized molecular docking in assessing the *in silico* effects of the BZ resistance mutations;

2. Studies that utilized molecular docking in assessing the *in silico* effects of the BZ resistance mutations.

From the included studies, papers that did not meet the following criteria were excluded:

1. Studies that did not report docking scoring functions (i.e., binding affinities) and/or binding free energies (e.g., MM-PBSA or MM-GBSA);

2. Studies that did not report the effects of BZ resistance mutations on the interaction between β-tubulins and benzimidazole drug ligand/s;

3. Studies done using non-helminth β-tubulins as macromolecules;

4. Studies that did not use commercially available benzimidazole drugs as ligands; and

5. Studies that utilized newly designed and synthesized benzimidazole derivatives.

6. Research not in the English language.

Risk of Bias Assessment

Due to the *in silico* and computational nature of the studies being reviewed, traditional checklists for laboratory experiments are not well-suited as the method of bias assessment. Hence, we developed a simple checklist that is based on the quality of the modeled β-tubulin macromolecule, ligand preparation, docking software, simulation quality, and data analysis utilized. The tool is in the form of a 13-item close-ended questionnaire (Supplementary Table 1). All included studies were evaluated using this tool.

Data Acquisition and Synthesis

The author's name, year of publication, software used in molecular docking and/or system used in molecular dynamics, helminth species of the β-tubulins used as the macromolecule, benzimidazole ligand used, BZ resistance mutation evaluated, docking scoring functions and/or binding free energies of the complex, description of the changes in interactions, and relative resistance-associated effects were the data acquired from the selected studies. Simple descriptive statistics, like counts and frequencies, were used to describe and synthesize the results of this scoping review.

Results

Characteristics of the studies included

A total of 37 hits were found in the three databases searched (Fig. 1). Sixteen of these were removed due to duplication. The full text of one article was not accessed. After the eligibility screening, two were removed for not reporting docking scoring function, eight were removed for not using helminth β-tubulins, three were dropped for using newly designed benzimidazole ligands, and one did not report the effects of the docked complexes. In total, six research papers were included in this scoping review. The six research papers included in this review studied several helminth species: *Haemonchus contortus, Trichinella spiralis, Ancylostoma duodenale, Ancylostoma caninum, Ancylostoma ceylanicum, Necator americanus, Trichuris trichiura, Trichuris suis, Anisakis simplex, Ascaris suum, Ascaridia galli, Parascaris equorum, Toxocara canis* and *Fasciola hepatica*. The benzimidazole resistance-associated mutations studied included F167Y (TTC, TTT → TAC, TAT), E198A (GAG, GAA → GCG, GCA), and F200Y (TTC, TTT → TAC, TAT).

In silico effects of BZ resistance-associated mutations

Table 1 summarizes computational studies investigating the molecular mechanisms underlying benzimidazole (BZ) drug resistance in various helminth species. The results highlight the crucial role of specific amino acid residues (e.g., F167, E198, F200) in BZ binding and the potential mechanisms through which mutations in these residues can confer resistance. Mutations in specific amino acid residues (F167, E198, F200) within β-tubulin proteins are frequently associated with BZ drug resistance. These mutations can disrupt the conformation of the β-tubulin active site, destabilize BZ binding, and reduce drug efficacy. Moreover, mutations, particularly at position 198, can lead to the loss of hydrogen bonding interactions between BZ drugs and β-tubulin, contributing to resistance. Further, mutations at positions 167 and 200 may interfere with the opening of the binding site or the internalization of BZ ligands.

The impact of mutations on resistance can vary across different helminth species. For example, mutations in F167Y may have a greater impact on strongyle parasites compared to other soil-transmitted helminths. Overall, the results suggest that BZ resistance is primarily attributed to alterations in the β-tubulin binding site, hindering the effective interaction between BZ drugs and their target protein.

STUDY CODE	AUTHORS	YEAR PUBLISHED	MOLECULAR DOCKING SOFTWARE	MOLECULAR DYNAMICS SYSTEM	HELMINTH SPECIES	β-TUBULIN EVALUATED	BZ LIGAND USED	DOCKING SCORING FUNCTION(Kcal/mol)	BINDI FREE ENERG (Kcal/
					Trichuris suis	Wild-type	Albendazole	-8.53	
S5	Olivares-Ferretti et al. (2023)	2023	AutoDock Vina program in PyRx software	N/A	*Fasciola hepatica*	Wild-type isotype 1	Triclabendazole	-6.87	N/A
						Wild-type isotype 2	Triclabendazole	-6.40	
						Wild-type isotype 3	Triclabendazole	-6.38	
						Wild-type isotype 4	Triclabendazole	-6.33	
						Wild-type isotype 5	Triclabendazole	-6.17	
						Wild-type isotype 6	Triclabendazole	-6.48	
S6	Yeshiwa et al. (2024)	2024	AutoDock Tools in MGL tools 1.5.6	N/A	*Haemonchus contortus*	Wild-type	Albendazole	-8.51	N/A

Effects of Mutations on Docking Scoring Function and Binding Free Energy

Based on the results of the included studies, the BZ resistance mutations often lead to a decrease in the docking scoring function and binding free energy, indicating a weaker interaction between the BZ drug and the mutated β-tubulin protein. However, the magnitude of the effect can vary depending on the specific mutation and the BZ drug involved. While F167Y mutations can sometimes decrease binding affinity, their effect is often less pronounced compared to mutations at other residues. Also, the impact of F167Y may vary across different helminth species. Meanwhile, the E198A mutation consistently leads to a marked decrease in binding affinity, suggesting a crucial role of this residue in BZ binding. The E198A mutation likely disrupts hydrogen bonding interactions between the BZ drug and β-tubulin. The F200Y mutations generally result in a moderate decrease in binding affinity. This mutation may alter the conformation of the β-tubulin active site, affecting drug binding. The combined effects of multiple mutations can further reduce binding affinity and enhance resistance. Likewise, the impact of mutations may vary depending on the specific BZ drug being considered. Overall, the results suggest that mutations in these key residues can disrupt the structural and electrostatic interactions between BZ drugs and β-tubulin, leading to reduced binding affinity and ultimately, drug resistance.

Discussion

This scoping review was conducted with the aim of unraveling the mechanism of BZ resistance based on published research on molecular docking and dynamics. MEDLINE via PubMed, Scopus, and Science Direct were searched. A total of six eligible studies were included, which encompassed research that utilized β-tubulins from several helminth species and numerous benzimidazole ligands. Of the BZ mutations studied, E198A showed that it can drive down the binding affinity of BZ ligand-β-tubulin interactions. The F167Y and F200Y also showed a similar effect that could vary based on the helminth species. The F200Y mutation can alter the conformation of the β-tubulin active site, negatively affecting drug binding.

The studies included in this scoping review assessed both wild type and mutated helminth β-tubulins. The three canonical BZ resistance mutations—F167Y, E198A, and F200Y—that are induced by SNPs were all evaluated (Furtado et al., 2016). These mutations have been reported in many helminth species of public and veterinary health concern from many parts of the world (Diawara et al., 2013; George et al., 2022; Jimenez Castro et al., 2021). Hence, their inclusion in computational studies was warranted. However, their actual contribution to conferring resistance has been put into question (Lacey and Gill, 1994). Also, several recent evidence of alternative resistance mechanisms, such as enzymatic biotransformation of drugs using UDP-glycosyltransferases and long non-coding RNA interference (Chen et al., 2024; Dimunová et al., 2022), have been reported. However, laboratory research using gene editing that encoded resistance-associated mutations in *Caenorhabditis elegans* showed their actual potential to confer resistance, as previously mentioned (Dilks et al., 2021, 2020). Particularly, varying mutations that have been reported in amino acid position 198 have been reported to make edited *C. elegans* significantly more resistant to benzimidazole treatment compared to their wild-type counterpart (Dilks et al., 2021). This result echoes the finding in this systematic review that the E198A mutation had a consistent negative effect on the binding of BZ drugs and helminth β-tubulins indicating their important role in conferring resistance.

This scoping review has several limitations. First, only a few accessible databases were searched, hence there might be other research not indexed in these databases that were missed. However, searching only known indexing databases, like MEDLINE via PubMed and Scopus, ensures that only quality research papers from reputable journals are included. Second, the *in silico* nature of the research targeted for this review may lead to conclusions of insufficient evidence for the conclusions that they present due to the lack of laboratory confirmation. However, advances in computational biology have assured that the predictions are of high quality and accuracy, particularly for molecular docking and dynamics studies (Santos et al., 2019; Singh et al., 2023).

Conclusion

The computational studies included in this scoping review provide valuable insights into the molecular mechanisms underlying BZ drug resistance in various helminth species. The results consistently demonstrate that mutations in specific amino acid residues within β-tubulin proteins, particularly F167, E198, and F200, play a critical role in conferring resistance. These mutations disrupt the structural and electrostatic interactions between BZ drugs and helminth β-tubulin, leading to reduced binding affinity and ultimately, drug resistance. While the impact of these mutations can vary depending on the specific helminth species and the BZ drug involved, the overall findings highlight the importance of targeting these residues for the development of novel anthelmintic strategies to address emerging drug resistance. Future studies could explore additional mutations, investigate the interactions between BZ drugs and other β-tubulin isoforms, or investigate the potential for combination therapies to overcome resistance.

Declarations

Supplementary Materials

Supplementary tables and figures are available at [DOI]

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Conflict of Interests

The author declares that there is no conflict of any financial or non-financial interests regarding the publication of this paper.

Ethics Approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Data Availability

Not applicable

References

Table 1
The key results of the studies included in this scoping review.

STUDY CODE	AUTHORS	YEAR PUBLISHED	MOLECULAR DOCKING SOFTWARE	MOLECULAR DYNAMICS SYSTEM	HELMINTH SPECIES	β-TUBULIN EVALUATED	BZ LIGAND USED	DOCKING SCORING FUNCTION(Kcal/mol)	BINDI FREE ENER (Kcal/
S1	Aguayo-Ortiz et al. (2013b)	2013	AutoDock 4.2	GROMACS 4.5.3	*Haemonchus contortus*	Wild-type	Albendazole	-8.10	-68.41
							Carbendazim	-6.98	-53.08
							Oxibendazole	-8.17	-67.27
							Parbendazole	-8.09	-65.59
							Luxabendazole	-9.46	-64.77
							Mebendazole	-9.43	-66.58
							Nocodazole	-9.36	-64.6
						F167Y	Albendazole	-8.07	N/A
							Carbendazim	-7.62	
							Oxibendazole	-8.09	
							Parbendazole	-8.15	
							Luxabendazole	-9.70	
							Mebendazole	-9.46	
							Nocodazole	-9.35	
						E198A	Albendazole	-7.03	
							Carbendazim	-6.37	
							Oxibendazole	-6.66	
							Parbendazole	-7.05	
							Luxabendazole	-8.82	
							Mebendazole	-8.76	
							Nocodazole	-7.99	
						F200Y	Albendazole	-8.32	
							Carbendazim	-7.19	
							Oxibendazole	-8.28	
							Parbendazole	-8.26	
							Luxabendazole	-9.62	
							Mebendazole	-9.48	
							Nocodazole	-9.35	
S2	Aguayo-Ortiz et al (2013a)	2013	AutoDock 4.2	GROMACS 4.5.3	*Trichinella spiralis*	Wild-type	Albendazole	-7.70	-53.67
							Carbendazim	-7.05	-21.74
							Oxibendazole	-7.41	-31.92
							Parbendazole	-7.93	-37.20
							Luxabendazole	-9.41	-44.71
							Mebendazole	-8.73	-45.04
							Nocodazole	-8.82	-34.34
S3	Jones et al (2022a)	2022	Autodock vina v1.1.2	Molecular Operating Environment (MOE) 2020.01	*Ascaris suum*	Wild-type	Albendazole	-8.46	N/A
						F167Y	Albendazole	-8.04	
						E198A	Albendazole	-8.53	
						F200Y	Albendazole	-8.16	
S4	Jones et al (2022b)	2022	Autodock vina v1.1.2	Molecular Operating Environment (MOE) version 2020.01	*Ancylostoma duodenale*	Wild-type	Albendazole	-8.55	N/A
						F167Y	Albendazole	-8.19	
						E198A	Albendazole	-8.14	
						F200Y	Albendazole	-7.72	
					Trichuris trichiura	Wild Type	Albendazole	-8.13	
						F167Y	Albendazole	-9.82	
						F198A	Albendazole	-7.78	
						F200Y	Albendazole	-10.48	
					Anisakis simplex	Wild type	Albendazole	-7.74	
					Ascaridia galli	Wild type	Albendazole	-8.25	
					Parascaris equorum	Wild type	Albendazole	-8.19	
					Toxocara canis	Wild-type	Albendazole	-8.82	
					Ancylostoma caninum	Wild-type	Albendazole	-7.64	
					Ancylostoma ceylanicum	Wild-type	Albendazole	-8.29	
					Necator americanus	Wild-type	Albendazole	-7.54	

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Figures

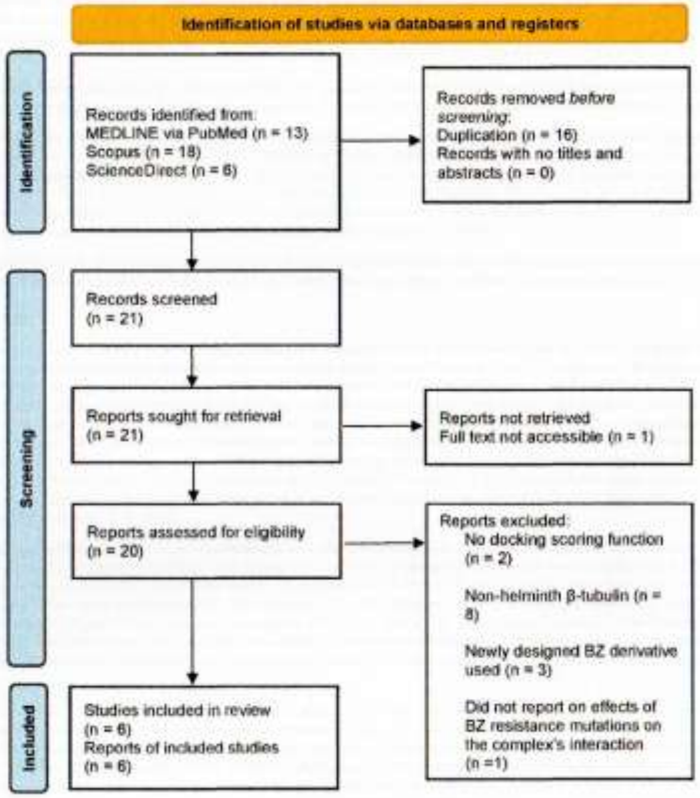

Figure 1

PRISMA-ScR (Preferred Reporting Items for Systematic Reviews and Meta-Analyses-Extension for Scoping Reviews) flowchart of the screening, selection, and eligibility selection in this study

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Letter to the Editor

Can resistant *Ancylostoma ceylanicum* arise, persist, and be transmitted between humans and animals?

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To the Editor

Ancylostoma ceylanicum is a zoonotic hookworm that is prevalent in Asia-Pacific region where it is predominant among humans and companion animals [1–3]. This hookworm is reported to affect nearly 30% to 50% of people in countries within Southeast Asia [2, 4, 5]. Among animals, particularly dogs and cats, prevalence rates are 35% but can be as high as more than 95% within the same region [5, 6]. Hence, it is now believed that this zoonotic hookworm is the number one cause of infections among animals and is second only to *Necator americanus* among humans [2, 7]. Transmission dynamics, as revealed by phylogenetic and population genetics analysis, show that there are three pathways that *A. ceylanicum* takes to proliferate between humans and animals (Figure 1). A larger group of the population circulates between humans and animals, while two smaller populations persist within both hosts [2, 6, 8]. The true zoonotic nature of this hookworm presents a serious One Health threat to human and animal populations cohabitating in endemic areas.

Control and elimination has primarily been through pharmaceutical interventions: Mass Drug Administration (MDA) with benzimidazoles in humans and routine veterinary deworming for pet animals [9–11] . Resistance to benzimidazoles, such as albendazole and fenbendazole, is caused by specific genetic variations known as Single Nucleotide Polymorphisms (SNPs) in the *β-tubulin isotype 1* gene [14]. These SNPs result in amino acid substitutions in the protein, altering its structure and hindering the drug's ability to bind to its target site [12]. The F167Y and Q134H mutations have been reported in the dog hookworm, *A. caninum*, in the United States [13, 14]. Moreover, the mutations have also been reported in the human hookworm, *Necator americanus* [15]. Among, *A. ceylanicum*, mutations have been found in laboratory specimens that have been induced to gain resistance against albendazole [16]. The documented emergence of drug resistance in other hookworm species, the aforementioned study of laboratory-induced resistance, and its potential for widespread distribution highlight the danger of resistance development in *A. ceylanicum*. Such resistance could significantly compromise our ability to control and eliminate this parasite, posing a serious risk to public health.

Can *A. ceylanicum* develop resistance to benzimidazole drugs, outcompete other parasite species after treatment, and be spread between animals and humans (Figure 2)? Given the potential transmission routes of zoonotic *A. ceylanicum* and the risk of drug resistance development from both animals and humans, repeated drug treatments could lead to the emergence of resistant hookworm populations that can spread between human and animal populations. If resistant *A. ceylanicum* populations become widespread in both humans and animals, it could have severe consequences for public health and veterinary medicine. One way to determine if drug-resistant *A. ceylanicum* populations have emerged is to analyze the genetic makeup of isolates from both humans and animals using the *β-tubulin isotype 1* gene as a marker. By analyzing the genetic makeup of these isolates, researchers can potentially trace the lineage and transmission routes of drug-resistant zoonotic hookworms. This analysis may reveal information about how genetic variations associated with benzimidazole resistance are transmitted between humans and animals. The occurrence of these mutations has been reported. While whole-genome sequencing could provide more comprehensive genetic information, it requires substantial resources and collaboration. In addition to genetic analysis, it will be crucial to monitor the emergence of drug resistance in both humans and animals following established guidelines from organizations like the World Health Organization and the World Association for the Advancement of Veterinary Parasitology [17–19].

In conclusion, the zoonotic nature of *A. ceylanicum* presents a significant One Health challenge due to its potential for transmission between humans and animals. The emergence of benzimidazole resistance poses a significant threat to public health and animal welfare. Population genetic analysis using the β-*tubulin isotype 1* gene can be employed to track the transmission dynamics of resistant hookworms and assess their potential for zoonotic circulation. By understanding the genetic structure and transmission pathways of these resistant populations, we can develop more effective control strategies and mitigate the risks associated with benzimidazole resistance. Effective control and preventative efforts against drug-resistant *A. ceylanicum* may include combination treatment, targeted treatment to the most at-risk populations, and monitoring resistance occurrence.

In the Asia-Pacific region, most *A. ceylanicum* infections are transmitted between humans and animals (Red), with fewer isolates occurring and persisting solely in humans (Green) or animals (Orange) [2, 4, 6, 8].

Figure 1. The transmission dynamics of *Ancylostoma ceylanicum* in the Asia-Pacific region.

Figure 2. Can resistant *Ancylostoma ceylanicum* isolates arise, persist, and be transmitted between humans and animals?

Drug resistance in *A. ceylanicum* can arise from both human and animal interventions, such as mass drug administration and veterinary deworming. Due to the parasite's zoonotic nature, resistant hookworms could potentially become dominant in either humans or animals and spread between species.

Conflict of Interest

The author has no conflict of interest to declare.

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FULL TEXT LINKS

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Anat Histol Embryol. 2024 Jan;53(1):e12970. doi: 10.1111/ahe.12970. Epub 2023 Sep 23.

Ultrastructural evidence of telocytes in the embryonic chick heart

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Abstract

The cardiac telocyte (TC) is a novel interstitial cell type with a unique ultrastructure and great potential in therapy. The present study examined its presence in the heart of chicken embryos ageing 7-15 days old (Hamburger-Hamilton [HH] stages 31-41) using transmission electron microscopy. TCs were identified across all stages in the atrial and ventricular myocardium, close to maturing cardiomyocytes, blood vessels and lymphatics. Early-stage TCs have immature features resembling mesenchymal cells. Late-stage TCs were distinct, possessing the cytoplasmic prolongations termed telopodes (Tps), which are very long and thin, usually 1-3 in number, and display a moniliform appearance and have an average thickness below 0.2 μm. TCs residing in the epicardium and endocardium were also detected. In the subepicardium near developing coronary vessels, they were localized in the cardiac stem cell niches, coexisting with cardiac stem cells and cardiomyocyte progenitors. Electron-dense structures and the release of extracellular vesicles were observed between embryonic TCs and surrounding structures, suggesting roles in intercellular communication, cardiomyocyte differentiation and maturation, angiogenesis, and stem cell nursing and guidance.

Keywords: chick; embryo; heart; morphogenesis; telocyte; telopode.

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HEAT STRESS: CAN ANIMALS BE STRESSED AND STILL BE HEALTHY?

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Abstract: In this era of rapid climate change, heat stress has emerged as a major problem for sheep farming. The quantity and quality of sheep production, as well as the welfare, are all shown to be negatively impacted by heat stress. Reduced cellular immune function in sheep exposed to high temperatures increases susceptibility to various diseases, although the underlying mechanisms are unclear. The imbalance between oxidants and antioxidants, known as oxidative stress, negatively impacts animal health due to heat stress-induced impairments in immune responses, increased production of reactive oxygen species, and/or a lack of antioxidants. Heat stress in livestock has negative effects on both the cell-mediated and humoral immune responses. However, the negative effects of heat stress on immune response in sheep can vary by breed. This review discusses how heat stress affects sheep's immunity. An overview of some molecular markers related to immunity, such as interleukin (ILs), Toll-like receptors (TLRs), and also the heat stress protein (HSPs) in sheep, is also discussed. Finally, recent research on the effects of antioxidant supplementation and other promising nutritional strategies for reducing heat stress and improving animal health is presented.

Introduction

Heat stress (HS) is one of the main problems faced by livestock amid the recent increasing intensity of global climate change. During the HS conditions, the livestock's body temperature rises, and they are unable to dissipate enough heat to maintain thermal equilibrium, leading to decreased productivity, reduced reproductive performance, weakened immunity, and increased mortality in some cases, as illustrated in Figure 1.
In terms of animal health, the increased risk of disease occurs due to weakened immunity, as during HS, the animal elicits several thermoregulatory activities, including behavioral, physiological, neuroendocrine, and cellular responses to maintain homeostasis and survival, during these, immune responses in the animal tend to be suppressed (Figure 2). HS can be mitigated by using exogenous antioxidants and salt supplementation in the diet, as presented in Table 1.

Discussions

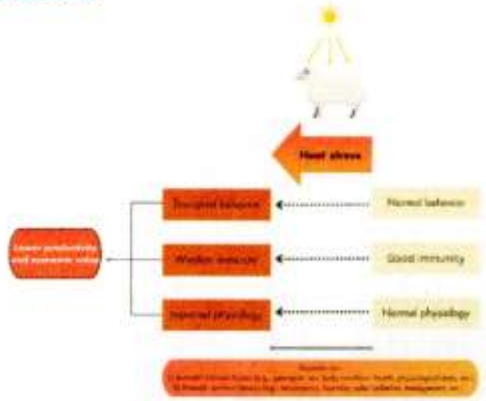

Figure 1. Heat stress deleterious impact to livestock through various ways

Table 1. Nutritional intervention in sheep that successfully reduce heat stress

Breed	Supplementation (per kg feed)	Ref
Malpura ewes	20 gr mineral and antioxidant mixture (164.0 mg zinc sulphate, 0.95 mg cobalt sulphate, 1.2 g chromium acetate, 0.1 mg selenium chloride and 40.0 mg vitamin E)	[1]
Merino × Poll Dorset crossbred ewes	100 IU vitamin E and 1.20 mg Selenium	[2]
Katahdin × Dorper ewe lambs	250 mg free ferulic acid	[3]
Crossbred lambs ((Merino × Border Leicester) × Dorset)	228 mg vitamin E and 1.16 mg Selenium	[4]
Katahdin × Pelibuey and Dorper × Pelibuey crossbred ewes	10 mg of zilpaterol hydrochloride	[5]
Australian Merino rams	0.8 mg Selenium and 150 mg vitamin E	[6]
Barki rams	4% Sargassum latifolium algae (contain β-carotene, fucoxanthin, and tocopherol)	[7]
Merino × Poll crossbred sheep	0.4 and 0.8 µg nano chromium picolinate	[8]
Chios cross-bred ewes	0.515 g cornus extract with oregano and thyme essential oils	[9]
Afshari × Chaal lambs	2% phytogenic-rich herbal mixture (cinnamon, turmeric, rosemary, and clove buds)	[10]
Ujumqin lambs	5-10 g chestnut tannins	[11]
Iraqi ewes	40 mg vitamin C	[12]

References
[illegible]

corrected for differences. In seasonal comparison, the values for winter were used as the calibrator, while for sex differences, the rams' values were used as the calibrator.

$$Gene\ expression\ ratio = \frac{(E_{GOI})^{\Delta Ct\ GOI}}{(E_{HKG})^{\Delta Ct\ HKG}}$$

(EGOI- corrected efficiency of the gene of interest (GOI); EHKG- corrected efficiency of the housekeeping gene (HKG); ΔCt GOI- delta cycle threshold value of the GOI; ΔCt HKG- delta cycle threshold value of the HKG)

Gene expression ratios means, standard deviation, and standard error were presented and graphically compared between the values for the summer and winter seasons and in-season comparison between rams and ewes.
Meteorological and gene expression data were downloaded and processed using Microsoft Excel (Microsoft Corporation, Redmond, WA), including tabular and graphical presentations [22-23].
For analysis, the differences of *IL-10* gene expression between seasons (summer and winter), between the breeds, and sexes (rams and ewes) in every season were determined through Student t-test [24] using the average cycle threshold (CT) values for the gene of interest. A difference with P<0.05 value was considered statistically significant. Data analysis was carried out using R program for Windows [25].

3 Results

3.1. Climatological variables

The 30-month climatological records, which span from 2018 to 2021, show the seasonal variations of meteorological variables such as temperature, relative humidity, and THI surrounding the study's sample collection period. Environmental temperature and air relative humidity were factors that affected THI. The highest THI values were observed during the summer months of June, July, and August, which correspond to high-temperature levels. Conversely, relative humidity falls during periods of high temperature, while it is high during low-temperature periods such as the winter season (Figure 1).

Figure 1. 30-month climatological record in the farm location. The mean THI values during sample collection days were highlighted in red (summer) and blue (winter) bars.

The data on the climatic condition during the collection of samples in summer and winter are presented in Table 2. The summer data collection day was characteristically hot and dry, with temperature recorded at 32.80°C and relative humidity at 34.50%. In contrast, the winter data collection day was cold and humid, with temperature recorded at -3.33 °C and relative humidity at 99.70%. The calculated THI during summer collection day was 79.0, indicating severe heat stress, while the THI during winter was 26.1, meaning there was no heat stress in the animals. Figure 2 shows the heat stress intensities that correspond with the THI values during the sample collection and entire study period.

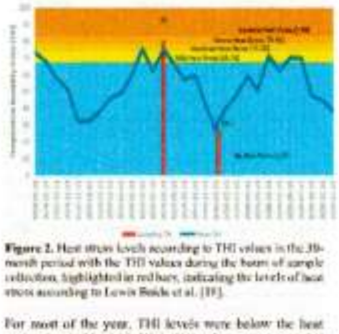

Figure 2. Heat stress levels according to THI values in the 30-month period with the THI values during the hours of sample collection, highlighted in red bars, indicating the levels of heat stress according to Lewis Baida et al. [19].

For most of the year, THI levels were below the heat stress threshold of 65. High THI values were recorded during the summer season, indicating heat stress conditions. Mean THI levels during the summer season show moderate heat stress, but there were times during the day that were marked with elevated THI values, and thus characterized heat stress to be severe. The hour of sample collection for summer shows that the animals were subjected to severe heat stress condition, with THI values at 79.0, several points higher than the mean THI during that day. On the contrary, the absence of heat stress was established during the winter sample collection with a THI of 26.1.

3.2. Expression of *IL-10* gene

Following qPCR analysis to determine the gene expression levels of *IL-10* in the experimental animals, relative expression was calculated using the Pfaffl method where the winter season values were used as calibrator. For all animals, mean gene expression ratio during the summer season was 16.82 with a standard deviation of 20.85 (Table 3). In contrast, the winter mean gene expression ratio was 1.18 with a standard deviation of 0.61. Relative gene expression ratios of *IL-10* in summer and winter seasons were presented in Figure 3 with winter season value as calibrator. A higher gene expression ratio of *IL-10* is found in the summer season as compared to the winter season ratio.

Table 2. Climatological data on the day of sample collection

Season	Sampling date	Sampling time	Temperature (°C)	Relative humidity (%)	THI	Heat Stress Rate
Summer	13/ 08/ 2019	12.00-13.00	32.80	34.50	79.0	Severe
Winter	22/01/ 2020	12.00-13.30	-3.33	99.70	26.1	No Stress

Table 3. Relative Gene Expression of *IL-10* in Hungarian Indigenous Tsigai and Dorper Sheep during summer and winter seasons

	Relative gene expression (Mean ± SD)	
Breed	**Summer**	**Winter**
Overall	16.82 ± 20.85	1.18 ± 0.61
Hungarian Indigenous Tsigai	12.3 ± 15.25	16.2 ± 18.53
White Dorper	1.1 ± 0.56	1.04 ± 0.35

SD: Standard Deviation

Breed-wise, the Hungarian Indigenous Tsigai sheep has an *IL-10* gene expression ratio mean of 12.3 and a standard deviation of 15.25 during summer. The White Dorper, on the other hand, has a mean of 16.2 and a standard deviation of 18.53 during summer. Here, the White Dorper showed a numerically higher ratio of gene expression during the summer seasons compared to the Hungarian Indigenous Tsigai.
The gene expression ratio of all the animals and the two breeds during the summer and winter are graphically presented and compared in Figure 3.
The average CT-values were used to statistically analyze differences among the gene expression in 2 seasons (Table 4), between the two breeds, and among sexes. In all animals, expression levels of *IL-10* were significantly higher (P=0.00019) during the summer season than those samples collected during the Winter season. Within-breed comparison of summer and winter CT-values showed significantly higher *IL-10* expression during the summer season in both breeds (Hungarian Indigenous Tsigai P= 0.015 & White Dorper P= 0.004). Between the two breeds, Hungarian Indigenous Tsigai and White Dorper, there is no significant difference in the CT-values during the summer and winter seasons. Similarly, no significant difference between ewes and rams was found during the two seasons in terms of relative gene expression ratio and CT-values (Table 5).

Table 4. Mean and standard deviation of CT-value of *IL-10* of Hungarian Indigenous Tsigai and White Dorper in summer and winter seasons.

	CT-value (Mean ± SD)			
Season	**Hungarian Indigenous Tsigai**	**White Dorper**	**Overall**	**P-value**
Summer	32.87 ± 1.94^a	32.62 ± 2.27^a	32.74 ± 1.92^a	*0.87*
Winter	29.37 ± 1.64^b	28.09 ± 0.34^b	28.66 ± 1.[illegible]^b	*0.12*
Overall	31.25 ± 2.29	30.35 ± 3.20	30.80 ± 2.75	
***P*-value**	*0.015*	*0.004*	*0.00019*	

SD: Standard Deviation, Note: The different superscripts show significantly different values (P < 0.05).

Table 5. Relative gene expression ratio and CT-value of *IL-10* of Ewe and Ram Hungarian Tsigai and White Dorper Sheep in summer and winter seasons.

	Hungarian Indigenous Tsigai			White Dorper		
Season	**Group**	**Relative Gene Expression Mean**	**CT-value Mean**	**Group**	**Relative Gene Expression Mean**	**CT-value Mean**
Summer	Ewe	0.01	34.205	Ewe	177.61	30.765
	Ram	0.06	31.53	Ram	1.09	34.475
Winter	Ewe	0.43	30.885	Ewe	0.6	30.005
	Ram	0.01	28.36	Ram	1.01	28.275

Continental vs. tropical breed: immunity comparison under heat stress conditions utilizing qRT-PCR technique

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Abstract. Understanding the responses of animals to seasonal heat stress on the genetic level has led to the identification of genes implicated in thermal stress reaction mechanisms. In this study, the relative gene expression of Interleukin-10 (*IL-10*), an anti-inflammatory cytokine and biomarker for heat stress-mediated immune modulation, was observed during the summer and winter seasons in continental and tropical sheep breeds, namely, Hungarian Indigenous Tsigai and White Dorper, employing quantitative real-time polymerase chain reaction (qRT-PCR). Temperature-humidity indices (THI) were calculated to assess heat stress levels. The results indicate that *IL-10* CT-values were significantly higher during the summer, when heat stress prevails, in both sheep breeds compared to winter. While the White Dorper exhibited a higher numerical value for the summer relative gene expression ratio (16.2) compared to the Hungarian Indigenous Tsigai (12.3), no significant differences in CT values were observed between breeds or among sexes. These findings suggest the immune-adaptive characteristics of the two sheep breeds during seasonal heat stress. The variation of *IL-10* gene expression levels between the two breeds can be attributed to their geographical origins; the White Dorper emerging from arid subtropical South Africa and the Hungarian Indigenous Tsigai Sheep thriving in the seasonally harsh Carpathian climate for centuries.

Keywords: gene expression, heat stress, Hungarian Tsigai, *IL-10*, White Dorper

1 Introduction

As a major sector in the livestock industry, sheep production is key to ensuring global food security for the rapidly growing population. Its importance to low-income families all around the world as a source of food and livelihood cannot be overlooked as well [1]. With climate change projections pointing to worse scenarios of increased global temperatures in the years to come, it is essential to tailor-fit the livestock industry to a warmer and harsher environment by selecting animals with tolerance and adaptation traits [2]. Equipped with a robust knowledge of genes related to heat tolerance and adaptation, the livestock industry can keep up with the climatological challenges and sustain productivity.

Heat stress presents direct challenges to the well-being of animals that lead to an array of detrimental effects on their physiology, behavior, overall production performance, reproduction, and health [3]. In response to heat stress, the animal attempts to maintain homeostasis and re-establish homeothermy by invoking compensatory and adaptive mechanisms through behavioral and physiological changes to promote welfare and ensure survival [4].

The immune system of animals, in general, is one of the well-studied aspects of the physiological responses of livestock to heat stress. The multifarious interplay of hormones and key immune system cells and factors during heat stress leads to complex immune responses that involve rapid inflammatory reaction, antagonistic immunosuppression, and shifting of immune regimes, among others [5-6]. Understanding and knowing these mechanisms and the factors involved usher in the determination of reliable biological markers of heat response in animals with respect to immunity.

Cytokines are some of the most prominent actors in the immune system and, in this regard, the immune response to heat stress. Of them, the Interleukin 10 (*IL-10*) plays a major role in modulating the other cytokines and effectors of the immune system that attempts to preserve its integrity and function while facing heat stress [7-8].

A deeper investigation of genes associated with heat tolerance and adaptation in livestock has caught the attention of researchers in recent years because of the increasing availability of genomic tools and capabilities. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is a reliable and key enabling technology for gene expression analysis. Its rapid, cost-effective, and robust approach in gene expression analysis has encouraged researchers to use it for many purposes, including the detection and measurement of the relative expression of genes associated with heat tolerance and adaptation [9].

The influence of seasons on the expression of several heat stress-related genes in livestock was investigated in dairy cattle [10-11], sheep [12], and pigs [13]. This study aims to bridge the scarcity of comparable studies in sheep that intend to investigate seasonal effects on the expression of heat tolerance genes such as *IL-10* as well as a comparison of tropical and temperate breeds on the same subject.

In the present study, the relative gene expression of *IL-10* was determined in the Hungarian Indigenous Tsigai sheep and White Dorper sheep in Hungary during the summer and winter seasons. The Tsigai sheep is an indigenous temperate triple-purpose breed in the Central-Eastern European region that has been bred and maintained as part of the Hungarian sheep sector for more than two centuries [14-15] while the White Dorper is regarded as a breed of Tropical origin that was developed in arid subtropical South Africa for meat purpose in the 1940s. The breed has just been introduced in Hungary in 2008 [16]. This study aimed at understanding the dynamics of the relative gene expression of *IL-10* during the two seasons, in the two breeds, and among the sexes, with particular emphasis on heat stress conditions and the further goal of expounding the adaptability of the two breeds to heat stress situations.

2 Materials and Methods

2.1. Sample Collection and Location

Four Hungarian Indigenous Tsigai sheep and 4 White Dorper sheep were utilized in this study as experimental animals. Of them, 2 are ewes, and 2 are rams for each breed. The animals were housed in conventional deep litter type housing with several pen separation devices and implements that allow adequate airflow and access to the immediate environment during the hot season and enclosure during the wet and cold seasons. Under the regular farm feeding regimen, the animals were provided clean drinking water. All animals that were part of the study were of similar age (2 to 3 years old), body weight (ewes: 45 to 55 kg; rams: 65 to 75 kg), and in optimal health, exhibiting no physical or anatomical abnormalities.

About 5 ml whole blood sample was obtained from the jugular vein of the same animals in RNAprotect Animal Blood Tubes (QIAGEN, Hilden, Germany) and preserved at -70°C until further analysis.

The animals used in this study were kept in the Kismacs Experimental Station of Animal Husbandry of the University of Debrecen, located at 4002 Debrecen, Kismacs Tanya 4, Hungary. The elevation is 127 m above sea level and site coordinates are 47.58° N and 21.58° E.

2.2. Climatological Data

Data on the climatic condition during the sampling day, which include temperature and relative humidity, were recorded from 12:00 to 13:00 in the afternoon and obtained from the Hungarian Meteorological Service station in the vicinity. To calculate the THI for each sampling day, the equation by Mader et al. [17] was used.

$$THI = (0.8 \times T_{db}) + \left[\left(\frac{RH}{100}\right) \times (T_{db} - 14.4)\right] + 46.4$$

T_{db}- Dry bulb temperature (°C), RH- Relative humidity (%)

The severity of heat stress in livestock is typically rated using the THI with ranges categorized as follows: no stress (≤67), mild (68–74), moderate (75–78), severe (79–83), and extreme (≥84) [18].

Additionally, in order to provide a broader and more objective overview of the climatic cycle and changes in meteorological variables as the seasons change in the area and to give background reference to the climatological data on sampling days, the 30-month climatological records (August 2018 to January 2021) [19] measured by the adjacent government meteorological station, were obtained from the Hungarian Meteorological Service.

2.3. Quantification of relative gene expression levels using RT-qPCR

Following the manufacturer's instructions, total RNA was extracted from sheep blood using the RNeasy Protect Animal Blood Kit (QIAGEN, Hilden, Germany). Using a NanoDrop ND-1000 Spectrophotometer, RNA quality and quantity were assessed (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA of about 100 ng was reverse transcribed into cDNA with specific primers (Table 1) by the qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, United Kingdom). Forward and reverse primers (Table 1) were created with Primer Express v3.0.1 software (Applied Biosystems, Foster City, CA, USA) and confirmed for target identity with Primer Blast from the National Center for Biotechnology Information (NCBI) (Ye et al., 2012). A 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to conduct quantitative real-time PCR (qPCR). The 10 ll reactions consisted of 5 ll PowerUpTM SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.6 ll each of 10 lM forward and reverse primers, 1.3 ml dH2O (MilliporeSigma, Burlington, MA, USA), and 2.5 ml cDNA (2 ng/ml). For relative expression studies, one housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) was amplified alongside the target genes (*IL-10*). Quantitative PCR was done in triplicate for each sample.

Table 1. Detail of primers for qPCR analyses of the target genes

Target gene	Gen Bank accession	Primers	Fragment size (bp)
IL-10	NC_056065	F: TGATGCCACAGGCTGAGAAC R: CAGAAAACTGATGACAGCGCC	116
GAPDH	NC_046254.1	F: CTGGCCAAGGTCATCCAT R: ACAGTCTTCTGGGTGGCAGT	86

2.4. Data and Statistical Analysis

The relative expression of the *IL-10* gene was determined by calculating the values using the equation proposed by Pfaffl (2001). The method compared the expression of *IL-10* gene with the reference gene GAPDH, with their amplification efficiencies both

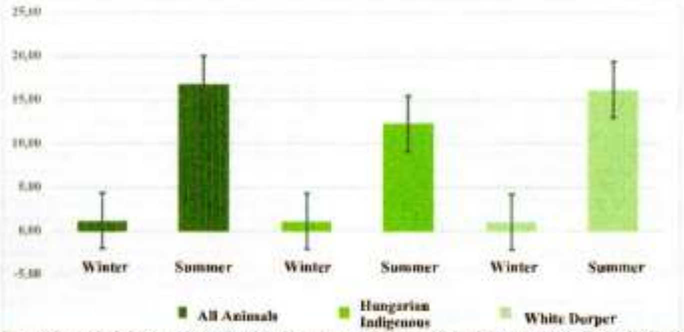

Figure 3. Bar graph of relative gene expression of *IL-10* in each season of the year with the winter season as the calibrator. The X-axis is the relative gene expression in the two seasons. The Y-axis is gene expression for all animals, and the two breeds studied. Different colors represent different breed.

4 Discussion

Maintaining thermoneutral conditions is one of the important requirements in ensuring the welfare and optimal production and reproduction performance of animals [26].

THI is considered as one of the most reliable measures and sensitive indicators of heat stress in animals, which integrates temperature and relative humidity parameters [27-28]. In this study, during the summer sample collection, THI was calculated to be high at 79.0, which indicates a state of severe heat stress. High temperature and low humidity are typical of summer seasons in the region. The average annual temperature in the immediate area was 11.66 °C for the period of 2011 to 2020 [29]. The average temperature recorded on the day of collection was 26.70 °C, a 136% deviation from the annual average. Factoring the daily maximum temperature and the relative humidity during the summer months of June to August [29], THI ranges from 69.67 to 88.80 for sheep, which indicates mild to extreme heat stress.

Consistent with the climatological data gathered and determined in this study, elevated THI in sheep during the summer season and hot periods that established a heat stress condition was also reported in Small tail Han sheep [30], Indian sheep [31], and Omani and Australian Merino sheep [32] where the animals exhibited increased respiratory rate, pulse rate, and rectal temperatures. Such signs are distinctive physiological indicators of heat stress response in animals [33-34].

High-THI conditions also influence metabolic and hematological parameters in sheep. Cortisol and biochemical parameters creatinine, zinc, and phosphorus were elevated during heat stress conditions and are highly correlated to THI levels in Suffolk sheep [35]. Sheep blood parameters chlorides, sodium, phosphorus, total protein, tetraiodothyronine, cholesterol, triglyceride, creatinine, cortisol, and glucose were likewise reported to be significantly increased by heat stress conditions in the study of Li et al. [30]. In the same way, values for red blood cell (RBC), hemoglobin (Hb%), and packed cell volume (PCV%) were significantly elevated in indigenous sheep subjected to heat stress [36].

The negative effects of heat stress on general welfare, comfort, and health could result in diminished immune function and increased incidence of diseases [37-38].

Primarily, the response of the immune system to abiotic stressors, such as heat stress, begins with the stimulation of primary and secondary lymphoid and myeloid organs that catalyze the synthesis and release of immune cells and agents [5]. Studies show that in acute heat stress (brief and intense), immune activation happens, while chronic heat stress causes immune suppression [39]. Elevated cortisol levels, as a result of the activation of the hypothalamic-pituitary axis (HPA), alter various immune function elements that may increase the animal's vulnerability to diseases [40]. This effect is demonstrated in the induction of inflammation in acute heat stress and the suppression of release and synthesis of inflammatory cytokines and the consequent changes to the dynamics of balance on the levels of other cytokines, immune cells, and substances in chronic situations [41-42].

Cytokines are key mediators of the immune system's response to stressors and pathogens. Proinflammatory cytokines work to activate the innate immunity for the body's early response against infectious agents and support the activities of the cell-mediated immune response of the adaptive immunity to synergistically combat the pathogen of concern. However, specific cytokines modulate the previous cytokines to suppress the immune response and shift the modality of the immune system into adaptive immunity. The most powerful and significant immunomodulatory cytokine of this effect is *IL-10* [43].

In the present study, overall *IL-10* gene expression in Hungarian Indigenous Tsigai and White Dorper sheep were shown to be significantly higher (P=0.015) in animals under severe heat stress (summer) compared to those that experienced no heat stress condition (winter). This result is in accordance with the findings of Caroprese et al. [44], who demonstrated high *IL-10* expression in sheep experiencing hyperthermia. Similarly, in cattle, *IL-10* levels were increased in heat-stressed Holstein cows [10, 45], Italian Friesian cows [46], and Sahiwal cows [11].

The expression of *IL-10* in heat stress situations has various physiological and immunological indications that help the animal adapt and restore equilibrium. While it suppresses the proinflammatory response, it leads the immune regime from TH1 to TH2 that activates the adaptive immunity [47-48]. Sustained *IL-10* levels during heat stress and the extended period after the heat stress situation and stressful activities have effectively controlled immune response to stress [49].

Ripley [50] has reported that *IL-10* activates heat shock protein 90 (HSP90). Heat shock proteins are molecular chaperones that are another important factor for protecting cells from damage, such as in heat stress situations and in antigen presentation to improve both innate and adaptive immunity [51]. *IL-10* expression by the experimental animals in this study suggests that there could also be accompanying HSP expression, which also aids in the immune response and endogenous protection against heat stress.

Furthermore, *IL-10*, in line with its anti-inflammatory actions, has also been associated with the inhibition of cyclooxygenase (COX) and, consequently, prostaglandin E2 (PGE2), the principal downstream mediators of fever that are induced by inflammatory cells [52]. This would mean that the expression of *IL-10* during heat-stress situations is thermo-modulatory and helps reduce heat load as it eliminates endogenous heat production by fever pathways.

Though all the sheep in this study show significantly higher expression of the *IL-10* gene during the summer season, the inter-breed comparison reveals a numerically higher gene expression ratio among the White Dorper (16.2) against the Hungarian Indigenous Tsigai (12.30). Statistical analysis of the CT-values, however, yielded no significant difference between the two breeds on the same subject. The two breeds, despite having different geographical origins, manifest comparable thermo-tolerance evident in the *IL-10* mRNA expression that bears no significant difference.

The Hungarian Indigenous Tsigai Sheep, being a temperate indigenous breed that thrived in the continental climate for centuries [15, 53], elucidated heat stress response in the distinctively harsh and hot summer environment of the region.

In a similar manner, the White Dorper sheep that is considered tropical in origin, maintained its heat stress responsive characteristic as it experienced the hot summer season in the study location where it was introduced a few years ago. Despite being present in Hungary for not a long time, the breed's tropical characteristics made acclimatization manageable for it to thrive during the hot summer months in the area.

Even though *IL-10* is a prominent immunological biomarker in heat stress-related studies in domestic animals, comparative studies of its mRNA expression across breeds, particularly between tropical and temperate breeds, are very scarce, especially on sheep.

In contrast to the findings of the present study, various related studies reported differences in *IL-10* gene expression among different breeds in domestic animals, such as cattle, during heat stress. Previous studies showed significantly higher *IL-10* gene expression in Sahiwal cows [11] and Karan Fries cows [54] compared to other breeds that include temperate ones. Citing other related thermotolerance immune biomarkers such as heat shock proteins (HSPs), Sahiwal, a tropical cattle breed, also exhibited higher mRNA expression of HSP90 [55] and HSP70 [56] compared to the temperate and tropical cross, Frieswal. Following this trend, the Caribbean sheep breed Pelibuey was found to be less susceptible to heat stress and with increased HSP70 concentration compared to Suffolk [57].

A comparative study in pigs revealed better cell viability in the tropical Creole pigs than the temperate Large White pigs, with a significant increase in the expression of HSPs. Interestingly, however, there was no difference in mRNA expression of HSP70.2 and HSP90 [58] in agreement with the findings of the current study.

The increase in *IL-10* concentration can result in the upregulation of HSPs gene expression in peripheral blood mononuclear cells, consequently leading to increased HSP concentration in circulation [56].

The equivalent heat tolerance in the Hungarian Indigenous Tsigai to the tropical breed White Dorper indicated by its *IL-10* gene expression in this study can be attributed to its indigeneity in the area that is shown in its hardiness and suitability to local environment. Its exposure to the local agro-climatic conditions across generations through centuries helped it adapt to the harsh summer months in the region. Its success in variable climatic conditions is proven by its wide distribution in the Central-Eastern European and Balkan regions being integrally used in sheep breeding programs confirmed by relatively low to moderate levels genetic variation among country populations [53, 59]. The tenacity of indigenous breeds in extreme climatic conditions can be ascribed to their physiological and genetic adaptations that have arisen over generations of slow modification and adaptation to environmental challenges [60, 28].

On another note, the excellent thermotolerant character of the White Dorper sheep, according to the parameters of this study, suggests its suitability for the harsh summer heat stress conditions in the area, despite its relatively short period of habitation. Tropical breeds can thrive comfortably in temperatures as high as 38 °C and produce adequately by virtue of thermoregulatory processes [61]. This characteristic was exhibited by Malpura ewes from the Indian semi-arid regions [62] and Santa Ines and Morada Nova sheep in Brazil [63]. Performance-wise, Dorper raised in the same area under Hungarian rearing conditions proved to have a good production and reproduction performance as reported in a study by Budai et al. [16].

In a pilot study evaluating reproductive performance and health indicators in the same area in Hungary, genotype differences were found in incidences of clinical mastitis (higher in Tsigai), lameness (higher in Dorper), and litter size (lower in White Dorper), while no differences were observed in conception and lamb survival rates [64]. It provides an overview of the variability of immune status among the breeds of interest, which merits the conduct of further investigation as to how heat stress could affect these incidences and the role of *IL-10* in such health challenges and reproductive performance.
Finally, in contrast with the findings of Li et al. [30], that reported better high-temperature tolerance in ewes over rams, the present study finds no sexual differences. The absence of sexual differences in the mRNA expression of *IL-10* in this study implies that thermotolerance and heat stress immune responses in both breeds are not influenced by sexual factors.

5 Conclusion

Overall, the study has demonstrated higher relative gene expression of the IL-10 gene during the summer season in both the Hungarian Indigenous Tsigai and White Dorper when heat the animals experienced heat stress conditions in contrast with the winter season. Implicated as a molecular biomarker for heat stress response, the significant relative gene expression of *IL-10* indicates immune modulation and shift to adaptive immune response. There has been no significant difference in the *IL-10* gene expression between the two breeds. While sexual factors do not affect *IL-10* gene expression, its expression can potentially enhance physiological adaptive mechanisms to avoid cellular damage during heat stress conditions in both breeds, regardless of sex.

Owing to their origins, the comparable heat stress response through the expression of the IL-10 gene proves that both breeds, continental and tropical in origin, are adaptive to the region's environmental conditions and could be utilized in breeding programs to improve the immunity, resiliency, and production performance of sheep, even in variable climatic conditions.

Further study on the links of genetic adaptability, physiological reactions, immune response, breeding programs, animal health, and productivity must be conducted to unravel complex mechanisms of homeostasis and animal performance in the face of heat stress, particularly involving *IL-10* and related biomarkers.

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CALABARZON, and Davao) in terms of live weight production; 2) to conduct economic cost-benefit analyses of the INSPIRE grant, which was given to the industry stakeholders in the three regions for 2022–2023; and 3) to estimate the needed national investment requirements from 2025 to 2028 to restore pig production to pre-ASF 2018 levels. The choice of study was designed to measure the impact of the pig repopulation program on the top two badly hit regions and one slightly hit region to determine pig repopulation impacts and to guide future pig repopulation policies. Before the ASF hit in 2019, Central Luzon and CALABARZON were the top two pig-producing regions. Davao Region is a major pig-producing region in the south. The findings of this study could contribute to policymaking, industry resilience, and long-term ASF mitigation strategies.

2. Materials and methodology

2.1. Model: specification and justification

The SARIMA model, an extension of the classical ARIMA approach, is designed to model time series data that exhibit both trend and seasonal patterns. While the standard ARIMA model captures autocorrelation and non-stationarity through autoregressive (AR), differencing (I), and moving average (MA) components, adds a seasonal layer, making it well-suited for data with periodic fluctuations.

Formally, a model is denoted as **ARIMA (p, d, q) (P, D, Q) [s]**, where:

- (p, d, q) represent the non-seasonal AR, differencing, and MA terms;
- (P, D, Q) represent the seasonal AR, differencing, and MA terms;
- s denotes the length of the seasonal period (e.g., 4 for quarterly data, 12 for monthly data).

According to Box et al. (2015) and [illegible] et al. (2023), the model extends the basic ARIMA formulation by incorporating seasonal autoregressive and moving average operators, which allows the model to better capture recurring seasonal behaviours observed in the data. This model was selected due to its ability to handle both non-stationarity and seasonal structures in time series data. This is particularly relevant for pig production data, which often shows strong seasonal patterns influenced by biological cycles, market demand, and environmental conditions. The model's strength lies in its ability to utilize historical values and errors—including those from previous seasons—to generate reliable forecasts. The adoption is also consistent with previous applications in livestock forecasting and epidemiological modelling (e.g., [illegible], 2022; Yadav & Akhter, 2021; [illegible] et al., 2022; [illegible] et al., 2023), where seasonal effects were found to significantly affect production and disease dynamics. Its flexibility and interpretability make it a powerful tool for understanding and predicting fluctuations in regional pig production, especially in the context of disruptions such as ASF.

2.2. Data description and study scenarios

This study employs quarterly regional pig production data from the Philippine Statistics Authority (PSA), covering the period from the first quarter of 2000 (2000Q1) to the first quarter of 2024 (2024Q1). As noted by Box et al. (1994), a minimum of 50 observations is recommended for reliable time series modelling. With over two decades of quarterly data, amounting to more than 90 observations, this study meets the necessary threshold for robust SARIMA forecasting.

To evaluate the impact of external shocks and policy interventions, two distinct analytical scenarios were developed:

1. No ASF Scenario—includes data from 2000Q1 to 2019Q2, representing a hypothetical baseline in which ASF had not yet emerged.
2. Pre-INSPIRE Scenario—includes data from 2000Q1 to 2021Q4, capturing the conditions prior to the launch of the INSPIRE program.

Before modeling, the dataset was preprocessed to detect anomalies, ensuring the accuracy and consistency of the time series. Regions were categorized as either ASF-affected or non-affected to facilitate comparative forecasting and policy evaluation between impacted and control groups. Supplementary data were also obtained from INSPIRE program accomplishment reports, providing essential context for interpreting government interventions and enabling the subsequent cost-benefit analysis.

2.3. Model estimation

Given the quarterly nature of the dataset (i.e., seasonal period $s = 4$), model estimation was conducted using the auto.arima function (Hyndman & Khandakar, 2008) from the forecast package in R (R Core Team, 2021) and R Studio (2023). This function automates the selection of optimal model parameters by searching across a wide grid of candidate models and identifying the best-fitting structure based on information criteria—specifically, the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). This approach ensures that the final model is both parsimonious and statistically robust.

Model diagnostics were conducted to validate adequacy. Residuals were examined for autocorrelation using autocorrelation function (ACF) and partial autocorrelation function (PACF) plots, and the Box–Ljung test was employed to confirm that the residuals approximated white noise. These procedures ensured that the selected models captured the underlying data structure effectively and were well-suited for generating reliable forecasts across different policy and outbreak scenarios.

2.4. Economic evaluation: cost-benefit analysis (CBA)

To evaluate the economic viability of the INSPIRE program, a Cost-Benefit Analysis (CBA) framework was employed. This approach compares the present value of projected benefits from pig repopulation against the present value of implementation costs.

$$Cost - Benefit\ Analysis\ Ratio = \frac{Present\ Value\ of\ Benefit}{Present\ Value\ of\ Cost} \quad (1)$$

A **Net Present Value (NPV)** calculation was also performed:

$$NPV = \sum_{t=1}^{n} \frac{NCF_t}{(1+r)^t} \quad (2)$$

where NPV = net present value, NCF_t = net cash flow generated by the proposed project in year t, and r = discount rate. These indicators help determine the financial justification for continued or expanded public investment in pig repopulation efforts.

The concept of CBA is rooted in early work by Dupuit ([illegible]), with further development in the field of agricultural economics ([illegible], 1995). In this study, CBA was informed by actual program data and pig production forecasts derived from ARIMA modeling, thus integrating empirical forecasting with economic assessment.

3. Results and discussion

Two sets of results were generated and compared with those of PSA production. First, the ARIMA forecasting results for a scenario with no ASF using datasets from 2000Q1–2019Q2 (78 data points): Central Luzon, CALABARZON, and Davao Regions. Second, a dataset from 2000Q1 to 2021Q4 (88 data points) was used to forecast the pig production scenario, representing the period before the implementation of the INSPIRE project. Fig. 1 shows the map of the three regions in the Philippines.

Fig. 1. Central Luzon, CALABARZON, and Davao Regions.
Source: DA-Information and Communications Technology Service (ICTS).

3.1. Results of the Augmented Dickey-Fuller (ADF) test for stationarity under No ASF and ASF scenarios

To ensure the appropriateness of SARIMA modeling, all the time series data set were subjected to the ADF test to assess stationarity. For most of the series, the null hypothesis of non-stationarity was not rejected at level, but was rejected after first differencing, indicating that the series became stationary upon differencing. ASF Scenario data were stationary after the second differencing (not shown). A summary of these results is presented in Table 1.

Table 1
The Augmented Dickey-Fuller (ADF) Test for Stationarity.

Variable	No ASF scenario		ASF scenario	
	At level	After 1st difference	At level	After 1st difference
Central Luzon	-1.1998	-3.0023**	-0.09157	-3.4307
CALABARZON	-2.4122	-4.1008***	-1.1571	-2.2073
Davao Region	0.7515	-4.0049***	1.1668	[illegible]

***, ** and * represent stationarity at 1, 5 %, and 10 % levels of significance respectively. Note: Central Luzon, CALABARZON, and Davao Region No ASF obtained stationarity at 1 % after 2nd difference.

3.2. Model estimation, diagnostic checking and forecasting

3.2.1. Central Luzon

For the Central Luzon no ASF scenario, the model chosen was SARIMA (1,1,1) (0,1,0), with a MAPE of 4.12 and an AIC of 1464.16 (Table 2). The ACF plot in Fig. 2 shows that only one line exceeds the range, which indicates a favorable model. As shown in Table 3, the Box-Ljung test's p-value of 0.57 indicated white noise (or no autocorrelation). A low p-value (typically less than 0.05) indicates that the null hypothesis of no autocorrelation is rejected, which suggests the presence of autocorrelation. On the contrary, a high p-value means there is not enough evidence to reject the null hypothesis, indicating no significant

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Ex-post impact analysis of the Philippine pig repopulation program

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ABSTRACT

The 3-year Integrated National Swine Production Initiatives for Recovery and Expansion (INSPIRE) program is the national repopulation and recovery program of the Philippine Department of Agriculture-National Livestock Program, offering a mix of interventions aimed at reviving the swine industry, which was significantly impacted by African Swine Fever (ASF). The study focused on the interventions provided to the three regions with the highest allocation from fiscal years (FYs) 2022 and 2023: Central Luzon, CALABARZON, and the Davao region. A combination of SARIMA modeling and financial analyses was employed to measure the impacts of the repopulation program.

Two forecasts were created: one assumes a situation without African swine fever outbreaks, using datasets from 2000Q1 to 2019Q2, whereas the other reflects the situation prior to the implementation of the INSPIRE program, which utilizes datasets from 2000Q1 to 2021Q4.

The study estimated the impact of the INSPIRE program in these three regions, revealing that Central Luzon and CALABARZON significantly benefited from the program. Without the INSPIRE program, pig production in Central Luzon would have been wiped out by the second quarter of 2022 (2022Q2), whereas CALABARZON would have faced a similar scenario by the first quarter of 2023 (2023Q1). In contrast, the INSPIRE program had a lesser impact in the Davao Region, as it was affected by ASF later.

The cost benefit analysis and net present value of the program investments yielded favorable ratios that indicate their financial viability and success. Based on the impact analysis, it is recommended that the swine repopulation program be continued beyond 2025 with increased funding.

This is the first study in the Philippines which utilizes time series SARIMA forecasting to evaluate the impact of a pig repopulation program.

1. Introduction

African swine fever (ASF) is a highly contagious viral disease that affects domestic and wild pigs and causes significant illness and mortality. It is not harmful to humans or other animal species, but it can cause severe economic losses in affected areas due to the death of pigs, restrictions on movement of pigs and their products, and added costs of control measures.

1.1. Global, Asian, Philippines and Philippine regional perspective of ASF

[illegible] et al. (2024) and [illegible] et al. (2023) reported on the current situation of ASF globally, Asia and in the Philippines. Other studies have given global ASF, Philippine context, and regional context such as [illegible] et al. (2023a, 2023b, 2024) in CALABARZON, Garcia et al. (2024) in Central Luzon and [illegible] et al. (2023) in Davao Region. [illegible] et al. (2024) provided a detailed global overview of ASF globally and in the Philippines. On the Philippine situation, the study reviewed current ASF surveillance, prevention, and control strategies in the Philippines. [illegible] et al. (2023a) reported a clear seasonal pattern with higher frequencies of ASF outbreaks occurring from August to October and lower frequencies during April to May. It added that in terms of space, disease was more severe in the northern island of Luzon, particularly during the second half of the year. They concluded that spatial and temporal patterns can inform disease prevention and control efforts in the future, and help authorities prepare for and mitigate the impact of potential ASF outbreaks. In another follow-up study, the findings of [illegible] et al. (2023b), [illegible] et al. (2023) suggested that swill or contaminated feed, inadequate biosecurity protocols, and movement of personnel were the top risk factors favoring ASF spread among farms in general. For commercial farms, they added that contaminated vehicles and personnel were also important, whereas for backyard farms, the introduction of new pigs, environmental contamination, and poor feeding quality were relevant risk factors. Garcia et al. (2023) gene sequenced 125 blood samples from Central Luzon, Philippines from January to April 2022. The results of their study revealed close phylogenetic relationships among ASF isolates from Vietnam, Malaysia, India and the rest of the Philippines. The results of the study revealed close phylogenetic relationships among ASF isolates from Vietnam, Malaysia, India and the Philippines.

In July 2019, the Philippines reported its first cases of ASF in Rizal province near Metro Manila ([illegible], 2019). The virus subsequently spread to other provinces, including Bulacan, Pampanga, Nueva Ecija, and Cavite. The ASF outbreak significantly impacted Philippine pork production, leading to the culling of more than 300,000 pigs. As a result, the growth rate of pork production dramatically decreased by 26 % in 2021 (Philippine Statistics Authority, 2021). In addition to its devastating effects on pig farmers, ASF also leads to higher pork prices, severely affecting vulnerable consumers and livelihoods ([illegible] et al., 2022). In response to the ASF outbreaks, the Philippine government implemented various policies and public health strategies ([illegible] et al., 2020), such as the National Zoning and Movement (NZM) plan for ASF, which designates different zones based on ASF risk levels and restricts movement between regions.

Lifting from the blueprint of the National Foot and Mouth Disease Eradication Program in the Philippines ([illegible] et al., 2011), a set of actions referred to as the 1–7–10 protocol was conceptualized and implemented to contain the spread of ASF. Recently, DA Administrative Circular (AC) 6, Series of 2024 revised 1 kilometer in the 1–7–10 protocol of DA Administrative Order (AO) No. 22, Series of 2020–500 m. The essential components of the revised protocol include the rapid culling of domestic pigs within the 500-meter radius of ASF-infected farms, implementing active surveillance activities and testing within the 7 km radius, and requiring pig farms within the 10 km radius to submit a mandatory report on disease surveillance. The stamping-out of pigs within 500 m from the infected premises (the quarantine zone) is mandated to be completed within five days or less, regardless of the population.

On 10 May 2021, Presidential Proclamation No. 1143 signed by President Rodrigo R. Duterte declared a State of Calamity throughout the country for one year, acknowledging the urgency of mitigating economic losses and ensuring food supply ([illegible], 2021). The proclamation allowed local government units (LGUs) to allocate funds for quick response activities and the armed forces of the Philippines to provide support in maintaining peace and order in affected areas.

Additionally, the Bantay ASF sa Barangay Program (BABay ASF), a National African Swine Fever Prevention and Control Program, was launched in 2021. The program aimed to prevent and control ASF through surveillance, monitoring, and repopulation efforts. It involved information dissemination, biosecurity measures, and collaboration with local government units.

1.2. Integrated National Swine Production Initiatives for Recovery and Expansion (INSPIRE) Program

To address the decline in pig production due to ASF, the INSPIRE program was conceptualized. The 3-year INSPIRE program is the national repopulation and recovery program of the Department of Agriculture-National Livestock Program (DA-NLP), which offers various interventions to jumpstart and revive the severely affected pig industry following the guidelines of the DA Memorandum Circular No. 24 series of 2020 and its subsequent amendments and revisions. The ASF crisis, however, presents the opportunity to reboot, modernize and restructure the industry for greater efficiency, sustainability and disease resiliency.

While the primary focus of the program is on increasing pig inventory and the breeder base, the program also pays particular attention to clustering and consolidation strategies, specifically for smallholder raisers, to facilitate technical support services and other interventions along the value chain. In support of the repopulation efforts, the program also includes the implementation of biosecurity plans anchored on the BABay ASF Programs.

Government financial institutions have been tapped to provide concessional and flexible loan terms to commercial pig raisers to accelerate recovery. According to the latest Philippine Statistics Authority (PSA) data, commercial pig raisers have lost approximately 30 % of their breeder animals.

Moreover, the program also focused on establishing an acceptable insurance system through the Philippine Crop Insurance Corporation to encourage and restore the confidence of pig raisers. For 2022, the INSPIRE program had a budget of PhP2.75 billion ($49 million), whereas for 2023, the budget was increased to PhP2.95 billion ($52 million) to implement its two major components: Calibrated Repopulation and Recovery of Breeder Base.

1.3. Review of related literature

Most impact analyses in scientific literature discuss animal health program strategies. There is no mention of impact analyses of animal repopulation programs using the time series Seasonal Autoregressive Integrated Moving Average (SARIMA) modelling (which is the unique contribution of this paper to scientific community). This research paper aims to contribute to the literature by addressing this gap and by proposing a new method of measuring impact using existing tools (e.g., SARIMA modeling and cost–benefit analysis). For animal health programs, [illegible] et al. (2022) developed a stochastic model combining transmission between wild boar and domestic pigs, which was calibrated to synthetic data corresponding to different phases describing epidemic progression. This model was then used to produce forward projections describing the likely temporal evolution of the epidemic under various disease management scenarios of ASF.

[illegible] et al. (1999) reported that a cost–benefit analysis based on significantly improved performance parameters demonstrated that Mycoplasma hyopneumoniae vaccination was economically attractive. [illegible] et al. (2016) conducted a cost-benefit assessment of Salmonella-control strategies in pigs reared in the United Kingdom. The study suggested that the cost of implementation outweighed the savings for all interventions, even if the effectiveness could be improved. In addition, to achieve a net cost benefit, reducing the cost of interventions is essential. [illegible] et al. (2008) reported that vaccination with a highly efficacious vaccine may be a cost-effective strategy to control FMD, especially when vaccinated animals are not subsequently slaughtered and there is no future adverse economic impact, such as trade restrictions. [illegible] et al. (2015) applied partial budget analyses and estimated the benefit-cost of vaccinating sow herds preventatively to control porcine reproductive and respiratory syndrome virus control.

[illegible] et al. (2017) conducted a quantitative assessment of the social and economic impacts of ASF outbreaks in northern Uganda. The study indicated that the causal relationship between the social and economic impacts of ASF outbreaks in smallholder systems is complex. Pigs are often kept as passive investments (as in other developing countries) rather than as active working capital, complicating economic analyses and challenging disease control arguments based on only standard economic models.

This paper examines the economic impact of pig repopulation. The purposes of this research paper are as follows: 1) to conduct an ex post evaluation of the impact of INSPIRE in three regions (Central Luzon,

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Table 2
Summary Results for the Central Luzon Forecast (No ASF Scenario).

SARIMA Model	AIC	BIC	MAPE
(1,1,1) (0,1,0)	1466.08	1471.06	4.15

autocorrelation is present in the data up to the specified lag.

Moreover, for Central Luzon with the ASF scenario, the model chosen was SARIMA (0,1,2) (0,1,1), with a MAPE of 10.12 and an AIC of 1804.08 (Table 4). The ACF plot in Fig. 3 shows that only one line exceeds the range, which indicates a favorable model. As shown in Table 5, the Box-Ljung test's p-value of 0.76 indicated white noise (or no autocorrelation).

Fig. 4 shows the resulting impact of INSPIRE in Central Luzon. If there was no INSPIRE project, zero pig production would be forecasted by 2022Q1. The INSPIRE project resulted in additional pig production of 127,269 live weight metric tons (MTs) in 2022 and 180,158 MTs in 2023. Furthermore, it is interesting to note that in a no ASF scenario, the industry would have continued a consistent trend of pig production volume with gradual annual growth. With the sweeping impact of ASF in 2019 and more drastically in 2020, the trend was interrupted, and a slump was recorded. Upon the implementation of the INSPIRE Program, the downward trend of pork production was terminated, and a gradual increase was recorded, showing a sign of recovery of production levels that could be attributed to government policy and limited interventions to the industry through INSPIRE. Despite a slow growth rate, the growth from the period of 2022-2024 has been consistently increasing, showing the program's impact on local industry momentum in terms of pork production. Central Luzon was the Philippine's leading pork-producing region with the presence of commercial-scale pig farms and a robust presence of backyard [illegible]. Being known as home to the country's culinary capital, pork consumption is also high in the area. Medium-scale and cooperative-based farms, though at present, are inferior in size and capacity in Central Luzon compared to corporate pig farms. INSPIRE catered only to these medium-scale and cooperative farms given the limitation provided by relevant implementing guidelines. There is an appreciable impact, as seen in the data, but this did not cause significant recovery back to pre-ASF levels in the time-period covered, because of the slower recovery efforts on the side of the commercial and corporate pig farms that are not covered by direct INSPIRE interventions.

3.2.2. CALABARZON

For the CALABARZON no ASF scenario, the model chosen was SARIMA (1,0,0) (0,1,1), with a MAPE of 5.48 and an AIC of 1416.75 (Table 6). The ACF plot in Fig. 5 shows that only one line exceeds the range, which indicates a favorable model. As shown in Table 7, the Box-Ljung test's p-value of 0.57 indicated white noise (or no autocorrelation).

Moreover, for the CALABARZON with ASF scenario, the model chosen was SARIMA (0,1,0) (0,1,0), with a MAPE of 5.34 and an AIC of 1662.22 (Table 8). The ACF plot in Fig. 6 shows that only one line exceeds the range, which indicates a favorable model. Based on the data in Table 9, the Box-Ljung test's p-value of 0.94 indicated white noise (or no autocorrelation).

Fig. 7 shows the resulting impact of INSPIRE on CALABARZON. If there was no INSPIRE project, zero pig production would be forecasted by 2023Q1. The INSPIRE project resulted in additional pig production of 137,735 live weight MT in 2022 and 233,204 MT in 2023. In a similar way to that of Central Luzon, the swine industry of CALABARZON was set to continue a trend of annual increase in growth but was halted with the ASF problem evident in the significant fall of production volume in the year 2020. Recovery efforts, both in the private and government sectors resulted in a slight increase in production in 2021. This trend, however, was punctuated with subsequent decrease in 2021 and the early half of 2022 because of episodes of resurgence of ASF outbreaks in the region. From there, with the INSPIRE intervention in full swing, the data show that there has been a gradual increase in pork production levels. The CALABARZON swine industry landscape is more dominated by small and medium-scale pig farms and cooperative-based swine farming. Although there exist commercial level farms in the region, farmer-level production is practiced widely because of a stronger entrepreneurial drive and capability among individuals and cooperatives. Despite the risk of ASF and reinfection, this entrepreneurial drive enabled farmers and cooperatives to manage the risk and proceed with the repopulation efforts as most of their livelihoods only center on pig farming. This regional landscape allowed INSPIRE to cater to more small hold, medium-scale and cooperative-based farmers that led to a higher growth in pig production levels within the period covered in this study. This landscape too, could be the reason for punctuated growth patterns because of sporadic recurrence of outbreaks given the inferior biosecurity capabilities among non-commercial raisers.

Table 3
Box-Ljung Test for the Central Luzon Forecast (No ASF Scenario).

X-squared	p-value
0.31	0.77

Table 4
Summary Results for the Central Luzon Forecast (with the ASF Scenario).

SARIMA Model	AIC	BIC	MAPE
(0,1,2) (0,1,1)	1804.08	1813.76	10.12

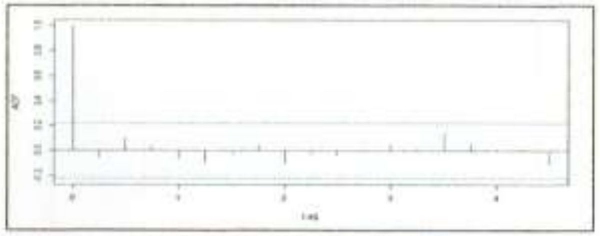

Fig. 2. ACF Results for the Central Luzon Forecast (No ASF Scenario).

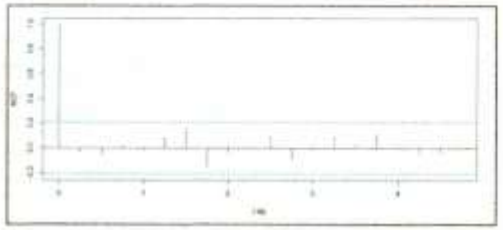

Fig. 3. ACF Results for the Central Luzon Forecast (with the ASF Scenario).

Table 5
Box-Ljung Test Results for the Central Luzon Forecast (with the ASF Scenario).

X-squared	p-value
0.09	0.79

3.2.3. Davao region

For the Davao Region no ASF scenario, the model chosen was SARIMA (0,0,0) (0,1,0), with a MAPE of 3.74 and an AIC of 1270.53 (Table 10). The ACF plot in Fig. 8 shows that only one line exceeds the range, which indicates a favorable model. As shown in Table 11, the Box-Ljung test's p-value of 0.42 indicated white noise (0,1,0).

Moreover, for the Davao region with the ASF scenario, the model chosen was SARIMA (2,0,0) (0,1,0), with a MAPE of 2.92 and an AIC of 1466.55 (Table 12). The ACF plot in Fig. 9 shows that only one line exceeds the range, which indicates a favorable model. Based on the data in Table 13, the Box-Ljung test's p-value of 0.92 indicated white noise (or no autocorrelation).

Fig. 10 shows the resulting impact of INSPIRE in the Davao Region. The forecasted Davao (with the ASF forecast) data does not differ much from the Davao PSA pig production data. Part of this reason could be that the region was hit by ASF later than the 2 other regions and to a lesser extent. Compared with the Davao PSA data, the 4th quarters of 2022 and 2023 of Davao (with the ASF forecast) are even higher, which suggests that INSPIRE negatively affected pig production during those two quarters. The ASF scenario crippled the supposed trajectory of growth of pig production in the Davao region. While the actual industry production managed to narrow the difference that ASF caused in terms of pig production levels vis-a-vis the normal expected trend, it is important to note that the characteristics of the swine industry in the Davao region are dominated by smallholder farms compared to the number of medium-scale and commercial-scale pig farms. According to Table 14, compared to the budget allocations that Central Luzon (PhP1.4 billion or $24.8 million) and CALABARZON (PhP898 million or

Table 6
Summary Results for CALABARZON Forecast (No ASF Scenario).

SARIMA Model	AIC	BIC	MAPE
(1,0,0) (0,1,1)	1416.75	1425.44	5.48

Fig. 4. Central Luzon Pig Production Comparison, 2018–24.
Source: PSA, authors' estimations

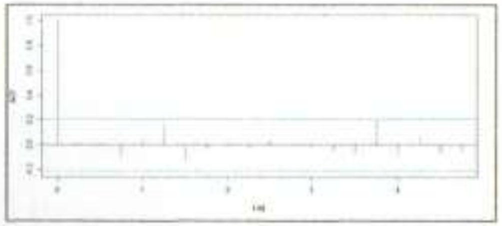

Fig. 9. ACF Results for the Davao Region Forecast (with the ASF Scenario).

Table 13
Box-Ljung Test Davao Region Forecast (with the ASF Scenario).

X-squared	p-value
0.01	[illegible]

including the Philippines ([illegible], 2011). Compared with Central Luzon and the Davao Regions, CALABARZON produced the highest returns. Moreover, the Davao region produced the lowest returns compared with the other two regions.

Table 14
Budget Allocation for the 3 Regions under INSPIRE (in PhP).

Regions	2022 Budget	2023 Budget	2024 Budget	Total
Central Luzon	836,300,000	600,064,000	0	1,436,364,000
CALABARZON	318,000,000	598,000,000	30,000,000	946,000,000
Davao Region	272,000,000	140,000	0	242,140,000

Source: DA-NLP Budget Utilization Report.
Note: The PhP-to-$ exchange rate for 2022–2023 is PhP56 = $1.

Table 15
Economic Analysis of INSPIRE Projects for 2022–2023.

Location	2022 INSPIRE	2023 INSPIRE
Central Luzon CB Ratio	29	36
Central Luzon NPV, 12 %	PhP20,376,565,018	PhP19,429,313,951
CALABARZON CB Ratio	42	112
CALABARZON NPV, 12 %	PhP32,032,319,978	PhP38,693,802,144
Davao Region CB Ratio	2	6
Davao Region NPV, 12 %	PhP314,246,034	PhP966,391,568

Note: The PhP-to-$ exchange rate for 2022–2023 is PhP56 = $1.

3.4. Investment requirements for pig production

Since 2025, there has been a need for Philippine pig production to increase by 6.6 % annually from 2025 to 2028 to return to the pre-ASF production of 2.32 million MT (Fig. 11). If the current NLP budget is maintained, there is even a decline in pig production because of recent and continuing ASF cases. The 2000Q1 to 2024Q2 datasets were used to forecast the remaining quarters until 2028Q4, with the assumption that the DA-NLP budget will remain at its current level. The chosen model was SARIMA (1,1,1)(1,1,1), with a MAPE of 2.22 and an AIC of 2093.69 (Table 16). The ACF plot in Fig. 12 shows that only one line exceeds the range, which indicates a favorable model. Based on the data in Table 17, the Box-Ljung test's p-value of 0.98 indicated white noise (or no autocorrelation).

Surprisingly, a downward trend in pig production was detected. If the current actual budget level to the repopulation program is maintained in the medium term, pig production may decline. This suggests that a higher budget (2–3 times the current budget) for the implementation of pig industry repopulation is needed to arrest the decline in pig production and achieve the goal of the INSPIRE program of restoring the Philippine pig sector to its pre-ASF 2018 levels.

Table 18 shows the investment requirements needed to increase pig production to 6.6 % annually until the end of 2028, when production presumably returned to pre-ASF 2018 levels. It is not necessary that the government shoulder all the investment requirements. The government merely acts as an enabler of the environment and as a jump starter to increase the confidence of commercial and backyard raisers in pig production. Pig farmers will raise pigs naturally if it is profitable for them to do so, as envisioned by the invisible hand of Adam Smith in the Wealth of Nations (1800).

The private sector has shown a willingness to invest towards pig production recovery as noted in the following announcements. In southern Philippines (Gingoog City, Misamis Oriental), Mabuhay Agro Ventures Corporation is partnering with Denmark-based Danbred to establish a large-scale genetic pig farm aimed at supporting repopulation efforts in the wake of ASF to the tune of PhP2.8 billion or $48.3 million (Prescilla, 2023). JG Agroventures, Inc., forged a long-term agreement with PIC, the global leader in swine genetics, to establish and operate a breeder nucleus and multiplier farm in Nueva Ecija (Central Luzon) that will significantly expand PIC's capacity to produce breeders locally and disseminate genetic improvement to help Filipino farmers rebuild and improve the industry (PIC, 2023). Apart from those announced in the media and those seeking endorsement for tax holidays from Department of Agriculture (DA), it is difficult to analyze private sector investments.

The unique contribution of this paper to the evaluation literature is the use of the time series SARIMA modelling to measure the impact of pig repopulation program (INSPIRE) in the Philippines by modelling data sets with ASF and without ASF scenarios. A related study is that of the Error-Correction Model used by Mutu et al. (2014) when they modelled Philippine chicken and beef prices with and without FMD. Other related studies are that of Levy (1988) and McGuire (1987). Levy (1988) used intervention analysis, a form of time series, to ascertain the effects of the program in the Philippines. McGuire (1987) applied multiple time series analyses which are applied to government decision inputs (the number of dredge and fill permits issued) and environmental quality outputs (the number of water quality violations) to test the use of multiple time series methods on natural resources policy data (aquatic preserve management).

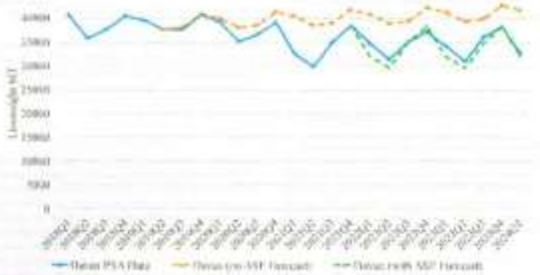

Fig. 10. Davao Pig Production Comparison.
Source: PSA, authors' calculations.

Table 16
Summary Results for the Philippine Pig Production Forecast (with the ASF Scenario).

SARIMA Model	AIC	BIC	MAPE
(3,1,1)(3,1,1)	3093.63	3106.33	3.33

4. Lessons learned

The lessons learned from this study can be summarized in two. First, time series ARIMA modelling using the with ASF and without ASF scenarios are useful in evaluating the impact of a pig repopulation program. Previous internal tools used DA to the animal repopulation impacts are Excel-based and simple forecasting. With the use of time series ARIMA modelling in R, the method becomes scientific. And lastly, there are regions that produce minimal results even with infusion of financial resources. Given the budget limitation and the need for the prudent use of taxpayers' money, the results of this paper will be very useful to the broader international evaluation community. Based on the results of Fig. 4, Fig. 7, Fig. 10, and Table 14, there are large economic impacts caused by the pig repopulation program to Central Luzon and CALABARZON, whereas it is minimal only in the Davao Region. This means that, whether there is INSPIRE (or any pig repopulation program) or not, the Davao Region will continue to produce pigs. Since the government is always scrutinizing the object and is always looking for value for taxpayers' money, there is a need to allocate scarce government resources where it will have the greatest economic impact or gain. In designing future pig repopulation programs, the Philippine government should first measure the economic impacts of the 2022–2023 INSPIRE on the 13

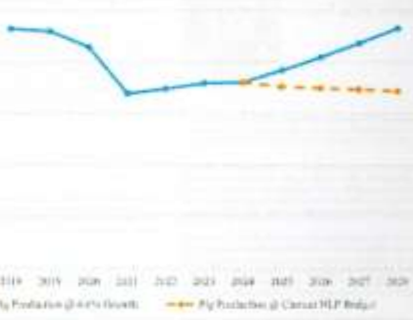

Fig. 11. Pig Production Higher Budget vs. Current NLP Budget.
Source: PSA, authors' forecasts.

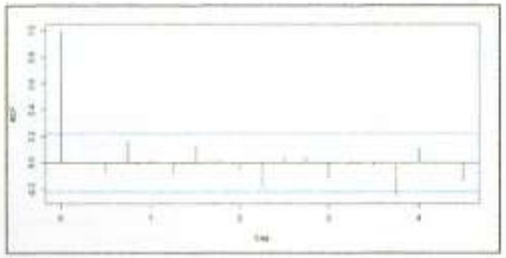

Fig. 5. ACF Results for CALABARZON Forecast (No ASF Scenario).

Table 7
Box-Ljung Test for CALABARZON Forecast (No ASF Scenario).

X-squared	p-value
0.02	0.97

Table 8
Summary Results for the CALABARZON Forecast (with the ASF Scenario).

SARIMA Model	AIC	BIC	MAPE
[illegible]	[illegible]	[illegible]	[illegible]

$14.8 million) received during the period for the implementation of the INSPIRE Program from 2022 to 2026. Davao Region received a lesser amount (PhP242 million or $4.2 million) which despite yielding outputs, are not that significant in making significant effects in the cumulative regional production levels. This could also be attributed to the continuous and active repopulation initiatives of the private sector in the medium-scale and commercial level farms.

Government programs for livestock industries that particularly involve subsidies, grants, and assistance to domestic animal production and stakeholders prove to have an impact on the production levels of livestock in the medium term. In Türkiye, government funding for livestock supports markedly increased over a 10-year period, from a mere 8.2 % ratio of the total agricultural budget in 2004–29.7 % in 2014 (Tas et al., 2016). This, in turn, resulted in a steady increase in the inventory and production volume of major livestock and poultry commodities in the same period, with growth extending even up to the 2030 forecasts ([illegible], 2021). Similarly, government support for farm investments and activities fostered significant farm growth in the livestock sector of Austria, which showed a rapid response to investments in terms of inventory and farm size (Kirchweger & Kantelhardt, 2015). Among highly self-sufficient countries in beef, an analysis of government expenditures and support revealed their effectiveness in maintaining and improving production levels ([illegible] et al., 2019).

Programs, and thereby the expenditures allocated by governments for the development of the livestock sector, result in appreciable gains in terms of growth in inventory and production levels. Like the impact of the INSPIRE program of the Philippine government, the pig sector benefitted to a certain extent, particularly in averting massive losses in pig inventory and pig-based livelihoods. The responsiveness of the sector to developmental interventions initiated by the government, demonstrated through production levels, is worth noting in the assessment of the success and effectiveness of the program. This study is the first to analyse the impact of a government program for pig industry repopulation in response to the damage of ASF that directly aided pig farmers with the introduction of farm structural reforms. More than policy and regulations, the Philippine government's approach to agricultural development heavily involves direct production support to farmers, and this is the main strategy of the INSPIRE Program. The study, therefore, is in a unique position to provide analysis of industry implications of government budget allocations and production support inputs directly given to farmers.

Table 9
Box-Ljung Test Results for the CALABARZON Forecast (with the ASF Scenario).

X-squared	p-value
0.04	0.84

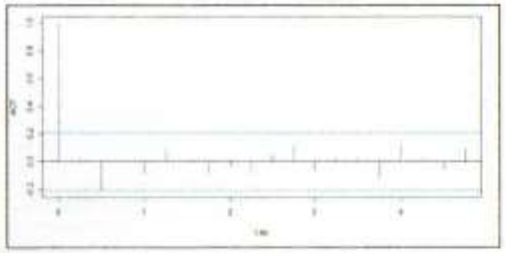

Fig. 6. ACF Results for CALABARZON Forecast (with ASF Scenario).

Fig. 7. CALABARZON Pig Production Comparisons.
Source: PSA, authors' calculations.

Table 10
Summary Results for the Davao Region Forecast (No ASF Scenario).

SARIMA Model	AIC	BIC	MAPE
[illegible]	[illegible]	[illegible]	3.74

3.3. *Economic analysis*

Table 17 shows the calculation methodology for the CBA and NPV. Since this is a 5-year measurement of the effects, it is easier to calculate as opposed to multiyear effects (CBA can also be used for multi-year calculations depending on the purpose). The discount rate used was 12 %. The Asian Development Bank has recommended the use of a 10–12 % discount rate for the appraisal of projects in its Member States.

Table 11
Box-Ljung Test for the Davao Region Forecast (No ASF Scenario).

X-squared	p-value
[illegible]	[illegible]

Table 12
Summary Results for the Davao Region Forecast (with the ASF Scenario).

SARIMA Model	AIC	BIC	MAPE
[illegible]	[illegible]	[illegible]	[illegible]

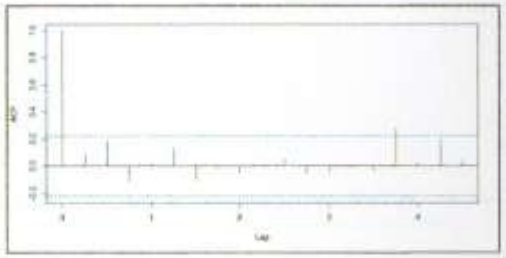

Fig. 8. ACF Results for the Davao Region Forecast (No ASF Scenario).

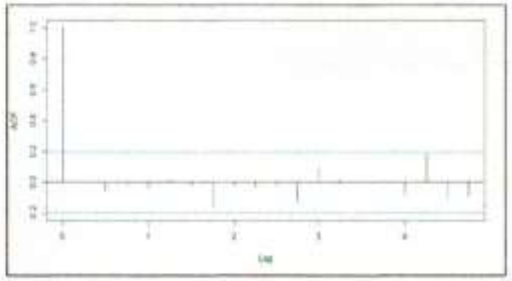

Fig. 15. ACF for the Philippine Pig Production Forecast (with the ASF Scenario).

Table 17
Box-Ljung Test for Philippine Pig Production Forecast (with the ASF Scenario).

X-squared	p-value
6.90	[illegible]

Table 18
Investment Requirements Needed to Grow Pig Production to pre-ASF 2019 PSA Figures.

	2025	2026	2027	2028
Additional fatteners in heads (@ 100 kgs per head)	[illegible]	[illegible]	[illegible]	[illegible]
Additional target production (liveweight MT)	[illegible]	[illegible]	[illegible]	[illegible]
Total target production based on PSA (liveweight MT)	[illegible]	[illegible]	[illegible]	[illegible]
Investment requirements (billion PhP)	[illegible]	[illegible]	[illegible]	[illegible]

Source: Authors' calculations
Note: The PhP to $ exchange rate for 2024 is PhP/$1 = [illegible].

other Philippine regions (except the National Capital Region where pig production is almost nil). Regions that have a relatively high economic CB ratio (at least 30) receive relatively high pig repopulation investments, whereas those with a low economic CB ratio receive minimal investments.

5. Conclusions

In this study, we examined the policy of pig repopulation considering ASF outbreaks. We also suggest a new approach for evaluating the impacts of pig repopulation programs by existing methods (e.g., SARIMA modeling, CBA, and NPV). We concluded that pig repopulation is a necessary food policy, as it shows that the INSPIRE program was restored and inspired confidence in pig production in 2022–2023, which otherwise would have declined steeply in Central Luzon and CALABARZON. However, pig repopulation investments in the Davao Region produced minimal effects. Moreover, this study was also able to demonstrate favorable cost-benefit ratios and economic values that indicate the viability and practicality of the interventions delivered to beneficiaries and the sector. However, there is still much to be done to return pig production to pre-ASF 2018 production. Hence, continuing the implementation of the pig industry repopulation program with an even higher budget allocation (3–4 times the current budget) is necessary. This is the first study in the Philippines which modelled time series SARIMA to determine the impact of a pig repopulation program and combined with CBA to determine investments needed to get back to pre-ASF pig production level.

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CRediT authorship contribution statement

Lary Nel Bilbao Abao: Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Formal analysis, Conceptualization. **Alvin Paul J. Dinula:** Writing – review & editing, Formal analysis, Conceptualization. **Roland Yap Fajardo:** Writing – review & editing, Supervision, Formal analysis, Data curation. **Ruth Miclat Sonaco:** Writing – review & editing, Supervision, Formal analysis, Data curation. **Miguel Valcerdes Gulogulo:** Writing – review & editing, Methodology, Formal analysis. **Joseph Frank Mgaya:** Writing – review & editing, Software, Formal analysis.

Declaration of Competing Interest

The authors declare no competing interests.

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Authors' contributions

All authors contributed to reviewing the topics, statistical analysis and writing the present article. All authors read and approved of the final manuscript.

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Table 1
Detailed list of primers for qRT-PCR analysis of target genes.

Target gene	GenBank accession	Primer	Size of amplified product (bp)
HSP70	NC_056071.1	F: [illegible] R: [illegible]	63
IL10	NC_056069	F: [illegible] R: [illegible]	110
TLR2	NM_001048231.1	F: [illegible] R: [illegible]	66
TLR4	NM_001135930.1	F: [illegible] R: [illegible]	77
TLR9	NM_001115881.1	F: [illegible] R: [illegible]	77
GAPDH	NC_040254.1	F: [illegible] R: [illegible]	80

cDNA equivalent to 5 ng of starting total RNA was used as template for each real-time PCR reaction. Forward and reverse primers (Table 1) were created with Primer Express v3.0.1 software (Applied Biosystems, Foster City, CA, USA) and confirmed for target identity with Primer Blast from the National Center for Biotechnology Information (NCBI) (Ye et al., 2012). Real-Time PCR System, Roche Light Cycler96 was used for qPCR with a 1-min denaturation, followed by 50 cycles of 95 °C for 15 s, 62 °C for 20 s and 72 for 15 s. High resolution melting analysis was performed for each run.

Reactions were set up with PowerUpTM SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturers' instructions. The amount of cDNA equivalent to 5 ng of starting total RNA was used as template for each real-time PCR reaction.

For relative gene expression studies, one housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) was amplified alongside the target genes (IL10, TLR2, TLR4, TLR9, and HSP70). Quantitative PCR was done in triplicate for each sample.

2.4. Statistical analysis

Using the Pfaffl method (Pfaffl, 2001) which requires primer efficiency for both gene of interest (GOI) and housekeeping genes using a standard curve and its cycle threshold (Ct) values and accounts for differences in primer efficiencies during the fold change calculation. The relative quantification of the target gene was determined by comparing the expression levels of GAPDH as a reference gene and GOI of HSP70, IL10, TLR2, TLR4, and TLR9 with Spring season value as the calibrator. The standard deviation (SD) of the relative gene expression was determined by calculating the mean value of the breed population's gene expression ratio per GOI, which then use to calculate the individual deviation within the breed. LinReg PCR version 2017.0 software (Ramakers et al., 2003) was used to calculate primer efficiency. Further, because the substantial variation in relative gene expression levels makes finding statistically significant differences between groups more difficult, Ct values with less fluctuation were employed to detect statistically significant differences between groups. Ct value data were analyzed by mixed Analysis of variance (ANOVA) with a general linear model (GLM) with repeated measurement in SPSS Version 25 (IBM Corp., Armonk, NY, USA), with breed, sex, season, breed*sex, breed*season, sex*season, and breed*sex*season as the mixed design factors. Mauchly's test was used to decide the assumption of sphericity in the repeated measurement, and Levene's test was based on the median for the equality of error variance within season measurement. Further, a post-hoc multiple comparison test was done using the Tukey test for breed and season as the factors. Visualization of the data was done using GraphPad Prism version 8.0.0 for macOS (GraphPad Software, San Diego, CA, USA). The results are shown as the mean ± SD. A difference with $p < 0.05$ was determined as statistically significant.

3. Results

3.1. Climatological conditions

The climatic data during the sampling day in each season of the year and the calculated temperature humidity index (THI) is presented in Table 2. The THI in all sampling days were within the thermoneutral zone, except for summer season which was 78.99.

3.2. CT value

The gene expression of all GOI (Table 3) in this study were significantly different ($p < 0.05$) in each season, showing the dynamic gene expression according to seasonal variation. The interaction between the season, breed, and sex were identified in IL10 gene expression ($p < 0.05$), indicating the changes in gene expression in each season were not equivalent in each breed and sex group. According to the between-group test, it was found that there were significant differences in gene expression ($p < 0.05$) in each breed group across the season for IL10 and TLR4 genes.

3.3. Relative gene expression

The relative expression was calculated using the Pfaffl method (Pfaffl, 2001) and is presented in Table 4 and visualized as Fig. 1 (A-E). The spring season was considered as a thermo-neutral season and used as a calibrator.

For HSP70, relative gene expression with different peak was observed: spring for Hungarian indigenous Tsigai (1.039 ± 0.326) and White Dorper (1.074 ± 0.441), summer (7.494 ± 11.932) for Hungarian Merino. In all GOI in this study, the lowest expression relative to the spring season was found in the autumn.

An increasing gene expression trend was identified for IL10 in the summer season. The highest relative gene expression means of IL10 were found in the summer season for White Dorper (17.355 ± 5.848) and Hungarian indigenous Tsigai (6.299 ± 0.412), but for Hungarian Merino, the highest expression was identified in the winter season (2.553 ± 2.128).

For the two Toll-like receptor genes, TLR2 and TLR4, in spring and autumn, the gene expression tend to be low and a higher relative gene expression was observed in winter and summer. Both genes were observed to be overexpressed during the summer time in all breeds, with the mean relative gene expression value of 12.053 ± 11.036, 3.517 ± 2.720, and 14.263 ± 13.447 for TLR2 in Hungarian indigenous Tsigai, Hungarian Merino, and White Dorper, respectively. While for TLR9 were 5.747 ± 5.481, 7.497 ± 6.507, and 6.910 ± 8.130, respectively.

Besides that, for TLR4, the highest expression was observed in the summer for Hungarian Merino (15.204 ± 19.950) and White Dorper (14.263 ± 13.417), but not for Hungarian indigenous Tsigai, which was observed in the winter (2.283 ± 1.817). But still, a lower expression was observed during the thermoneutral seasons; spring and autumn.

4. Discussion

The health and well-being of animals are determinant factors in achieving maximum productivity. The seasonal stressor is one of the major components in achieving this optimum profit in livestock farming, as has been proven by many previous findings

Table 2
The THI in each sampling season.

Season	Date	Time	Temperature (°C)	Relative humidity (%)	THI
Spring	20/04/2020	11.00–11.30	19.11	61.00	64.54
Summer	13/08/2019	11.00–11.00	32.85	34.50	78.99
Autumn	19/11/2020	11.00–11.30	7.84	76.84	46.15
Winter	22/01/2020	11.00–11.30	1.51	80.39	34.65

Table 3
Mean and standard deviation of CT value of IL10, TLR2, TLR4, TLR9, and HSP70 of Hungarian Indigenous Tsigai, Hungarian Merino, and White Dorper in each season of the year.

Gene	Season	CT-Value (Mean ± Std. Deviation)			
		Hungarian Indigenous Tsigai	Hungarian Merino	White Dorper	Overall
HSP70	Summer	22.540 ± 0.473	22.500 ± 0.279	22.566 ± 0.628	22.536 ± 0.462^{ab}
	Autumn	24.875 ± 1.032	24.411 ± 1.266	23.940 ± 0.758	24.393 ± 0.002^{c}
	Winter	22.582 ± 1.046	22.371 ± 0.055	21.536 ± 0.597	22.053 ± 0.673^{a}
	Spring	22.837 ± 0.634	23.203 ± 0.106	22.885 ± 0.861	23.015 ± 0.763^{b}
	Overall	23.446 ± 1.271^{a}	23.067 ± 1.013^{a}	22.712 ± 1.006^{a}	23.084 ± 1.163
IL10	Summer	32.493 ± 1.581	31.857 ± 1.055	32.122 ± 1.696	32.160 ± 1.665^{a}
	Autumn	31.215 ± 0.757	30.137 ± 0.624	30.145 ± 0.441	30.648 ± 0.752^{b}
	Winter	28.808 ± 1.486	28.524 ± 0.095	28.125 ± 0.446	28.494 ± 1.218^{c}
	Spring	29.755 ± 0.881	25.473 ± 0.351	28.100 ± 0.527	29.441 ± 0.742^{c}
	Overall	30.813 ± 1.722^{a}	30.250 ± 1.761^{ab}	29.658 ± 1.802^{b}	30.246 ± 1.876
TLR2	Summer	26.927 ± 1.259	26.486 ± 1.003	26.115 ± 1.267	26.487 ± 1.201^{a}
	Autumn	27.528 ± 1.370	25.576 ± 0.801	23.698 ± 0.548	26.395 ± 1.140^{a}
	Winter	24.195 ± 1.214	24.113 ± 1.229	23.837 ± 0.243	24.075 ± 0.860^{b}
	Spring	24.453 ± 0.779	22.840 ± 0.167	24.095 ± 0.807	24.180 ± 0.716^{b}
	Overall	25.798 ± 2.022^{a}	24.663 ± 1.261^{ab}	24.430 ± 1.262^{b}	25.261 ± 1.539
TLR4	Summer	24.017 ± 0.721	24.161 ± 0.548	23.502 ± 0.608	23.920 ± 1.000^{a}
	Autumn	26.613 ± 1.676	25.690 ± 1.521	25.720 ± 0.620	26.061 ± 1.640^{b}
	Winter	23.605 ± 0.275	22.787 ± 1.278	22.483 ± 0.451	22.396 ± 0.677^{a}
	Spring	24.189 ± 0.767	23.617 ± 0.277	23.095 ± 0.539	23.647 ± 0.581^{a}
	Overall	24.727 ± 1.367^{a}	24.481 ± 1.382^{a}	23.768 ± 1.292^{a}	24.226 ± 1.385
TLR9	Summer	26.572 ± 1.185	27.030 ± 1.494	25.873 ± 1.534	26.366 ± 1.310^{a}
	Autumn	25.651 ± 0.585	24.830 ± 1.126	24.398 ± 0.075	25.081 ± 1.062^{a}
	Winter	23.600 ± 1.538	23.670 ± 1.653	23.048 ± 0.557	23.367 ± 1.199^{b}
	Spring	23.381 ± 0.767	23.353 ± 0.626	22.562 ± 0.537	22.918 ± 0.638^{b}
	Overall	24.858 ± 1.708^{a}	24.428 ± 1.616^{ab}	23.656 ± 1.539^{b}	24.305 ± 1.598
GAPDH	Summer	26.540 ± 2.218	25.011 ± 2.806	25.685 ± 3.166	26.032 ± 2.616
	Autumn	22.677 ± 1.181	21.456 ± 5.218	23.962 ± 0.367	22.203 ± 0.056
	Winter	21.830 ± 1.724	21.787 ± 1.986	21.167 ± 1.462	21.511 ± 1.467
	Spring	21.983 ± 0.757	21.716 ± 0.295	21.640 ± 0.054	21.663 ± 0.541
	Overall	23.240 ± 2.581	22.456 ± 2.513	22.650 ± 2.590	22.857 ± 2.386

SD: Standard deviation; ^{a,b}: different superscript showed significant difference ($p < 0.05$).

Table 4
Relative gene expression calculation with Pfaffl method [23] of IL10, TLR2, TLR4, TLR9, and HSP70 of Hungarian indigenous Tsigai, Hungarian Merino, and White Dorper in each season of the year relative to spring season as a calibrator.

Gene	Season	Relative gene expression (Mean ± SD)		
		Hungarian Indigenous Tsigai	Hungarian Merino	White Dorper
HSP70	Summer	0.777 ± 0.242	7.494 ± 11.932	0.657 ± 0.419
	Autumn	0.327 ± 0.192	0.378 ± 0.303	0.391 ± 0.003
	Winter	1.030 ± 0.089	2.487 ± 2.042	0.524 ± 0.408
	Spring	1.039 ± 0.326	1.054 ± 0.365	1.074 ± 0.441
IL10	Summer	6.299 ± 0.412	1.976 ± 1.642	17.355 ± 5.848
	Autumn	0.578 ± 0.175	0.304 ± 0.126	1.302 ± 0.108
	Winter	0.830 ± 0.518	2.553 ± 2.128	4.992 ± 1.364
	Spring	1.124 ± 0.576	1.026 ± 0.254	5.498 ± 6.417
TLR2	Summer	12.053 ± 11.036	3.517 ± 2.720	14.263 ± 13.447
	Autumn	0.380 ± 0.213	0.300 ± 0.054	0.415 ± 0.716
	Winter	7.062 ± 7.176	1.835 ± 2.528	1.644 ± 1.605
	Spring	1.029 ± 0.361	1.039 ± 0.231	1.307 ± 0.432
TLR4	Summer	6.779 ± 6.100	15.204 ± 19.950	14.263 ± 13.417
	Autumn	6.428 ± 0.360	0.124 ± 0.201	0.419 ± 0.176
	Winter	2.283 ± 1.817	3.414 ± 4.013	1.649 ± 1.605
	Spring	1.021 ± 0.242	1.038 ± 0.366	1.167 ± 0.403
TLR9	Summer	5.747 ± 5.481	7.497 ± 6.507	6.910 ± 8.130
	Autumn	0.257 ± 0.085	0.842 ± 0.353	0.320 ± 0.088
	Winter	1.593 ± 1.874	8.637 ± 9.523	0.868 ± 1.160
	Spring	1.063 ± 0.496	1.271 ± 1.078	1.186 ± 0.597

SD: Standard deviation.

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Original article

Hungarian indigenous Tsigai, a promising breed for excellent heat tolerance and immunity

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ABSTRACT

The adverse effects of climate change on sheep farming have become more noticeable in recent decades. Extensive efforts have been made to untangle the complex relationship between heat tolerance, animal health, and productivity, also to identify a resilient and economically suitable breed for selection that can be resilient to future climate change conditions. Using quantitative real-time polymerase chain reaction (qRT-PCR), we observed the seasonal variations in the expression of several important genes related to heat stress and immunity (HSP70, IL10, TLR2, TLR4, and TLR8) in three of the most widely kept sheep breeds in Hungary: The indigenous Tsigai, Hungarian Merino, and White Dorper. We found that the seasonal stressor affected the relative gene expression of all genes in this study. Notably, The Hungarian indigenous Tsigai was the most robust breed adapted to the Hungarian continental (hot summer, cold winter) environment, with excellent thermotolerance and immunity. Furthermore, despite suffering from heat stress in the summer, Hungarian Merino maintained their robust immune system well throughout the year.

1. Introduction

Sheep (Ovis aries) have been thought to be one of the most robust livestock that has inhabited a wide range of geographical areas since their domestication due to their exceptional adaptability to various types of diets and tolerance to adverse climatic conditions (Kijas et al., 2009; Sawyer and Narayan, 2019). However, in these constantly changing climate conditions, sheep are facing additional stressors (Abdul Niyas et al., 2021; Michaba et al., 2021). These challenges have significant economic consequences as well. Farmers, of the one of the major sheep exporters, New Zealand, for example, have been facing impacts of climate change for more than a decade, which unfavourably determine the profitability of their production (Wreford et al., 2009). Heat stress seriously endangers reproduction of certain sheep breeds and thus appears as a notably risk for efficient sheep management (Van Wettere et al., 2021).

Many aspects of cellular behaviour must change in response to environmental stressors, one of them is the changes in gene expression which are a fundamental component of stress reactions (de Nadal et al., 2011) as are changes in metabolism, cell cycle progression, protein homeostasis, cytoskeletal architecture, vesicular trafficking, and enzymatic activity modification, which requires more energy expenditure and may compromise animals' growth potential and productive capacity (Richter et al., 2010). Excessive heat exposure triggers multiple cellular responses and induces transcription and translation of several genes and modifications in protein synthesis, such as increased expression of heat shock proteins (HSPs), which function as intracellular chaperones in heat-stressed animals, preventing protein and cell damage (Hooper et al., 2018).

The expression of HSPs is a possible sign of animal response to intense environmental stress. On the basis of their molecular size and similarity in amino acid sequence, HSPs are divided into multiple families (Morange, 2006). Genetic and biochemical research indicates that the 70-kDa heat shock protein (HSP70) family assists in the folding of proteins during translation in the cytoplasm of both prokaryotic and eukaryotic cells and is primarily found in cytosol and nucleus. There are constitutively expressed and inducible versions of HSP70 that are activated by stress stimuli (Hansen, 2004). Its expression is a possible indicator of an animal's capacity to adjust to intense environmental stress; a strong association exists between the development of HSPs and the induction of thermotolerance through the inhibition of stress kinase activity (Dangi et al., 2014).

Despite the disruption in HSP cellular mechanism, the alteration in immune-related gene regulation to either compromise or enhance immune cell activity is also induced during heat shock (Kumar et al., 2020). Interleukin-10 (IL10) is a major inflammatory regulator released by macrophages, regulatory T cells, dendritic cells and certain epithelial cells (Mallikarjunappa et al., 2020). In sheep, IL10 has been linked to susceptibility to various diseases, such as Peste des petits ruminants virus (PPRV) (Wani et al., 2018), bluetongue virus (BTV) (Sánchez-Vizcaíno et al., 2015), and *Haemonchus contortus* infection (Gass, 2012). Another one is the Toll-like receptors (TLRs), an extensively studied immune-related gene expressed on numerous cell types, including mucosal surface cells and tissue immune cells (Díaz-Tamayo et al., 2021). TLRs are a collection of evolutionarily conserved pattern-recognition receptors (PRRs) that can detect a variety of pathogen-associated molecular patterns (PAMPs) from a wide range of microorganisms and activate innate immune responses (Janeway and Medzhitov, 2002) and are found to be essential for host resistance to gastrointestinal parasitic infection (Benavides et al., 2016) and classical Scrapie in sheep (García-Martínez et al., 2022).

With the threat of unavoidable climate change and its harmful effects, effort are constantly made to thoroughly comprehend how environmental conditions generated some coping mechanisms in the animal's body. Many studies has reported the seasonal effect on heat-stress related gene expressions in livestock (Singh et al., 2014; Archana et al., 2018; Kumar et al., 2018). Given the scarcity of research on studies of seasonal gene expression in sheep, this study aims to describe the seasonal changes of relative expression level of the following heat stress and heat stress-related genes: *HSP70, IL10, TLR2, TLR4,* and *TLR8* in three sheep breeds reared in Hungary today (Hungarian indigenous Tsigai – indigenous breed in Central-Eastern-Southern Europe; Merino – worldwide spread sheep breed, its dispersal started in the 18th century in Hungary; White Dorper – tropical origin, was introduced in Hungary 10 years ago), with the goal of observing the adaptability of the three breeds to Hungarian temperate climatic conditions, also to contribute with valuable knowledge on biological response to seasonal stressors in sheep.

2. Materials and methods

2.1. Sample collection and location

Total blood sample was initially collected from 24 animals (12 ewes and 12 rams) from three different breeds: Hungarian Merino, Hungarian indigenous Tsigai, and White Dorper, four rams and four ewes per breed. All three breeds represent the major breeds reared in Hungary. Hungarian indigenous Tsigai is a natural breed belonging to the Tsigai group, indigenous to the Eastern and Central Europe. The Hungarian Merino is the result of Merino breeding in Hungary beginning in the late 17th century and continuing into the 18th century, when it began to be crossed with local breeds. White Dorper is a relatively new breed in Hungary imported from South Africa. All animals included in the study were relatively the same age (2 to 3 years old), body weight (ewes: 45 to 55 kg; rams: 65 to 75), and in excellent health condition with no physical and anatomical abnormalities. The animals were kept in the Kismacs Experimental Station of Animal Husbandry of the University of Debrecen, located at 127 m above sea level (47.58° N and 21.59° E). The average annual maximum and minimum ambient temperature ranges from −7.5 to 28.0 °C with annual precipitation of 550 to 600 mm. The sampling was done in four different seasons in 2019–2020: spring (April), summer (August), autumn (November) and winter (January). All animals received the same management during the experiment period. The breeds were kept and fed together throughout the year, segregated by sex in sheep shed technology, which included an enclosure. The exception was the autumn breeding season, when they were kept in harem. All animals are feed ad libitum a diet which consisted of hay + fodder 0.4 kg/sheep/day, with free access to clean water. Fodder contained 50% corn, 50% oats. During lambing, ewes receive an extra 1 kg of alfalfa hay. 4 weeks before insemination and during insemination, they were additionally given alfalfa silage 1 kg/sheep/day. Selenium lick blocks were available to the animals for 365 days.

Due to the lengthy research period, several animals had to be culled (dead or removed from the farm) from the target individuals during implementation. In spring, all 24 samples were collected, in summer 21 animals (1 Hungarian indigenous Tsigai ewe, 1 Hungarian indigenous Tsigai ram, and 1 Merino ram died), in autumn 17 animals (2 Merino ewes died and 2 White Dorper were removed from the farm), and in winter 15 animals (1 Merino ram died and 1 Merino ram was removed from the farm). At the end of the research period, 15 animals had completed four-season sampling: Hungarian Merino (2 ewes and 1 ram), Hungarian indigenous Tsigai (3 ewes and 3 rams), and White Dorper (2 ewes and 4 rams). In every peak season of the year, about 3 ml blood sample was obtained from the jugular vein of the same animals in Tempus™ Blood RNA Tube (Applied Biosystem) and preserved at −70 °C until further analysis.

2.2. Climatological data

The climatic conditions during the sampling day were recorded at every hour. The THI was calculated for each sampling day using the following equation by (Marai et al., 2001). The severity of heat stress in livestock is typically rated using the THI, which ranges from 0 (no stress) to > 84 (extreme heat stress), categorized as follows: no stress (≤67), mild (68–74), moderate (75–78), severe (79–83) and extreme (≥84) (Lewis Baida et al., 2021).

$$THI = (0.8 \times T_{db}) - \left[\left(\frac{RH}{100}\right) \times (T_{db} - 14.4)\right] + 46.4$$

T_{db}- Dry bulb temperature (°C); RH- Relative humidity (%).

2.3. Quantification of gene expression levels using qRT-PCR

The total RNA was isolated from 3 ml of total blood using Tempus Spin RNA Isolation Kit (Applied Biosystems, USA) following the manufacturer's instructions and treated with DNase (Qiagen, catalog number: 79256).

RNA quality and quantity assessment were done using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 300 ng total RNA was reverse transcribed into cDNA with specific primers (Table 1) by the qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, United Kingdom). The amount of

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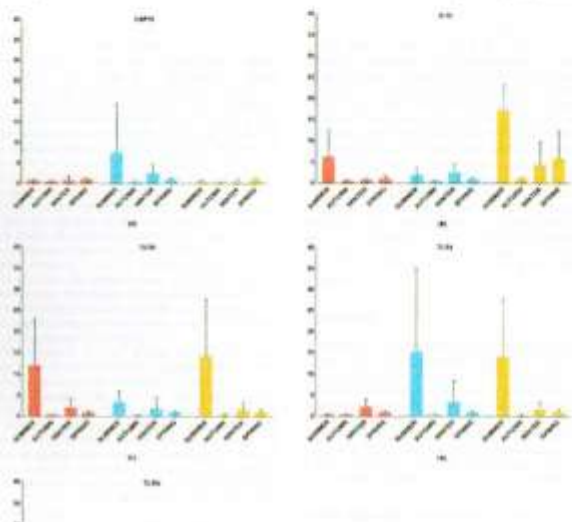

Fig. 5. Bar graph of relative gene expression of (A) HSP70, (B) IL10, (C) TLR2, (D) TLR4, and (E) TLR8 in each season of the year with the spring season as the calibrator. The X-axis is the relative gene expression in different season of the year. The Y-axis is gene expression for each different breeds in the study. Different colours represent different breed; red for Hungarian indigenous Tsigai, blue for Hungarian Merino, and yellow for White Dorper.

(Lara and Rostagno, 2013; Hedges et al., 2018). The seasonal variation shows temperature, humidity, rainfall, and daylight gradients across the year, which may affect the livestock's homeostasis directly through changes in physiological and biological processes, also behavior adjustment (Maurelli et al., 2019; Berihulay et al., 2019). Understanding the livestock's seasonal adjustment is critical for successful and profitable livestock farming, especially in an era where climate change is the most pressing issue, exposing animals to temperatures beyond their comfort zones and compensating for their production ability.

The thermal comfort zone for sheep varies among breeds, which correlates to their morphological characteristic facilitating the body's heat exchange with the environment, for example the coat colour and characteristics or the physical characteristic of the hair/wool (Fasin et al., 2014). This morphological diversity in livestock provides a potent system for genetic dissection of traits with complex inheritance, which also demonstrates their adaptation to their environment and is an indication of natural selection driving greater diversity (Boyko et al., 2018). In this study, the three most common sheep breeds in Hungary were observed: the Hungarian indigenous Tsigai; the commercial breed with a long history of crossbreeding, the Hungarian Merino, which both are cold tolerant breeds; and the South African commercial breed White Dorper, which is a breed with a high tolerance to heat. All of the breeds in this study have a dominating white coat colour, with both the Hungarian indigenous Tsigai and Hungarian Merino having long wool and White Dorper having short wool. The aim was to see if there is any difference in adaptability to the Hungarian environment between the three different breeds, although all of them have been acclimatized to it as these breeds have been widely kept in Hungary.

Generally, sheep have an upper critical between 25 and 31 °C depending on factors such as breed, age, and physiological state. Heat stress occurs when the effective temperature of the environment is higher than the upper critical temperature (Hopkins et al., 1978). To assess livestock productivity responses to climate change, the simplest method is to use the THI, which is calculated by combining the average temperature (°C) and relative humidity (%) of a given location (Ratchamak et al., 2021). It is undeniable, however, that THI may not entirely portray the thermal environment in which animals live because it does not take into consideration such factors as wind velocity, solar radiation, the shade provided to animals, or the availability of water. According to the THI calculation of each sampling season, almost-severe heat stress was observed in the summer season (THI = 79.89), which generated some gene expression changes inside the sheep body, explained as follows.

Thermal acclimation and adaption in small ruminants are invariably correlated with increased HSP concentrations. The HSPs have been the core of molecular responses in livestock to heat stress, but its stress tolerance mechanism is complex and not fully understood. With high HSP concentrations, cells can respond to heat stress better, while low levels of HSP make them more vulnerable (Rout et al., 2016; Joy et al., 2020). As it was observed in this study, Hungarian Merino, the cold tolerant breed with a high susceptibility to heat stress, the relative gene expression of *HSP70* was upregulated in the summertime, indicating heat-stress induced *HSP70* expression. As explained by Alemu et al. (2018) and Hooper et al. (2018), a relatively higher *HSP70* gene expression level suggests a greater need to protect the conformation of proteins on the cells from heat stress damage such as denaturation and aggregation. A previous study on goats showed *HSP70* gene is essential in goat environmental stress tolerance and adaptation, and its expression increases during heat stress (Banerjee et al., 2014; Archana et al., 2018) since it is a mechanism to protect proteins from degradation and facilitates their refolding, thereby enhancing cell survival (Dangi et al., 2014).

The expression pattern of the *HSP70* gene is species- and breed-specific for goats, as shown by research by Banerjee et al. (2014), possibly due to variances in thermal tolerance and adaptability to different climatic circumstances. They also confirmed that summertime expression of *HSP70* gene is more remarkable in Indian cold-adapted goats (Gaddi and Chegu) than in heat-adapted goats (Sirohi and Barbari). The spike of *HSP70* expression due to heat stress in other livestock has also been reported, e.g., cattle (Bharati et al., 2017) and buffalo (Yadav et al., 2021). In comparison, the Hungarian indigenous Tsigai and White Dorper *HSP70* expression in this study showed a small range of relative gene expression during the season of the year.

The Hungarian indigenous Tsigai, as expected, has good adaptability to the Hungarian environment (Gáspárdy et al., 2006) and Kusza et al. (2010, 2011, 2015) explained that the Hungarian indigenous Tsigai sheep is an ancient breed that has its origins traced to Asia Minor, located in modern-day Turkey. This breed was introduced to Hungary during the latter half of the 1700s. The breed has gained importance in Hungary owing to its resilience and capacity to adapt to harsh climatic and the breed has a historical significance, contributing to the country's agricultural heritage and economy. While, the White Dorper has a better thermotolerance due to the advantageous morphological characteristics such as short hair, thin skin, and a low number of hair follicles per unit area that help in combating heat stressors by facilitating heat dissipation (Gautwier, 2011) without necessitating them to make physiological regulation such as increasing heart rate and respiration rate to help to release the internal heat or reducing feed intake and body weight to decrease the endogenous heat production (Marai et al., 2007). Many findings have proven their extraordinary thermoregulation and resistance to heat stress (Almeida et al., 2013; Joy et al., 2020).

The colour and characteristics of an animal's coat can have a significant impact on its ability to acclimate to changing temperatures. Wool not only have significant economic value, but also according to a review by Ashiti et al. (2022) acts as a protective barrier that helps regulate body temperature by preventing heat loss in cold environments and blocking excessive heat gain in hot environments. Nevertheless, the thickness and type of wool can also impact an animal's ability to regulate its body temperature. For instance, wool sheep with thicker wool may have a lower thermoregulatory capacity due to the insulation effect of the wool, which inhibits body heat from escaping. In contrast, animals with thinner wool may have improved thermoregulation due to air flexibility within the fleece, which reduces heat loss via convection (McManus et al., 2020). The heritability of wool traits is moderate to low as they are influenced by multiple genetic and environmental factors. The wool-growing process in the epidermis involves intricate coordination among numerous genes and cell types, such as Ubiquitin conjugating enzyme E2 E3 (UBE2E3) and Rhophilin Rho GTPase binding protein 2 (RHPN2), which play crucial roles in keratinocyte differentiation and cell proliferation (Zhao et al., 2021), Follistatin (FST) genes which regulates hair follicle morphogenesis and cycling (Ma et al., 2017), also Keratin (KRTs) and Keratin-associated protein (KRTAP) which also plays important role in epithelial cell's stress response and apoptosis (Sulayman et al., 2018). The genetic and cellular mechanisms underlying the relationship between wool characteristics of sheep and their thermal adaptation remain obscure and complex. The incomplete understanding of the complexities of these biological processes contributes to a continuing lack of clarity on the subject.

Aside from its direct role in molecular protection against heat stress (intercellular function), the external *HSP70* also plays a vital role in the immune system. One is by signaling immune cells against invading pathogens via increased neutrophils and macrophages (Oglefald et al., 2005), in collaboration with host pattern recognition molecules like toll-like receptors (TLR) through the pathogen-associated molecular pattern (PAMP) to activate immune response (Hassan et al., 2019). The other is through the intervention of extracellular inflammatory signaling pathways, such as in the production of interleukin-10 (IL10), the foremost anti-inflammatory and immunosuppressive cytokine (Borges et al., 2012).

Heat stress causes an imbalance in the immune system by shifting the adaptive immune function from the normal cell-mediated to humoral immunity, which is a significant contributor to the poor health that results from it. Heat stress has been shown to reduce sheep's immunity, making them more susceptible to disease (Sophia et al., 2016; Shi et al., 2020). It is widely thought that heat-stress-induced glucocorticoids impair immunological homeostasis via altered cytokine production. However, the specific mechanism is unknown (Rogath et al., 2019). In this study, the expression of immune-related genes (*IL10*, *TLR2*, *TLR4*, and *TLR8*) was also studied to reveal the relationship between heat stress and immune deprivation in sheep.

The expression of *IL10* was significantly different in each season and was not equivalent in each breed. In White Dorper, it was overexpressed in the summertime, as was the case in Hungarian indigenous Tsigai but with a lower peak. However, in Hungarian Merino, peak expression was seen only during the winter, and seasonal variation was minimal. The previous study on various cattle breeds: Kocan Fries (Sheikh et al., 2016), Sahiwal cows (Gowraf et al., 2021), and Jersey (Kim et al., 2020) showed a higher *IL10* expression when facing heat stress. Also in sheep, an increased plasma *IL10* production was reported during the hyperthermia (Caroprese et al., 2014), which was explained as a mechanism to increase immune tolerance under heat stress condition. Meanwhile, Rashamol et al. (2019) showed no significantly different expression of *IL10* in heat stress and non-heat stress Malabari goats, demonstrating strong resilience to heat stress, notably in the ability to maintain the innate immune response, which is consistent with the expression of *IL10* in Hungarian Merino in this study. Further explained by [illegible] et al. (2022) that Merino was initially brought to Hungary in the 17th century, giving them plenty of time to acclimatize to the country's semi-arid environment and pastures, although as a imported multipurpose breed, Hungarian Merino has undergone numerous improvements programs since then by crossing them with various Merino and Merino-derived breeds. In contrast, because of its historical value, the Hungarian indigenous Tsigai has been included in the national gene conservation program in order to preserve its original qualities, particularly the traits that allowed them to thrive in a specific Hungarian environment. Nonetheless, the Hungarian Merino population has been declining recently, possibly as a result of the introduction of imported international breeds, which have become more popular among farmers in Hungary.

Pathogen identification by the highly specialized innate immune system relies heavily on Toll-like receptors (TLRs). When under stress, the immune system can either be enhanced or suppressed. In the hot summer, *TLR2*, *TLR4*, and *TLR8* expression was elevated in all breeds, except for *TLR4* in the Hungarian indigenous Tsigai, which showed a minimum expression fluctuation during the year with the highest expression in winter. Similarly, the *TLR2* expression in Merino also showed a minimum fluctuation. This finding is in accordance with the observation in Bengal goats (Paul et al., 2015), Malabari goats ([illegible] et al., 2019), and Tharparkar cattle (Bharati et al., 2017) in their case *TLR* genes were overexpressed in summertime.

TLRs are involved in the first line of defence against stress by detecting endogenous ligands such as HSPs. Researchers have already established that HSPs can stimulate the *TLR2* and *TLR4* pathways (Beg, 2002). HSPs improve antigen-presenting capacity via binding to TLR and HSPs' exposure to these TLRs may lead to the activation of dendritic cells and macrophages and the generation of immune-enhancing cytokines critical to host infection survival (Gobert et al., 2004; Paul et al., 2015). Increased *TLR2*, *TLR4*, and *TLR8* expression and signalling in immune cells can in turn enhance thermo-tolerance by increasing the innate immune response to PAMPs via a distinct immune response mechanism (Zhao et al., 2007).

The Hungarian indigenous Tsigai excelled in preserving its immune response while being subjected to hyperthermic conditions, as shown by the breed's lower expression of *TLR4* compared to other breeds and the downregulation of *TLR4* in the summer. According to research conducted by Gavojdian et al. (2015), the Hungarian indigenous Tsigai is less susceptible to lameness and pneumonia than the Dorper and White Dorper. Similar results were demonstrated in the spleen of Osmanabadi goats (Sophia et al., 2016), indicating the highly adaptive nature of this breed and their improved innate immune response against heat shock proteins when exposed to heat stress. It was also noted by Bharati et al. (2017) that *TLR4* could be crucial for providing a long-term immunological response to reduce the harmful effects of heat stress (inflammation and tissue injury) and confer thermo-tolerance under prolonged heat stress. It also identifies the Gram-negative bacteria-specific lipopolysaccharide (LPS) (Bulgari et al., 2017).

In this study, the Hungarian Merino was subjected to heat stress during the summer, as indicated by the relative expression of *HSP70*, but they maintained an effective immune response by maintaining high levels of *IL10* and *TLR2* gene expression throughout the year. It should come as no surprise that the indigenous Hungarian indigenous Tsigai has remarkable resilience to its natural environment and keep its resistance to seasonal stressors intact, while the immigrant White Dorper is only moderately suited to Hungarian climate. Through our research on genes for heat resistance and immunity, we confirmed that these three breeds are optimal for the Hungarian environment, given the risks posed by current and projected climate change. We recognize that our study is limited by the small and unequal sample size for each breed, which may reduce the study's statistical power and reliability, but we are confident that the results are indicative. It is essential to recognize the intricate nature of the five genes analyzed in this study, as well as their complex interactions with numerous other genes. The complexity of their functions and interrelationships is not to be disregarded, necessitating a more in-depth investigation into the relationship between sheep productivity and genetic adaptability.

5. Conclusion

This study confirmed the relative expression of genes involved in heat stress and immunity; *HSP70*, *IL10*, *TLR2*, *TLR4*, and *TLR8* are varied with the seasons in all three studied breeds of sheep, evidencing that seasonal stressors affects the thermo-balance and immunity of the sheep. The thermotolerance of the Hungarian Tsigai (indigenous breed) and White Dorper (tropical breed) is higher than that of the Hungarian Merino. However, the Hungarian indigenous Tsigai and, secondarily, the Hungarian Merino appear to have advantages over the White Dorper in terms of strength to maintain immunity under heat stress conditions.

CRediT authorship contribution statement

Putri Kusuma Astuti: Visualization, Writing – original draft. **Zoltán Bagi:** Writing – review & editing. **Lilla Bodrogi:** Methodology, Writing – review & editing. **Tímea Pintér:** Methodology, Writing – review & editing. **Gabriella Skoda:** Methodology, Writing – review & editing. **Roland Fajardo:** Writing – review & editing. **Szilvia Kusza:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethic declarations

All methods were carried out in accordance to the European Union's Animal Experimentation Directive (2010/63/EU) and ARRIVE guidelines.

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