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Widespread benzimidazole resistance and *β-tub-1* F200Y SNP in gastrointestinal nematodes of sheep across Portugal

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ABSTRACT

Anthelmintic resistance (AR) to benzimidazoles (BZs) in gastrointestinal nematodes (GINs) of sheep was assessed across 35 farms in northern (Trás-os-Montes, TM), north-central (Beira Interior, BI), central (Estremadura, ES), and southern (Alentejo, AL) Portugal. Pooled fecal samples were tested for AR by egg hatch tests (EHTs) with albendazole (ABZ) and thiabendazole (TBZ). Effective concentrations inhibiting 50% egg hatch (EC_{50}) were calculated, with $EC_{50} > 0.13$ μg/mL (ABZ) and > 0.10 μg/mL (TBZ) indicating AR. ABZ resistance was detected on 69% of farms, while TBZ, never administered, showed resistance on 59% of farms, suggesting wide BZ cross-resistance. EC_{50} values ranged from 0.05 to 0.68 μg/mL (ABZ) and 0.03–0.70 μg/mL (TBZ). Nemabiome metabarcoding identified 10 GIN species, predominantly *Haemonchus contortus* (41%), *Teladorsagia circumcincta* (33%), and *Trichostrongylus colubriformis* (19%). Less common species included *Bunostomum trigonocephalum* and *Oesophagostomum venulosum*. Species richness (3–6 per farm) was highest in TM, with unique occurrences of *B. trigonocephalum* and *Cooperia* sp. *H. contortus* dominated in TM and BI, while *T. circumcincta* prevailed in AL, where AR was most severe. Higher EC_{50} values correlated with increased *T. circumcincta* and *T. colubriformis* prevalence. The BZ-resistance F200Y SNP in the isotype-1 *β*-tubulin gene of *H. contortus* was detected on 85% of farms, with resistant allele frequencies inferring predominantly homozygous resistant populations (A/A; > 90% resistant allele) on some farms, strongly correlating with higher EC_{50} values, particularly in AL. Widespread BZ resistance underscores the need for enhanced AR surveillance, prudent anthelmintic use, and integrated management to sustain sheep health in Portugal, especially in Alentejo.

1. Introduction

Gastrointestinal nematodes (GINs) are major parasitic pathogens of ruminants, including sheep (*Ovis aries*), causing significant health and economic challenges in small ruminant farming (Zajac and Garza, 2020). Predominant GIN families include Trichostrongylidae, Strongylidae, Molineidae, and Ancylostomatidae, encompassing abomasal species (*Haemonchus contortus*, *Teladorsagia* spp., *Trichostrongylus axei*), small intestinal species (*Trichostrongylus* spp., *Cooperia* spp., *Nematodirus* spp., *Bunostomum* spp.), and large intestinal species (*Oesophagostomum* spp., *Strongyloides* spp., *Trichuris* spp.). GINs follow direct life cycles: adult females produce eggs, excreted in feces, which develop into infective L3 larvae in the environment, are ingested during grazing, and mature into egg-laying adults within weeks (Roeber et al., 2013; Taylor et al., 2015). Pathogenesis results from mucosal penetration by both larval and adult stages, leading to nutrient malabsorption, intestinal

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barrier disruption, inflammation, and, in hematophagous species like *H. contortus*, anemia and hypoproteinemia (Roeber et al., 2013). In sheep, heavy infections cause growth retardation, reduced meat, milk, and wool production, and increased mortality, contributing to annual economic losses across Europe estimated at €1.8 billion (Charlier et al., 2022).

GIN control relies heavily on anthelmintics, but widespread, often indiscriminate use has driven anthelmintic resistance (AR) in major GIN species, threatening sustainable livestock production (Kaplan, 2023). AR was first reported in sheep in 1957, with *H. contortus* resisting phenothiazine, followed by resistance to benzimidazoles (BZs), such as thiabendazole (TBZ), by the 1960s (Drudge et al., 1957, 1964). In Europe, AR prevalence is well-documented, with BZ resistance affecting over 80% of flocks in some regions, driven by over-treatment, under-dosing, and limited integrated parasite management (IPM) (Rose Vineer et al., 2020; Charlier et al., 2022). Geographic variation in AR underscores the need for region-specific studies to inform sustainable worm control (SWC) strategies (Charlier et al., 2022).

In Europe, AR prevalence in sheep GINs has been well-documented over the past two decades, with studies from the United Kingdom, France, Italy, Greece, Spain, and more, reporting high resistance to multiple drug classes, including BZ resistance (Rose Vineer et al., 2020; Charlier et al., 2024; Castagna, et al., 2024). Additionally, potential for cross-transmission of AR strains of GIN from wild-host reservoirs to domestic sheep, and *vice versa*, in sympatric habitats is known (Beaumelle et al., 2023; Barone et al., 2020).

To date, Portugal is under-represented in GIN AR research and surveillance, compared to other European nations. The Portuguese sheep industry supports over 2 million animals across 52,000 farms (Trading Economics, 2025), generates €32–118 million annually from meat, dairy, and wool (Eurostat, 2023). Rising treatment failures highlight an urgent need for AR assessment, with GIN-related costs estimated at €3.5–5.7 million annually (Eurostat, 2023). Limited studies in Portugal, primarily in Santarém and Beira Baixa, confirmed BZ resistance in *H. contortus*, *Trichostrongylus colubriformis*, and other GINs, accompanying high infection rates (Antunes et al., 2022). Other published studies quantified GIN burden in the Beira Baixa region, with *Strongyloides papillosus* identified to species, and other GINs to genus, including *Teladorsagia, Cooperia, Trichostrongylus, Oesophagostomum, Haemonchus, Chabertia,* and *Bunostomum* (Matos et al., 2023). GIN infection of sheep in the Centre region of Portugal was recently reported to be at 79.9%, but AR was not assessed. (Pereira et al., 2024).

BZs exert their anthelmintic effect by binding to β-tubulin subunits in helminth microtubules, disrupting polymerization, cellular integrity, nutrient transport, and metabolism (Martin, 1997). Resistance can arise from mutations in the isotype-1 β-tubulin gene (β-*tub-1*), reducing BZ binding affinity. Three non-synonymous single nucleotide polymorphisms (SNPs) at codons 167 (F167Y), 198 (E198A), and, most commonly, 200 (F200Y) are strongly associated with BZ resistance (Kwa et al., 1994; von Samson-Himmelstjerna et al., 2007; Dilks et al., 2020; Kaplan et al., 2023).

This study investigates BZ resistance prevalence across sheep farms in northern (Trás-os-Montes, TM), north-central (Beira Interior, BI), central (Estremadura, ES), and southern (Alentejo, AL), Portugal using the egg hatch test (EHT) (Coles et al., 2006). Nemabiome metabarcoding of ribosomal ITS2 sequences identified GIN species (Avramenko et al., 2015) with regional species diversity assessed using established metrics (Magurran, 2021). Presence and frequencies of β-tubulin SNPs associated with resistance were analyzed in *H. contortus* from 20 farms (Avramenko et al., 2019). Our findings reveal widespread BZ resistance and high resistant allele frequencies, signaling an urgent need for effective SWC strategies by the Portuguese sheep industry.

2. Materials and methods

2.1. Geographic origin and fecal sample collection

General climatic conditions at sample-collection sites, relevant to GIN ecology and transmission dynamics, varied across regions: Trás-os-Montes (TM) has a harsh continental climate, cold winters (snow and freezing) and hot, dry summers; Beira Interior (BI; Serra da Estrela area) has a mountainous climate with cold, snowy winters (sub-zero temperatures) and milder, warm summers, significantly cooler at higher elevations; Estremadura (ES; Sintra area) has a cooler microclimate influenced by proximity to the Atlantic Ocean, with milder temperatures, higher humidity, frequent fog/mist, and rainy winters; and Alentejo (AL) displays a hot-summer Mediterranean climate characterized by extremely hot (as high as 45ºC), dry summers and mild winters with low annual rainfall. Collectively, these sites spanned a transition from cooler, wet northern/continental interiors and mountains to hotter, drier southern plains (Britannica, 2026).

Fecal samples were collected from 67 sheep farms across four regions of Portugal: TM (northern, 18 farms), BI (Sintra area: north-central, 4 farms), ES (Serra da Estrela area, 8 farms), and AL (southern, 37 farms). Sampling occurred from mid-May to late July 2024. Sheep had not received anthelmintic treatment for at least 3–4 months prior to collection. Records of previously administered anthelmintics included closantel, mebendazole (MBZ), fenbendazole, albendazole (ABZ), and ivermectin. Sheep breeds included Merino, Suffolk, Île-de-France, Churra da Terra Quente, Galega Bragançana, Campaniça, and crossbreeds of Merino with INRA or Campaniça. Samples were collected from 10 to 50 sheep per farm, randomly selected from grazing flocks, regardless of age, sex, or breed. Fecal samples were obtained rectally only once per farm, at various times during the day, pooled and homogenized. Samples were vacuum-packed, transported on ice to the Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Oeiras, Portugal, divided into 5 g subsamples, vacuum-sealed, and stored at 5°C until egg isolation and analysis (Rinaldi et al., 2014). Due to insufficient eggs per gram (EPG <400) or DNA availability, only 35 farms (Fig. 1) were analyzed for EHT (Section 2.4) and 20 for metabarcoding (Section 2.7), excluding ES for metabarcoding.

2.2. Quantification of GIN eggs per gram (EPG) of feces

GIN egg counts were determined using the Mini-FLOTAC technique (Rinaldi et al., 2014). Briefly, 5 g subsamples from pooled fecal samples (Section 2.1) were homogenized in 45 mL saturated NaCl (specific gravity 1.2) within a Mini-FLOTAC device (University of Naples Federico II). Two, 1 mL aliquots were loaded into the sample chambers, allowed to stand for 10 min, and floating eggs transferred to the reading disc. Eggs were counted under a light microscope at 10 × magnification, and EPG was calculated as the mean count multiplied by 5 (dilution factor). Only 35 of the 67 farms met the 400 EPG threshold required for assessing AR using the EHT (Section 2.3). High levels of detritus in some samples required repeated washing and filtration, rendering preparation impractical for some farms.

2.3. Egg isolation for EHT

Eggs for EHT were isolated following methods adapted from Coles et al. (2006), von Samson-Himmelstjerna et al. (2009), and Demeler et al. (2012), with modifications. Five-gram subsamples of pooled fecal samples (Section 2.1) were homogenized in 45 mL saturated NaCl and filtered through a 500 μm sieve. The filtrate was centrifuged at 2000 rpm (Hettich Rotina 380, Tuttlingen, Germany) for 3 min, the supernatant discarded, and the pellet rinsed with deionized water over a 25 μm sieve. Retained eggs were resuspended in a small volume of deionized water, transferred to a centrifuge tube, and centrifuged again at 2000 rpm for 3 min. After discarding the supernatant, eggs in 20 μL

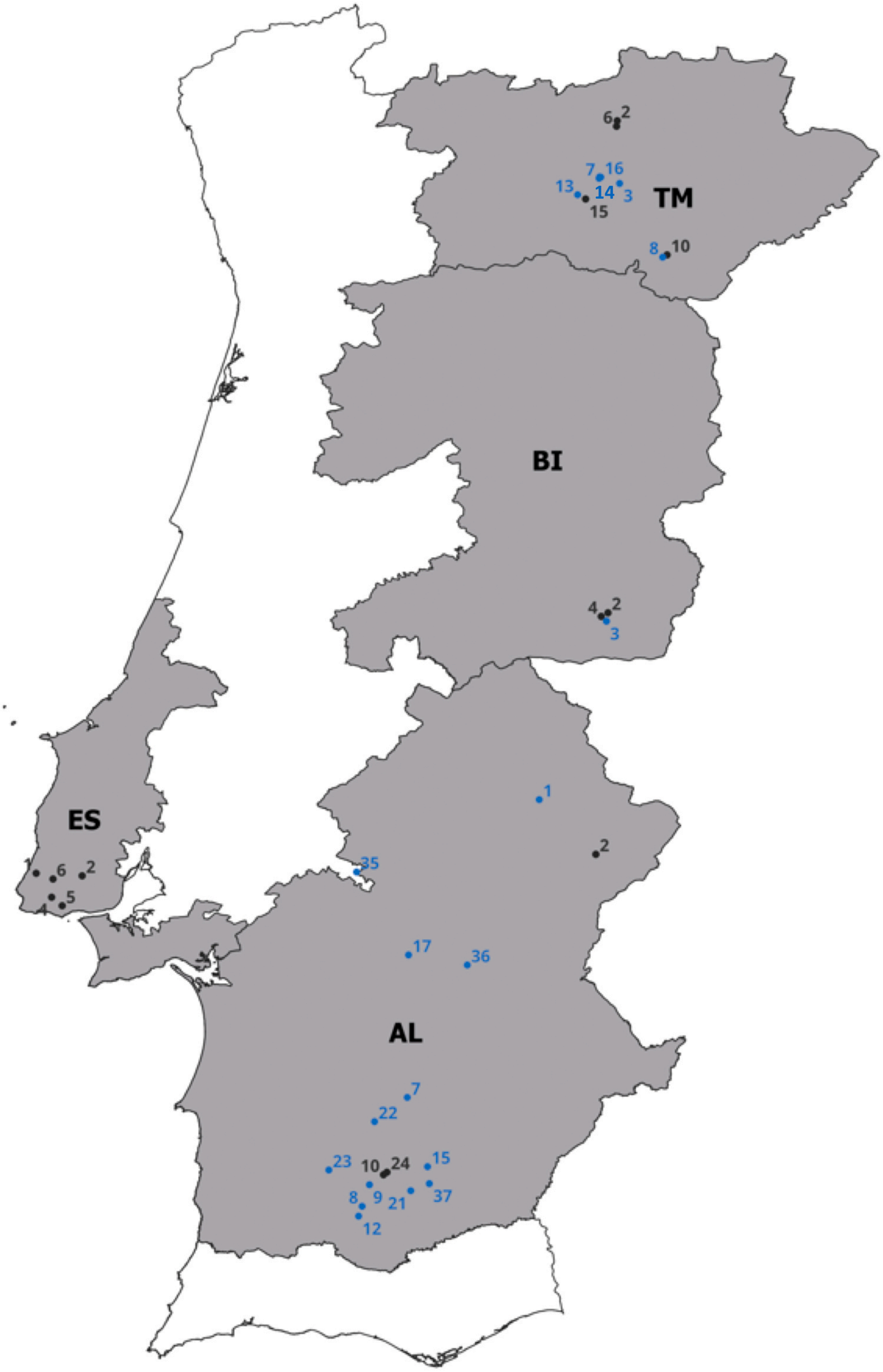

Fig. 1. Geographic Distribution of Sampled Sheep Farms in Portugal. Map illustrating locations of 35 sheep farms sampled for gastrointestinal nematodes (GIN) from May to July 2024 across regions Trás-os-Montes (TM), Beira Interior (BI), Estremadura (ES), and Alentejo (AL). All farms had pooled fecal egg counts exceeding 400 eggs per gram (EPG). Eggs from all farms were used in egg hatch tests (EHT) to assess ovicidal resistance to benzimidazoles (Section 2.3). Additionally, for 20 farms (indicated by blue dots), DNA was isolated from eggs for ITS2 metabarcoding to identify GIN species and isotype-1 β-tubulin (β-*tub-1*) metabarcoding to detect benzimidazole resistance-associated single nucleotide polymorphisms (SNPs) in *Haemonchus contortus* (Sections 2.5–2.7). The remaining farms are marked with black dots. Farm numbers are provided for reference to other figures and tables.

subsamples were counted under an inverted microscope at 10 × magnification. If an insufficient number of eggs was detected, additional fecal subsamples were processed, pooling eggs until 20 μL aliquots contained 100–200 eggs, as required for each microplate well in the EHT assay (Section 2.4).

2.4. *Assessment of anthelmintic resistance via EHT*

The EHT is an *in vitro* assay validated for assessing BZ resistance (Demeler et al., 2010) and suitable for large-scale testing of AR across geographically diverse samples, unlike the *in vivo* fecal egg count reduction test (FECRT) (Levecke et al., 2012; Morgan et al., 2022). The EHT was used to evaluate AR to ABZ and TBZ, which inhibit GIN egg embryonation and hatching. Procedures followed Coles et al. (1992; 2006), von Samson-Himmelstjerna et al. (2009), and Demeler et al. (2012). Assays were conducted in 24-well microplates. Stock solutions [0.5 µg/µL ABZ and TBZ in dimethyl sulfoxide (DMSO)] were serially diluted (2-fold) to create substocks. Each 2 mL microplate well contained 1.97 mL phosphate buffer (pH 7.2), 10 µL BZ substock (final concentrations: 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04 µg/mL), and 20 µL egg suspension (100–200 eggs; Section 2.3). Control wells received 10 µL DMSO instead of BZ substock. Assays, run in duplicate, were incubated at 25°C for 48 h and terminated with 10 µL Lugol's iodine per well. Unhatched eggs and L1 larvae were counted under an inverted microscope at 10 × magnification. The mean egg hatch proportion (EH) was calculated as:

EH = [number of larvae / (number of eggs + larvae)] / 2.

%Egg hatch inhibition (%EHI) was determined as:

$\%\mathrm{EHI} = [(\mathrm{EH}_{\mathrm{control}} - \mathrm{EH}_{\mathrm{treated}}) / \mathrm{EH}_{\mathrm{control}}] \times 100$

Effective concentrations inhibiting 50% hatch (EC_{50}) and 95% confidence intervals (95% CI) were derived via probit analysis, with a resistance threshold of 0.5 µM for each BZ (EC_{50}: ABZ >0.13 µg/mL; TBZ >0.1 µg/mL) (Coles et al., 2006).

2.5. Extraction and purification of GIN genomic DNA

GIN DNA was purified from eggs isolated from the nemabiome of pooled fecal samples (Section 2.1) as (Demeler et al., 2012). Eggs were isolated, as in Section 2.3, up to the final centrifugation. Five mL of the egg suspension, in the lower part of the tube, were layered onto sucrose gradients (10%, 25%, 40% w/v), centrifuged at 2000 rpm for 3 min, and the egg-band collected. Eggs were washed with deionized water, resuspended in 250 µL water, and stored at −20°C. Genomic DNA was extracted from egg suspensions standardized to yield 5 ng/µl high quality DNA. Exact egg counts per extraction were not recorded but input was normalized by fluorometric quantification prior to PCR to ensure comparability across farms (Avramenko et al., 2015). DNA was extracted using the NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany) according to manufactureŕs protocol, with purified DNA stored at −20°C.

2.6. Molecular identification and relative prevalence of GIN species by metabarcoding

2.6.1. ITS2 sequence generation

Species and relative prevalence of GINs were determined by metabarcoding using next-generation sequencing (NGS) of the nemabiome from pooled fecal samples collected at each farm (Section 2.1; Avramenko et al., 2015). Genomic DNA extracted from GIN eggs (>5 ng/µL; Section 2.5) was shipped on dry ice to Stab Vida, Genetics Laboratory (Caparica, Portugal). DNA quality was assessed for concentration and integrity to ensure suitability for library preparation, with integrity verified by agarose gel electrophoresis in TAE buffer and quantification performed using a Qubit 2.0 fluorometer with the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The trichostrongylid internal transcribed spacer 2 (ITS2) region was amplified by PCR using strongylid-conserved primers (Bisset et al., 2014):

Forward (5′–3′): CACGAATTGCAGACGCTTAG
Reverse (5′–3′): GCTAAATGATATGCTTAAGTTCAGC

PCR reactions (20 µL) contained 10 µL AmpliTaq Gold™ 360 Master Mix (Applied Biosystems), 5.84 µL nuclease-free water, 0.16 µL bovine serum albumin (20 mg/mL), 2 µL of 5 µM mixed forward and reverse primers, and 2 µL DNA template. Thermal cycling conditions were: 95°C for 10 min; 35 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1 min; followed by 72°C for 7 min. ITS2 amplicon libraries were prepared following a proprietary protocol from Stab Vida, adapted from Illumina's 16S Metagenomic Sequencing Library Preparation guide (15044223 Rev. B). Sequencing was performed on an Illumina MiSeq platform (MiSeq Reagent Kit v3, 300 bp paired-end). Post-sequencing quality control using FastQC software confirmed > 60% of bases exceeded a Q30 score at 2 × 300 bp, with sequence read counts per sample ranging from 138,258 to 302,004.

2.6.2. Bioinformatic analysis of ITS2 sequences

Raw ITS2 sequence reads were processed using the DADA2 plugin in QIIME2 (version 2024.10). Reads were denoised, trimmed, and truncated to remove low-quality regions (quality score ≥20, length ≥300 bp). Dereplication, chimera filtering, and clustering into amplicon sequence variants (ASVs) were performed. A rarefaction curve was generated by subsampling sequences at varying depths to assess ASV diversity. Taxonomic classification was conducted using a scikit-learn classifier trained on the ITS2 UNITE database (Release 9.0; Supplementary File 1). ASVs with ≥ 10 sequence reads were considered significant for species identification and relative abundance estimation. All analyses were performed within the QIIME2 2024.10 framework.

2.7. Nemabiome deep amplicon sequencing of *β*-tubulin isotype-1 gene (*β-tub-1*)

To characterize the genotypic basis of BZ resistance in GINs, deep amplicon NGS was used to estimate the prevalence and frequency of non-synonymous single nucleotide polymorphisms (SNPs) in isotype-1 *β*-tubulin gene (*β-tub-1*) of *H. contortus* (Avramenko et al., 2019). Fecal samples were collected from 20 sheep farms across three regions in Portugal: Trás-os-Montes (6 farms), Beira Interior (1 farm), and Alentejo (13 farms). Genomic DNA was extracted from nemabiome eggs using established protocols (Section 2.5). *β-tub-1* was amplified using *H. contortus*-specific PCR primers (Avramenko et al., 2019):

Forward (5′–3′): CGCATTCYTTGGGAGGAGG
Reverse (5′–3′): GTGAGTTTYAAGGTGCGGAAG

PCR reactions (20 µL) followed the reagent protocols for ITS2 amplification (Section 2.6.1), with thermal cycling conditions: 95°C for 10 min; 40 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s; followed by 72°C for 7 min. Amplicon purification, quantification, and library preparation followed the ITS2 protocol (Section 2.6.1). Sequencing was performed on an Illumina MiSeq platform (MiSeq Reagent Kit v3, 300 bp paired-end). Post-sequencing quality control using FastQC software confirmed that > 85% of bases exceeded a Q30 score at 2 × 300 bp.

Metabarcoding of isotype-1 *β*-tubulin of *H. contortus* was performed using quality-filtered reads aligned to the *H. contortus* isotype-1 *β*-tubulin reference sequence (GenBank Accession No. X67489) using GLC Genomics Workbench 12.0.3, with no evidence of cross-amplification from other species. Reads were then processed with FreeBayes for variant calling, at the F200Y site. Because DNA was extracted from pooled eggs representing a mixed population of diploid worms, FreeBayes was used to estimate resistant allele frequency per farm. Farms were then categorised by inferred population-level zygosity using the following conservative thresholds (adapted from Avramenko et al., 2019 and Baltrušis et al., 2020, to reflect near-fixation of alleles in resistant populations while accounting for sequencing noise): > 90% resistant allele = predominantly homozygous resistant (A/A); 10–90% = heterozygous/mixed (A/T); < 10% = susceptible (T/T). This provides a practical population-level classification while acknowledging exact per-worm genotype distributions cannot be resolved from pooled data. Inferred zygosity (A/A, A/T, T/T) and allele frequencies were determined per farm. Other polymorphic sites within exons were not assessed.

2.8. Statistical analysis

2.8.1. Probit analysis

%EHI data (Section 2.4) were analyzed to determine EC_{50} values for each BZ using probit analysis (Finney, 1971). The %EHI values from two replicates were averaged for each concentration (2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04 μg/mL) and converted to proportions. Probit analysis was performed using a generalized linear model (GLM) with a probit link function, modeling log10-transformed concentrations against inhibition proportions, in R (version 4.4.3; R Core Team, 2023). The EC_{50} was calculated as the BZ concentration corresponding to 50% inhibition. A nonparametric bootstrap method in base R was used to calculate 95% confidence intervals (CI) around the EC_{50}.

2.8.2. Correlation and comparative analyses

To assess relationships between anthelmintic resistance and GIN nemabiome populations, Pearson correlation and linear regression analyses were conducted to evaluate associations between EC_{50} values (between ABZ and TBZ) and the percent composition of *H. contortus*, *Teladorsagia circumcincta*, and *T. colubriformis*, the predominant species identified across 35 Portuguese sheep farms (Table 1). Beira Interior farms were excluded from TBZ analyses due to missing EC_{50} data. These analyses were performed using Python (version 3.11; Python Software Foundation, 2022) with the SciPy library (version 1.10.0; Virtanen et al., 2020).

Table 1

Farm-by-Farm Assessment of Gastrointestinal Nematode (GIN) Anthelmintic Resistance.

FARM	ABZ EC_{50}	Status	TBZ EC_{50}	Status
TM2	0.68	R	0.08	S
TM3	0.46	R*	0.68	R
TM6	0.08	S*	0.07	S
TM7	0.22	R	0.06	S
TM8	0.18	R	0.11	R*
TM10	0.14	R*	0.05	S
TM13	0.14	R*	0.14	R
TM14	0.12	S*	0.06	S
TM15	0.05	S	0.06	S
TM16	0.28	R	0.10	S*
BI2	0.14	R*	NM	-
BI3	0.11	S*	NM	-
BI4	0.12	S*	NM	-
ES1	0.11	S*	0.08	S*
ES2	0.19	R	0.23	R
ES3	0.14	R	0.29	R
ES4	0.09	S	0.06	S
ES5	0.07	S	0.03	S
ES6	0.23	R	0.22	R
AL1	0.16	R*	0.20	R
AL2	0.31	R	0.43	R
AL7	0.26	R	0.31	R
AL8	0.41	R	0.49*	R*
AL9	0.15	R*	0.11	R*
AL10	0.13	S*	0.09	S*
AL12	0.19	R	0.19	R
AL15	0.54	R	0.74	R
AL17	0.13	S*	0.04	S
AL21	0.37	R	0.63	R
AL22	0.38	R	0.70	R*
AL23	0.14	R*	0.11	R*
AL24	0.26	R	0.53	R*
AL35	0.08	S	0.09	S*
AL36	0.29	R	0.60	R
AL37	0.28	R*	0.49	R*

Effective concentrations (EC_{50}) of albendazole (ABZ) and thiabendazole (TBZ) inhibiting 50% egg hatch in the egg hatch test (EHT) for GIN eggs from sheep fecal samples across 35 Portuguese farms meeting the > 400 eggs per gram (EPG) threshold. Farms span regions, north to south, Trás-os-Montes (TM), Beira Interior (BI), Estremadura (ES), and Alentejo (AL). Resistance (R) is defined as $EC_{50} > 0.13$ μg/mL for ABZ or > 0.10 μg/mL for TBZ; susceptible (S); NM = not measured (insufficient sample). * indicates farms where 95% confidence interval (CI) for EC_{50} overlaps resistance threshold. Location of farm corresponds to region and farm-number in Fig. 1.

For *H. contortus*-specific analyses, Spearman's rank correlation coefficient (ρ) was used to assess relationships between EC_{50} values (ABZ: 20 farms; TBZ: 19 farms, excluding BI3 due to missing TBZ data), species diversity (H′; Magurran, 2021) of GIN nemabiomes (Table 2), prevalence of *H. contortus*, *T. circumcincta*, and *T. colubriformis* (Table 3), and F200Y resistant allele frequency in β-*tub-1* (Fig. 2; see supplementary Table 4 for detailed metabarcoding results), due to non-normal data distribution. EC_{50} values were compared across F200Y zygosity categories [homozygous resistant (A/A, $n = 11$), heterozygous (A/T, $n = 6$), no SNP (T/T, $n = 3$)] using the Kruskal-Wallis test, with post-hoc Dunn's tests for pairwise comparisons. Spearman's rank correlation was also used to evaluate the relationship between F200Y resistant allele frequency and EC_{50} values.

To assess regional trends in anthelmintic resistance, Kruskal-Wallis tests were used to compare EC_{50} values for ABZ (across TM, BI, ES, and AL) and TBZ (TM, ES, AL) across farms. The Cochran-Armitage trend test was applied to evaluate a north-to-south trend in dual resistance prevalence (farms resistant to both ABZ and TBZ) and inferred F200Y homozygosity (A/A genotypes) regional trends from north to south. (Fig. 2). Regional differences in A/A genotype proportions and resistance prevalence (ABZ and TBZ) across TM, BI, and AL were tested using chi-square tests for independence. Sub-analyses excluded farms TM3 (northern anomaly, A/A), BI3 (central, A/T), and T/T farms (TM8, TM14, AL9) to focus on farms with F200Y SNPs. All statistical analyses were performed in R (version 4.4.3; R Core Team, 2023), with significance set at $p < 0.05$.

3. Results

3.1. Anthelmintic resistance to ABZ and TBZ based on egg hatch inhibition

Fecal samples from all 67 farms contained GIN eggs, with an EPG range of 20 (one farm in Estremadura) to 5620 (one farm in Beira Interior). Thirty-five farms met the > 400 EPG threshold for EHT. Of these, 24 of 35 farms (69%) exhibited resistance to ABZ (EC_{50} range: 0.05–0.68 μg/mL, median: 0.16 μg/mL) (Table 1). For TBZ, 19 of 32 farms (59%; EC_{50} range: 0.03–0.74 μg/mL, median: 0.17 μg/mL) showed resistance. All farms susceptible to ABZ were also susceptible to TBZ.

Regionally, TM showed 70% ABZ and 30% TBZ resistance, BI had one of three farms resistant to ABZ (TBZ not assayed), ES had 50% ABZ and 50% TBZ resistance, and AL exhibited highest resistance prevalence, with 81% ABZ and 75% TBZ resistance. A significant north-to-south regional gradient of increasing resistance was observed for both BZs, with ABZ median EC_{50} values: TM (0.14 μg/mL) ≈ BI (0.12 μg/mL) ≈ ES (0.125 μg/mL) $<<$ AL (0.225 μg/mL) (Kruskal-Wallis, $H = 12.294$, $p = 0.006$) and TBZ values following a stronger trend: TM (0.075 μg/mL) < ES (0.15 μg/mL) $<<$ AL (0.255 μg/mL) (Kruskal-Wallis, $H = 14.529$, $p = 0.001$). Additionally, the percentage of farms with dual BZ resistance significantly increased regionally southward: TM (30%) < ES (50%) < AL (75%) (Cochran-Armitage trend test, $Z = 2.677$, $p = 0.004$). Moreover, EC_{50} values between ABZ and TBZ were significantly correlated across all farms (Pearson's $r = 0.805$, $p < 0.001$).

3.2. GIN species composition, relative abundance, and diversity

Analysis of GIN species composition, relative abundance, and Shannon's diversity index (H′) was conducted on 20 farms due to limited DNA availability (Table 2). Ten GIN species were identified, with *H. contortus*, *T. circumcincta*, and *T. colubriformis* predominant across TM, BI, and AL. In TM (6 farms), *H. contortus* ranged from 6.7% (TM3) to 97.5% (TM7), *T. circumcincta* from 0.5% (TM7) to 41.6% (TM8), and *T. colubriformis* from 0.6% (TM13, TM14) to 54.3% (TM3). Other species

Table 2

GIN Species Composition, Relative Incidence, and Diversity Across Sheep Farms.

Farm	Region	GIN Species	Incidence (%)	H′
TM3	Trás-os-Montes	*H. contortus*	6.7	1.14
		T. circumcincta	24.1	
		T. colubriformis	54.3	
		O. venulosum	14.4	
TM7	Trás-os-Montes	*H. contortus*	97.5	0.47
		T. circumcincta	0.5	
		T. colubriformis	1.4	
TM8	Trás-os-Montes	*H. contortus*	42.7	1.08
		T. circumcincta	41.6	
		T. colubriformis	13.6	
		O. venulosum	0.7	
		C. erschowi	1.0	
TM13	Trás-os-Montes	*H. contortus*	36.6	1.49
		T. circumcincta	21.0	
		T. colubriformis	0.6	
		B. trigonocephalum	23.6	
		T. axei	5.4	
		Cooperia sp.	12.3	
TM14	Trás-os-Montes	*H. contortus*	76.4	0.82
		T. circumcincta	7.1	
		T. colubriformis	0.6	
TM16	Trás-os-Montes	*H. contortus*	31.6	1.49
		T. circumcincta	25.1	
		T. colubriformis	9.2	
		B. trigonocephalum	28.9	
		T. axei	1.0	
		Cooperia sp.	3.6	
BI3	Beira Interior	*H. contortus*	50.8	1.15
		T. circumcincta	32.4	
		T. colubriformis	6.3	
		T. axei	0.1	
		C. erschowi	0.7	
		T. vitrinus	9.1	
AL1	Alentejo	*H. contortus*	0.7	0.36
		T. circumcincta	91.6	
		T. colubriformis	3.2	
		T. axei	4.2	
AL7	Alentejo	*H. contortus*	25.9	0.77
		T. circumcincta	69.7	
		T. colubriformis	0.3	
		O. venulosum	2.0	
		T. axei	1.7	
AL8	Alentejo	*H. contortus*	2.1	0.47
		T. circumcincta	85.5	
		T. colubriformis	12.0	
AL9	Alentejo	*H. contortus*	97.3	0.09
		T. circumcincta	0.7	
		T. colubriformis	0.9	
AL12	Alentejo	*H. contortus*	48.7	1.02
		T. circumcincta	36.7	
		T. colubriformis	13.5	
		C. erschowi	0.7	
AL15	Alentejo	*H. contortus*	2.0	0.82
		T. circumcincta	27.2	
		T. colubriformis	65.9	
		O. venulosum	0.8	
		C. erschowi	2.9	
		T. vitrinus	0.9	
AL17	Alentejo	*H. contortus*	73.6	0.79
		T. circumcincta	11.5	
		T. colubriformis	12.9	
		O. venulosum	1.2	
AL21	Alentejo	*H. contortus*	0.3	0.68
		T. circumcincta	62.2	
		T. colubriformis	37.2	
		N. filicollis	0.1	
AL22	Alentejo	*H. contortus*	2.0	0.95
		T. circumcincta	42.6	
		T. colubriformis	50.2	
		O. venulosum	3.9	
		C. erschowi	0.8	
AL23	Alentejo	*H. contortus*	40.0	1.17
		T. circumcincta	19.7	
		T. colubriformis	37.0	
		O. venulosum	0.5	
		C. erschowi	2.6	
AL35	Alentejo	*H. contortus*	71.9	0.75
		T. circumcincta	7.0	
		T. colubriformis	20.7	
AL36	Alentejo	*H. contortus*	69.8	0.73
		T. circumcincta	25.7	
		T. colubriformis	4.1	
AL37	Alentejo	*H. contortus*	0.2	0.75
		T. circumcincta	70.6	
		T. colubriformis	25.1	
		O. venulosum	2.5	
		C. erschowi	1.3	

Species composition, relative abundance (%), and Shannon's diversity index (H′) of gastrointestinal nematode (GIN) populations from 20 sheep farms from regions, north to south, Trás-os-Montes (TM), Beira Interior (BI), and Alentejo (AL).

Table 3

Farm Comparison of GIN Resistance Status and Predominant Species Composition.

Farm	ABZ Status	TBZ Status	Hc (%)	Tc (%)	Tcol (%)
TM3	Resistant	Resistant	6.70	24.1	54.3
TM7	Resistant	Susceptible	97.50	0.5	1.4
TM8	Resistant	Resistant*	42.70	41.6	13.6
TM13	Resistant*	Resistant	36.60	21.0	0.6
TM14	Resistant	Susceptible	76.40	7.1	0.6
TM16	Resistant	Susceptible*	31.60	25.1	9.2
BI3	Resistant	NM	50.80	32.4	6.3
AL1	Resistant*	Resistant	0.70	91.6	3.2
AL7	Resistant	Resistant	25.90	69.7	0.3
AL8	Resistant	Resistant*	2.10	85.5	12.0
AL9	Resistant	Susceptible*	97.30	0.7	0.9
AL12	Resistant	Resistant	48.70	36.7	13.5
AL15	Resistant	Resistant	2.00	27.2	65.9
AL17	Resistant*	Susceptible	73.60	11.5	12.9
AL21	Resistant	Resistant	0.30	62.2	37.2
AL22	Resistant	Resistant*	2.00	42.6	50.2
AL23	Resistant	Resistant*	40.00	19.7	37.0
AL35	Susceptible	Susceptible*	71.90	7.0	20.7
AL36	Resistant	Resistant	69.80	25.7	4.1
AL37	Resistant*	Resistant*	0.20	70.6	25.1

Comparison of anthelmintic resistance status for albendazole (ABZ) and thiabendazole (TBZ) (Table 1) with percent abundance of predominant gastrointestinal nematode (GIN) species *Haemonchus contortus* (Hc), *Teladorsagia circumcincta* (Tc), and *Trichostrongylus colubriformis* (Tcol) (Table 2) across 20 sheep farms in Trás-os-Montes (TM), Beira Interior (BI), and Alentejo (AL), listed from north to south. Resistance (R) is defined as EC50 > 0.13 μg/mL for ABZ or > 0.10 μg/mL for TBZ; susceptible (S). NM = not measured (insufficient sample). * indicates farms where the 95% confidence interval (CI) for EC50 overlaps resistance threshold.

in TM included *Bunostomum trigonocephalum* (max 28.9%, TM16), *Cooperia* sp. (max 12.3%, TM13), *Trichostrongylus axei* (max 11.6%, TM14), *Oesophagostomum venulosum* (max 14.4%, TM3), and *Chabertia erschowi* (max 1.0%, TM8). In BI (1 farm, BI3), *H. contortus* (50.8%), *T. circumcincta* (32.4%), and *Trichostrongylus vitrinus* (9.1%) were detected, with *T. vitrinus* also found in AL (max 0.9%, AL15). In AL (13 farms), *H. contortus* dominated six farms (e.g., 97.3%, AL9), *T. circumcincta* five (e.g., 91.6%, AL1), and *T. colubriformis* two (e.g., 65.9%, AL15). Other AL species included *O. venulosum* (max 3.9%, AL22), *C. erschowi* (max 2.9%, AL15), *T. vitrinus* (max 0.9%, AL15), and *Nematodirus filicollis* (max 0.1%, AL21).

Species richness ranged from three (TM7, AL9) to six (TM13, TM16, AL15) taxa per farm. H′ ranged from 0.09 (AL9, near-monospecific with 97.3% *H. contortus*) to 1.49 (TM13, TM16, high diversity with six species). TM farms averaged $H' = 1.08$, with high diversity ($H' \geq 1.08$) in five farms (e.g., TM13) and low diversity ($H' = 0.47$) in TM7 (97.5%

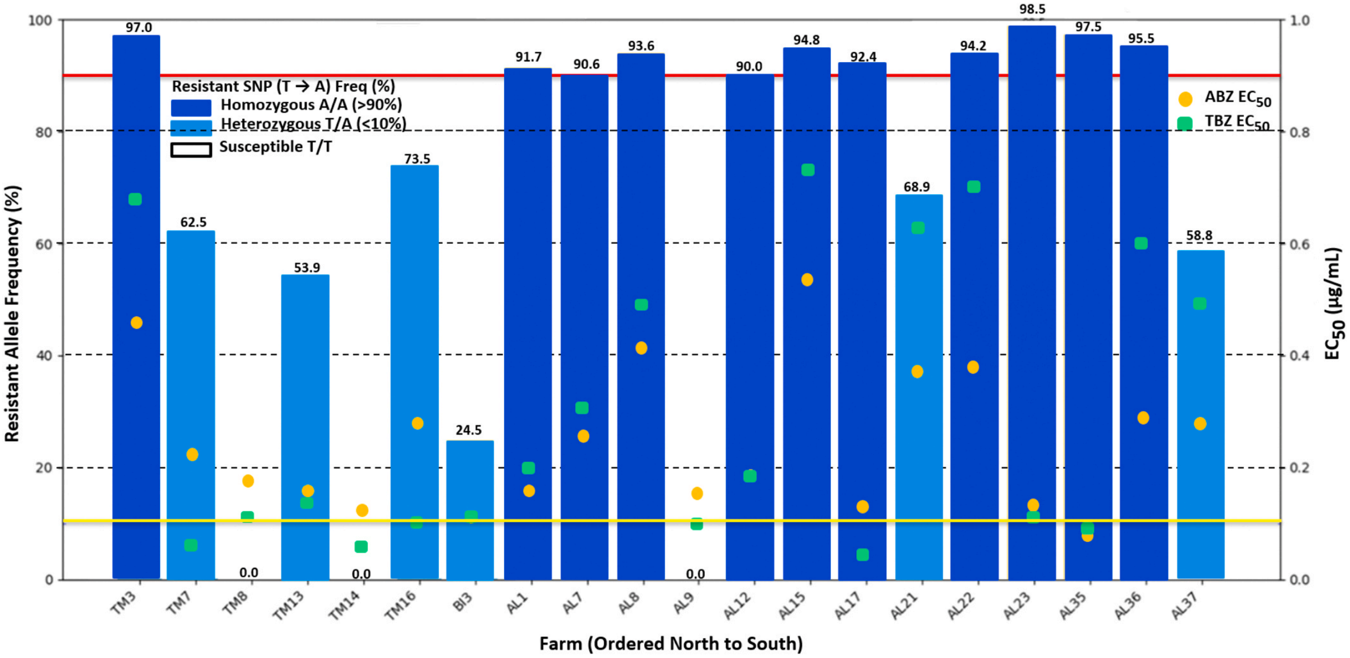

Fig. 2. Frequency of Benzimidazole-Resistant F200Y Allele and EC_{50} Values for Albendazole and Thiabendazole in *Haemonchus contortus* Populations from Portuguese Sheep Farms. Metabarcoding analysis of *β*-tubulin isotype 1 (β-*tub-1*) gene in *Haemonchus contortus* detected only the F200Y (T→A at nucleotide 619, exon 5) single nucleotide polymorphism (SNP) associated with benzimidazole (BZ) resistance across 20 sheep farms in Trás-os-Montes (TM), Beira Interior (BI), and Alentejo (AL) regions, ordered from north to south. Bars represent resistant allele (A) frequency (%), with values displayed above each bar. Bars are colored dark blue for farms inferred as predominantly homozygous resistant (A/A) to emphasize high-resistance populations, or light blue for heterozygous/mixed (A/T). No bar shows no SNP was detected, inferring predominantly susceptible (T/T) populations. Population-level zygosity was inferred as follows: $>$ 90% resistant allele = predominantly homozygous resistant (A/A); 10–90% = heterozygous/mixed (A/T); $<$ 10% = predominantly susceptible (T/T). Red horizontal line marks 90% threshold for inferred homozygous resistance. Yellow dots indicate egg hatch test EC_{50} values (μg/mL) for albendazole (ABZ), and green squares for thiabendazole (TBZ). Yellow horizontal line at 0.1 μg/mL denotes EC_{50} threshold above which resistance to BZs is indicated. Detailed data on F200Y zygosity, resistant allele frequency (%), *H. contortus* abundance (%Hc), and EC_{50} values for ABZ and TBZ are provided in Supplementary Table 4.

H. contortus). BI3 had H′ = 1.15 (six species). AL farms averaged H′ = 0.74, ranging from low (AL9, H′ = 0.09) to moderate (AL23, H′ = 1.17, five species). Farms with H′ $<$ 0.5 (TM7, AL1, AL8, AL9) had a predominant species comprising $>$ 85% of the population.

3.3. Relationships between anthelmintic resistance and GIN species composition and diversity

Correlations between AR EC_{50} values for ABZ and TBZ and GIN species composition and diversity were analyzed across 20 farms with overlapping data (Tables 1–3). Higher percentages of *T. circumcincta* and *T. colubriformis* were significantly correlated with increased EC_{50} values for ABZ (*T. circumcincta*: Pearson's r = 0.540, p = 0.013; *T. colubriformis*: r = 0.488, p = 0.029) and TBZ (*T. circumcincta*: r = 0.512, p = 0.024; *T. colubriformis*: r = 0.535, p = 0.017). In contrast, *H. contortus* showed no significant correlation with EC_{50} values (ABZ: r = -0.321, p = 0.162; TBZ: r = -0.280, p = 0.247). Species diversity (H′) and richness showed no significant relationship with AR (H′: Spearman's $\rho = -0.19$, p = 0.426), with resistance observed in both low-diversity (e.g., AL9, H′ = 0.09) and high-diversity (e.g., TM13, H′ = 1.49) farms.

3.4. Metabarcoding analysis of SNPs and benzimidazole resistance in Haemonchus contortus

Metabarcoding analysis of the isotype-1 *β*-tubulin gene in *H. contortus* targeted three SNPs associated with BZ resistance: F167Y (exon 4), E198A, and F200Y (exon 5) (Ghisi et al., 2007). Only the F200Y SNP (T→A) was detected in our study, across the 20 sheep farms analyzed (TM: 6 farms; BI:1 farm; AL: 13 farms), with probability scores of 1 and quality scores of 200 for all detected SNPs, indicating high-confidence genotyping (Fig. 2 and Supplementaty Table 4). The F200Y SNP was detected in *H.contortus* in 17 of these farms: 11 with inferred homozygous resistant (A/A) genotypes (TM3, AL1, AL7, AL8, AL12, AL15, AL17, AL22, AL23, AL35, AL36), six with heterozygous (A/T; TM7, TM13, TM16, BI3, AL21, AL37), and three with no SNP (T/T; TM8, TM14, AL9). Resistant allele frequencies ranged from 24.5% (BI3) to 98.5% (AL23) in SNP-positive farms, with T/T farms assigned 0% frequency.

A north-to-south regional gradient in F200Y SNP prevalence was observed, with significant regional differences in inferred A/A genotype proportions (chi-square, χ^2 = 6.45, df = 2, p = 0.040). AL had the highest A/A prevalence (10/13 farms, 76.9%; mean allele frequency = 91.4%, range = 58.8–98.5%), followed by two A/T farms and one T/T farm. TM showed greater variability: one A/A farm (16.7%; 97.0%), three A/T farms (mean allele frequency = 63.0%, range = 53.9–73.5%), and two T/T farms. BI had one A/T farm (24.5%) of the one farm assessed. The north-to-south increase in inferred A/A genotypes was significant (Cochran-Armitage, Z = 2.50, p = 0.012). Excluding TM3 (A/A anomaly), BI3, and T/T farms, the 14 remaining farms (TM: 3 A/T; AL: 10 A/A, 2 A/T) showed a stronger trend toward increased inferred homozygosity southward (Cochran-Armitage, Z = 3.15, p = 0.002).

Inferred zygosity and resistant allele frequency were significantly associated with EC_{50} values. EC_{50} values for ABZ (0.08–0.54 μg/mL) and TBZ (0.04–0.74 μg/mL) were significantly higher in A/A farms (median ABZ: 0.27 μg/mL; TBZ: 0.31 μg/mL) than in A/T (ABZ: 0.22 μg/mL; TBZ: 0.12 μg/mL) and T/T farms (ABZ: 0.15 μg/mL; TBZ: 0.10 μg/mL) (Kruskal-Wallis, H = 8.66, p = 0.013 for ABZ; H = 9.70, p = 0.008 for TBZ). High allele frequencies ($>$90%) typically corresponded to elevated ABZ EC_{50} values (e.g., AL15: allele frequency = 94.8%, EC_{50} = 0.54 μg/mL; TM3: 97.0%, 0.46 μg/mL), with exceptions (e.g., AL35: 97.5%, 0.08 μg/mL). TBZ EC_{50} showed similar trends but greater variability (e.

g., AL17: 92.4%, 0.04 μg/mL). Higher resistant allele frequency was significantly correlated with greater EC_{50} values (Spearman's $\rho = 0.58$, $p = 0.007$ for ABZ; $\rho = 0.62$, $p = 0.005$ for TBZ). No significant correlation was observed between *H. contortus* prevalence (%*H. contortus*, 0.2–97.5%) and inferred F200Y zygosity, with high % *H. contortus* in both A/A (e.g., AL17: 73.6%) and T/T farms (e.g., AL9: 97.3%). It should be noted that EC_{50} values, derived from nemabiome egg hatch assays, reflect the entire GIN community (Tables1, Supplementary Table 4), whereas allele frequencies only pertained to *H. contortus*.

4. Discussion

Anthelmintic resistance (AR) to benzimidazoles (BZs) in gastrointestinal nematodes (GINs) poses a significant challenge to sheep production in Portugal, where systematic surveys have lagged behind other European countries, despite widespread sheep farming (Eurostat, 2023). This study assessed BZ resistance using *in vitro* egg hatch tests (EHT) for albendazole (ABZ) and thiabendazole (TBZ) across 35 farms in Trás-os-Montes (TM), Beira Interior (BI), Alentejo (AL), and Estremadura (ES), Portugal (Table 1). Additionally, metabarcoding of the nemabiome across 20 farms (limited to TM, BI, AL due to DNA availability), characterized GIN species composition (Table 2) and BZ resistance-associated single nucleotide polymorphisms (SNPs) in isotype-1 β-tubulin gene of *H. contortus* (Fig. 2).

EHT assays revealed widespread AR, with 69% of farms resistant to ABZ, > 50% to TBZ, and 56% exhibiting dual resistance in their GIN communities (Table 1). In Europe, ABZ resistance often exceeds 80% of farms (Rose Vineer et al., 2020). In our study, seven additional farms showed emerging resistance (S*, upper 95% CI of EC_{50} overlapped resistance thresholds; Table 1). A significant positive correlation between ABZ and TBZ EC_{50} values (Pearson's $r = 0.805$, $p < 0.001$; Subsection 3.1), despite no prior TBZ administration, suggests cross-resistance, likely pervasive across Portugal. Regionally, Alentejo farms exhibited the highest median EC_{50} values (ABZ: 0.27 μg/mL, TBZ: 0.31 μg/mL), followed by intermediate values in BI and ES, and the lowest in TM (Cochran-Armitage, $Z = 2.38$, $p = 0.017$; Subsection 3.1), indicating a north-to-south of increasing gradient in BZ resistance. Higher ABZ resistance may reflect historical overuse, consistent with global patterns of frequent BZ administration (Kaplan et al., 2023). Anomalously high EC_{50} values in some farms (e.g., TM2: ABZ EC_{50} = 0.68 μg/mL) suggest localized intensive BZ use or introduction of resistant GINs (Calvete et al., 2012). Based on visual observation, EPG of feces did not correlate with EC_{50} values for either BZ (Subsection 3.1). This was likely due to variables such as worm fecundity, sample complexity (e.g., detritus), pre-patent infections, fecal moisture, density-dependent effects, and species-specific resistance (Morgan et al., 2022).

Metabarcoding identified 10 GIN species, including *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus colubriformis*, *T. axei*, *T. vitrinus*, *Oesophagostomum venulosum*, *Chabertia erschowi*, *Bunostomum trigonocephalum*, *Cooperia* sp., and *Nematodirus filicollis* (Table 2), expanding on prior Portuguese studies that identified fewer species via morphology (Matos et al., 2023; Antunes et al., 2022). The predominance of *H. contortus*, *T. circumcincta*, and *T. colubriformis* aligns with Mediterranean sheep helminthiasis patterns (Bosco et al., 2020; Castagna et al., 2024). Higher *T. circumcincta* and *T. colubriformis* abundance significantly correlated with elevated resistance values (EC_{50}) (*T. circumcincta*: $r = 0.540$, $p = 0.013$ for ABZ; $r = 0.512$, $p = 0.024$ for TBZ; *T. colubriformis*: $r = 0.488$, $p = 0.029$ for ABZ; $r = 0.535$, $p = 0.017$ for TBZ; Subsection 3.3), unlike greater variability in EC50s of *H. contortus* (Spearman's $\rho = -0.32$, $p = 0.162$), suggesting these two species have evolved highest levels of resistance in Portugal. For example, *T. circumcincta*-dominant farms (e.g., AL1, AL8) and *T. colubriformis*-dominant farms (e.g., AL15, TM3) often had EC_{50} values > 0.4 μg/mL (Table 3). This aligns with reports of BZ resistance linked to species-specific β-tubulin SNPs and frequent BZ use (Kaplan et al., 2023; Rose et al., 2015).

The F200Y SNP in *H. contortus* β-*tub-1* was significantly associated with BZ resistance, with farms showing higher inferred population-level homozygosity (A/A) and resistant allele frequency presented higher EC_{50} values (median ABZ: 0.27 μg/mL; TBZ: 0.31 μg/mL) than heterozygous (A/T) or susceptible (T/T) farms (Kruskal-Wallis, $H = 8.66$, $p = 0.013$ for ABZ; $H = 9.70$, $p = 0.008$ for TBZ; Subsection 3.4). Resistant allele frequency strongly predicted resistance (Spearman's $\rho = 0.58$, $p = 0.007$ for ABZ; $\rho = 0.62$, $p = 0.005$ for TBZ). The north-to-south increase in inferred A/A genotypes (TM: 16.7%; AL: 76.9%; $\chi^2 = 6.45$, $p = 0.040$) suggests stronger BZ selection pressure in Alentejo's warmer, drier climate, favoring *H. contortus* fitness (Rose et al., 2015). This resistance profile is further underscored by presence of F200Y SNP, detected in 85% of the studied farms in Portugal, with 55% exhibiting inferred homozygous and 30% heterozygous genotypes. Higher A/A prevalence was reported in Bosnia and Herzegovina (77%; Kapo et al., 2024) and Sweden (88.5%; Baltrušis et al., 2020). Exclusive detection of F200Y, consistent with global patterns (Kotze et al., 2014; Chaudhry et al., 2015), underscores its role as the primary BZ resistance driver in Alentejo. High A/A classification, therefore, indicates near-fixation of the resistant allele in the *H. contortus* population on that farm, consistent with phenotypic resistance observed in EHT assays.

Anomalies, such as AL35 with high A/A F200Y (97.5%), but susceptibility to ABZ (EC_{50} = 0.08 μg/mL) and TBZ (EC_{50} = 0.09 μg/mL), suggest additional factors difficult to explain, like variable drug uptake or host responses (Rufener et al., 2009; Whittaker et al., 2017). Farms lacking F200Y (TM8, TM14, AL9) but showing EC_{50} values near resistance thresholds (Fig. 2) may involve resistance in other GINs in the neambiome or alternative mechanisms, such as other β-tubulin mutations, cytochrome P450 detoxification, or polygenic resistance (Velík et al., 2004; Whittaker et al., 2017; Doyle et al., 2020). EHT assessment of the entire nemabiome may amplify contributions from resistant *T. circumcincta* or *T. colubriformis* in these farms (von Samson-Himmelstjerna et al., 2009). A recent study in Sinaloa, Mexico of sheep GINs reported the F200Y (all heterozygous) as the sole BZ resistance SNP in *H. contortus* and a GIN species composition of *Haemonchus* sp., *Cooperia* sp. and *Trichostrongylus* sp. (Solis-Carrasco et al., 2025).

Dual resistance in 56% of farms limits treatment options, necessitating non-BZ anthelmintics (e.g., macrocyclic lactones, imidazothiazoles) and IPM strategies like pasture rotation and selective treatment (Torres-Acosta et al, 2012; Charlier et al., 2020). Susceptible farms in TM offer opportunities to preserve BZ efficacy through cautious use. The warm, dry climate of Alentejo and high sheep density, similar to Northeast Spain (Calvete et al., 2012), likely enhance GIN fitness and resistance (Papadopoulos et al., 2001; Rose Vineer et al., 2020). Routine surveillance for resistance-associated SNPs, region-specific strategies, such as refugia-based approaches in Alentejo (Kenyon et al., 2009), and integrated management in TM, are critical to mitigate AR and sustain Portuguese sheep production.

5. Conclusion

This study reveals widespread benzimidazole (BZ) resistance in gastrointestinal nematodes (GINs) across 35 Portuguese sheep farms, with 69% resistant to albendazole (ABZ), 59% to thiabendazole (TBZ), and 56% exhibiting dual resistance. Resistance was most severe in Alentejo, where *Haemonchus contortus* showed high F200Y β-*tub-1* SNP resistant allele frequencies (mean 91.4%) and inferred homozygous resistant (A/A) genotypes in 76.9% of farms. A north-to-south gradient highlighted Alentejo's elevated resistance compared to partial susceptibility in Trás-os-Montes. Metabarcoding identified 10 GIN species, including *H. contortus*, *Teladorsagia circumcincta*, *Trichostrongylus colubriformis*, and *Chabertia erschowi*, with *T. circumcincta* and *T. colubriformis* abundance linked to increased resistance. A warm, dry climate, similar to Northeast Spain (Calvete et al., 2012), in Alentejo likely enhances GIN fitness. Pervasive resistance to ABZ and TBZ across all four Portuguese regions, alongside reported resistance to ABZ and mebendazole, in a separate study, in the Lisbon/Tagus Valley (Antunes et al., 2022), aligns with European trends (Charlier et al., 2024). Alentejo requires urgent adoption of IPM, including selective treatment and rotational grazing to curtail cross-transmission of resistant genotypes among sympatric domestic flocks and wild caprines, potentially serving as reservoirs (Beaumelle et al., 2023; Francis and Šlapeta, 2023). Further research should assess resistance to larvicidal anthelmintics (e.g., macrocyclic lactones, such as ivermectin), expand regional sampling, and incorporate farm-level management data to refine sustainable parasite control strategies for Portuguese small ruminant production.

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Ethical statement

Rectal fecal samples were collected as part of veterinary diagnostic screening for helminths. No additional handling or harm was caused to the animals for research purposes.

CRediT authorship contribution statement

Joel Antunes: Resources. **Ana Rita Simões:** Resources. **Paulo Almeida:** Formal analysis. **Faria Campbell Natalia:** Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Helga Waap:** Writing – review & editing. **Helena Monteiro:** Resources. **Humberto Pires:** Resources. **Hélder Quintas:** Resources. **Helder Cortes:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Natalia Faria Campbell reports financial support was provided by Mediterranean Institute for Agriculture Environment and Development. Natalia Faria Campbell reports financial support was provided by Foundation for Science and Technology. If there are other authors, they 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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2026.110782.

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