Enhancing Faecal Egg Count Reduction Tests with ITS2 Nemabiome

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Highlights

- Integrated ITS2 Nemabiome with FECRT to enhance assessment of species-level reductions.
- Hidden BZ and LEV resistance and susceptibility detected across multiple farms.
- LEV retained a higher efficacy than BZ across most farms and species.
- Enables the surveillance of emerging resistant species and targeted treatment

Abstract

The accelerating development of anthelmintic resistance among gastrointestinal nematodes (GIN) threatens the long-term sustainability of effective parasite control in Merino sheep. The faecal egg count reduction test (FECRT) remains the gold standard for assessing anthelmintic efficacy by calculating the GIN population reduction through the 95% efficacy threshold. However, its accuracy is compromised in mixed-species infections, where morphologically identical eggs are collectively included in the population reduction estimate. Internal Transcribed Spacer 2 (ITS2) Nemabiome represents a deep amplicon sequencing tool that complements FECRT by providing species-specific reductions in mixed-species infections. By integrating these two techniques, we demonstrated how the addition of ITS2 Nemabiome enhances the diagnostic output of FECRT in capturing resistance patterns at the species level rather than at the population level. Three pools of faecal samples were collected across twenty-one Merino sheep farms: a pre-treatment group, a post-Benzimidazole (BZ) group and a post-Levamisole (LEV) group. We conducted faecal egg counts and amplicon sequencing

of third-stage larvae to determine species composition and abundance of each treatment. Integration of ITS2 Nemabiome and FECRT involved scaling the ITS2 proportion reads to FECRT data to estimate and visualise individual species reductions across the two treatments. *Haemonchus contortus* was identified as the predominant species whereas LEV achieved a greater treatment efficacy than BZ. ITS2 Nemabiome revealed cases of hidden susceptibility and resistance in 63% of farms, providing diagnostic enhancement that would have otherwise remained undetected by FECRT alone. This work demonstrates the value of integrating ITS2 Nemabiome with FECRT to enhance the detection of emerging GIN species and anthelmintic resistance within flocks. It also represents an informative diagnostic framework for farmers that aim to refine their GIN control methods with a targeted and sustainable approach. In line with this, future research should aim to develop species-specific efficacy thresholds that address the differences in fecundity and prepatency, aligning with the species-specific reductions revealed through ITS2 Nemabiome.

Graphical Abstract:

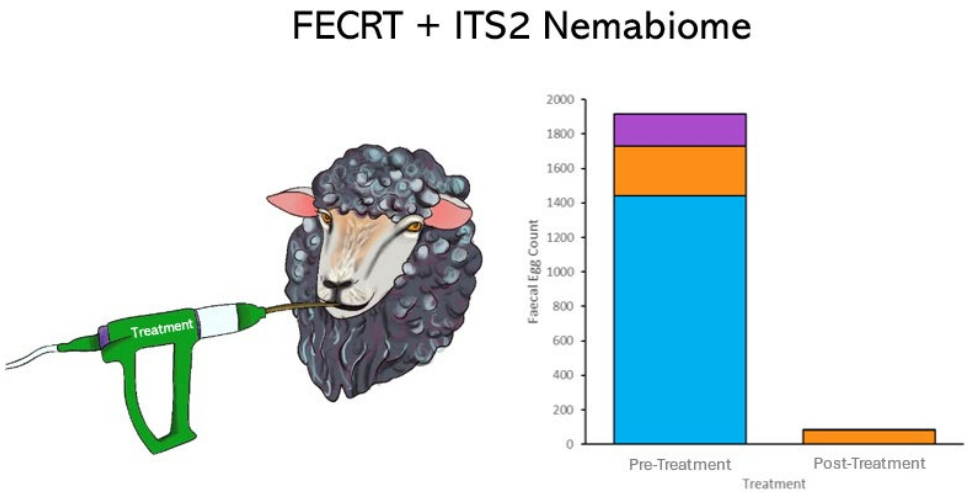

Image created by author

Keywords

Gastrointestinal nematodes; Anthelmintic resistance, FECRT, ITS2 Nemabiome; Targeted Parasite Control

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1. Introduction:

Control of gastrointestinal nematodes (GIN) in Merino sheep heavily rely on effective anthelmintics to maintain animal health and productivity. However, the accelerating development of anthelmintic resistance, particularly multiple-drug resistance in GIN, threatens the long-term sustainability of these treatments and concurrently the health and welfare of Merino sheep.

The faecal egg count reduction test (FECRT) is a traditional, field-based diagnostic test that remains the gold standard for assessing anthelmintic drug efficacy against gastrointestinal nematodes (GIN) (Nielsen, 2021). The test quantifies the percent reduction in GIN egg output following the administration of treatment with reductions greater than 95% indicating a susceptible GIN population (treatment efficacy) and reductions below 95% indicating resistance (treatment inefficacy) (Kaplan et al., 2023).

The primary advantages of FECRT lie in its statistical foundation and on-farm applicability, making it a practical and convenient diagnostic tool in livestock systems. The continued recognition of FECRT as the gold standard essentially stems from its validation through the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P). The W.A.A.V.P guidelines provide protocols, procedures and an explanation of the 95% efficacy threshold, ensuring standardisation across all anthelmintic classes in multiple host and

parasite species (Kaplan et al., 2023). Despite this robust foundation, FECRT incurs limited sensitivity and interpretive power when pre-treatment egg counts or sampling sizes are low (Dobson et al., 2012). It also cannot differentiate between GIN species in mixed-species infections as Strongylida produce morphologically indistinguishable eggs (Avramenko et al., 2015). This limits its ability in providing species-specific diagnoses and may obscure the detection of resistant or susceptible species that are concealed in low abundance (Morgan et al., 2022).

The Internal Transcribed Spacer 2 (ITS2) Nemabiome approach represents a molecular diagnostic tool that employs DNA metabarcoding to detect and identify GIN communities in pooled third-stage larval (L3) cultures. This technique targets the ITS2 ribosomal DNA region through pan-nematode primers, amplifying a conserved locus that permits interspecific variation across multiple GIN taxa, namely the Strongylida (Avramenko et al., 2015). Through this, ITS2 Nemabiome enables high-throughput sequencing of complex mixed-species infections, offering quantification of individual GIN species by mapping their relative proportions (Redman et al., 2019). This species-specific resolution allows for a more informed assessment of GIN infections by distinguishing species-specific resistance patterns and encouraging the onset of sustainable parasite control through the reduction of unnecessary anthelmintic use (Von Samson-Himmelstjerna et al., 2002). However, species representation bias is a significant limitation. While the ITS2 region is a universal genetic marker for a range of GIN, differences in the number of ITS2 tandem repeats between species may lead to the over-amplification of certain species, manipulating the relative abundance and true species composition within the sample (Avramenko et al., 2015; Von Samson-Himmelstjerna et al., 2002).

In this critical backdrop of increasing anthelmintic resistance and shift towards targeted and sustainable parasite control, diagnostic techniques serve as fundamental measures of both the

quantitative and qualitative components of GIN infections. However, existing diagnostic methods lack the ability to accurately quantify species composition in mixed-species infections, thereby limiting their capacity to guide informative control strategies that evaluate the anthelmintic performance at the species-level rather than across the entire GIN population. Diagnostic tools must, therefore, evolve to balance these aspects with precision and practicality, which can be achieved through the integration of FECRT with ITS2 Nemabiome. By employing FECRT's statistically validated quantitative assessment with ITS2 Nemabiome's species-specific proportional data, this integrated approach has the potential to provide a more comprehensive approach to GIN detection, identification and interpretation to better inform treatment outcomes for farmers.

Accordingly, the present study aims to discover whether the application or addition of ITS2 Nemabiome will enhance the diagnostic output of FECRT by comparing species-specific reductions to the whole population reduction. Furthermore, it aims to evaluate the value of ITS2 Nemabiome in the context of providing an informed diagnosis that strengthens the reliability of capturing species-specific resistance patterns in GIN infections.

2. Materials and Methods:

2.1 Sample Collection and Treatment

Faecal samples were collected from 21 Merino sheep farms across New South Wales. Each farm was pooled into 3 groups: pre-treatment, post-treatment following benzimidazole administration (BZ) and post-treatment following levamisole administration (LEV) (n = 63). Post-treatment samples were collected after 14 days following anthelmintic administration in accordance with W.A.A.V.P guidelines (Kaplan et al., 2023). These faecal samples were

transported to the NSW Department of Primary Industries for faecal egg counting and were refrigerated under appropriate conditions.

2.2 Faecal Egg Counts and Faecal Egg Count Reduction Test

Faecal egg counts were conducted by the NSW Department of Primary Industries using the mini-FLOTAC method. Faecal egg counts were determined for each treatment (n=63) per farm and tabulated in a spreadsheet. Pre-treatment samples below 150 eggs per gram (EPG) were excluded from the FECRT to ensure the test had sufficient statistical power and sensitivity to detect a valid reduction in GIN population (Kaplan et al., 2023).

For the FECRT, we expressed anthelmintic efficacy as a percentage reduction in the egg counts between the post-treatment (BZ and LEV) and the pre-treatment samples, using the W.A.A.V.P guidelines (Kaplan et al., 2023):

$$Reduction\ (\%) = 1 - \left(\frac{(Pre\ Treatment\ FEC - Post\ Treatment\ FEC)}{(Pre\ Treatment\ FEC)}\right) \times 100$$

Reductions > 95% were classified as susceptible populations and reductions < 95% were classified as resistant populations. Reductions that were exactly 95% were classified as an inconclusive result as the confidence intervals around this estimate may overlap the resistance threshold and skew the interpretive power of FECRT (Dobson et al., 2012, Kaplan et al., 2023). We tabulated the FECRT values for each farm's treatment in a spreadsheet for further analysis.

2.3 Third-stage Larvae Preparation

Larval cultures of each faecal sample were prepared according to the Australian and New Zealand Standard Diagnostic Procedures (ANZSDP) (Hutchinson, 2009). The faecal cultures were prepared and placed in 400 mL glass jars and incubated at 27 °C for 7 days, permitting the development of third-stage larvae (L3). In order to ensure the L3s grew under optimal conditions, temperature, humidity and sources of contamination and fungal growth were regularly monitored. Following incubation, the L3 were recovered by filling the culture jars with warm water (30 °C) and inverted over a petri dish. The L3 were allowed to stand overnight (approximately 9 hours) to enable larval migration. The water the L3s were placed in were then transferred into 15 mL Falcon tubes and centrifuged at 500 *g* for 3 minutes to obtain a concentrated larval pellet at the bottom. The samples were stored at 4 °C for downstream DNA isolation.

2.4 DNA Isolation

Before DNA was extracted from the larval samples, the suspensions were examined microscopically to estimate abundance. Only samples with ≥ 2000 larvae were processed to ensure sufficient DNA yield for ITS2 Nemabiome metabarcoding.

The larval samples were centrifuged at 1000 *g* DNA for 2 minutes before the genomic DNA was extracted from the larval pellets using the Monarch Genomic DNA Purification Kit following the manufacturer's instructions for animal tissue specimens (New England Biolabs, Australia). The DNA was eluted from all larval isolates into a final volume of 100 µL of elution buffer and stored in the freezer at -20˚C.

2.5 Serial Dilution

Extracted genomic DNA (100 µL) was serially diluted in distilled water to obtain a concentration of 1:1000. To reduce the effects of PCR inhibitors and errors, this was conducted through a stepwise dilution series where 45 µL of distilled water was mixed in

each stage (1:10, 1:100, 1:1000). Pipette tips were changed with each stepwise dilution and contact with the microcentrifuge tubes was avoided. The dilutions were stored in the freezer at -20 ˚C.

2.6 Real-time Polymerase Chain Reaction (PCR) for quantification and validation of GIN genomic DNA

All real-time PCRs were prepared with 10 µL of SensiFAST™SYBR® No-ROX mix (Meridian Bioscience, Australia), 0.8 µL of NC1 primer (forward), 0.8 µL of NC2 primer (reverse), 6.4 µL of water and 2 µL of the DNA template from the prepared 1:1000 serial dilution samples. Real-time PCR was conducted to quantify and validate the genomic DNA of representative GIN species. The ribosomal DNA of the ITS2 region was amplified using a pair of 'pan-nematode' primers: Strongyl_ITS2_F [S0865] (5′- ACG TCT GGT TCA GGG TTG −3′) and Stongyl_ITS2_R [S0866] (5′- ATG CTT AAG TTC AGC GGG TA-3′), generating amplicons that were approximately 240 base pairs in length (Francis and Šlapeta, 2022). PCRs were processed on the CFX Opus 96 Real-Time PCR Detection System where each well of the 96 well plate contained DNA template from a separate diluted sample, one negative control and one blank (water) (BioRad, Australia). The process involved an initial denaturation at 95 ˚C for 2 minutes, followed by 40 cycles of 95 °C for 5 seconds, 60 ˚C for 15 seconds, 72 ˚C for 15 seconds and a final melt curve analysis (Francis and Šlapeta, 2022).

2.7 ITS2 Nemabiome Metabarcoding

ITS2 Nemabiome metabarcoding was conducted to determine the composition of GIN species populations in the pre- and post-treatment samples. Genomic DNA lysates from the L3 cultures were submitted to the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) for library preparation and Illumina MiSeq sequencing. In summary, a fragment of approximately 311 to 331 base pairs of the ITS2 ribsosomal DNA

region was amplified using primer pairs containing Illumina sequencing adaptors and random nucleotides to offset the primers and the fluorescence signal during the process of sequencing (Francis and Šlapeta, 2022). Library preparation at the Ramaciotti Centre entailed the attachment of dual index barcodes and sequencing adaptors using the Nextera XT Index Kit V2 (Illumina Inc., USA). The indexed libraries were quantified, normalised, pooled and sequenced on the Illumina MiSeq platform with 2 × 250 base pairs paired-end reads (Francis and Šlapeta, 2022).

2.8 Bioinformatics and Data Analysis

Raw sequence data included paired forward and reverse reads in the form of FASTQ files. The data was processed and analysed through R using the DADA2 bioinformatic pipeline. The forward and reverse reads underwent primer removal, quality filtering, denoising, merging of the paired-end reads and chimera removal. The remaining amplicon sequence variants (ASVs) were assigned to their respective species through a published trichostrongylid nematode ITS2 rDNA database from nemabiome.ca using the 'assignTaxonomy' function (Workentine et al., 2020).

The read counts (from DADA2's ASV table output) for each GIN species per sample were exported to Microsoft Excel and tabulated in a custom spreadsheet. The ITS2 reads of each species were converted into relative proportions within the sample per farm before they were scaled to relative egg counts. The ITS2 proportion reads were scaled to the relative egg counts through the corresponding faecal egg count data from the NSW Department of Primary Industries. From this, samples with pre-treatment egg counts ≤150 eggs per gram (EPG) were excluded from analysis in accordance with W.A.A.V.P guidelines (Kaplan et al.,2023).

The final calculation involved a reduction map. Pre-treatment counts were set as 100% abundance and the post-treatment values (BZ and LEV) were expressed as percentage reductions relative to the pre-treatment count. Species with reductions < 95% were classified as resistant and species with reductions > 95% were classified as susceptible. Species with reductions as 95% were classified as an inconclusive result.

The ITS2 relative proportions, relative egg counts using FECRT data and the reduction map were visualised as clustered column graphs and a connected dot plot for each farm.

3. Results

In total, 21 farms were recruited for this study however, after the W.A.A.V.P prerequisite criteria of the 150 EPG pre-treatment count was applied, 16 farms were included for analysis (Kaplan et al., 2023).

3.1 Faecal Egg Count Reduction Tests reveal high Benzimidazole Resistance compared to Levamisole Resistance across farms

The FECRT was performed using benzimidazole (BZ) and levamisole (LEV) as the anthelmintic treatments for each farm. Across all farms, the mean pre-treatment egg count was 1334 EPG, mean post-treatment (BZ) count was 367 EPG and mean post-treatment (LEV) count was 188 EPG (Fig.1; Appendix A1). For BZ treatment, 14 out of 15 farms (Farm 1 had no BZ data) displayed reductions below 95%, indicating resistance to treatment. Farm 4 showed an increase rather than a decrease following BZ treatment suggesting substantial anthelmintic failure (Fig.1). For LEV, 8 out of 16 farms were resistant to treatment. The mean reduction for BZ treatment across all farms was 72% while the mean reduction for LEV treatment was 79%, demonstrating that LEV was generally more effective at reducing GIN populations than BZ.

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230 3.2 ITS2 Nemabiome integrated with FECRT demonstrates *Haemonchus contortus* as the
231 Predominant Species and confirms greater Levamisole Efficacy

232 A total of 70 unique ITS-2 Amplicon Sequencing Variants (ASVs) were generated across 62
233 samples. The depth of sequencing varied significantly with an average of 354 reads per
234 sample and a range of 25 reads to 1066 reads. There were 10 GIN species identified and 2
235 identified at the genus level: *Haemonchus contortus, Trichstrongylus colubriformis,*
236 *Telodorsagia circumcinta, Haemonchus placei, Trichstrongylus rugatus, Chabertia ovina,*
237 *Cooperia oncophora Oesophagostomum venulosum, Trichostrongylus axei and*
238 *Trichostrongylus vitrinus* (Table. 1). The read counts per species revealed a dominance of
239 *H.contortus*, followed by *T.circumcincta* and *T.colubriformis*. There were some uncommon
240 species identified including *C.ovina* and *C.oncophora* however, they were present at lower
241 abundances (< 100) and were therefore not retained for further analysis. A threshold for the
242 number of sequencing reads was not applied during this stage. Instead, a threshold was
243 applied post-hoc, whereby samples were excluded if their ITS2 proportional egg counts did
244 not exceed 150 EPG.

245 The ITS2 Nemabiome analysis revealed the presence, proportional abundance and reduction
246 patterns of GIN species for each farm (Fig.2; Appendix A2). *Haemonchus contortus* was the
247 predominant species identified across 14 farms, followed by *Telodorsagia circumcincta* in 7
248 farms *and Trichostrongylys colubriformis* in 3 farms (Fig.2; Appendix A2). In mixed GIN
249 infections (≥2 species), farms 3, 5, 7, 9, 10 and 14 displayed significant proportional changes
250 between the post-treatments (Fig.2; Appendix A2).

251 ITS2 proportion reads integrated with the faecal egg counts conveyed how individual species'
252 abundance changed in response to treatment. As the ITS2-derived egg counts were scaled

from the proportions of the ITS2 reads, the reductions following BZ and LEV treatment reflected the dominating species per treatment. This trend was further exemplified by the reduction map, which applied a 95% threshold to highlight the GIN species that were resistant or susceptible to treatment.

Farms 1, 3 and 4 exhibited GIN species that increased rather than decreased in abundance post-treatment, indicating substantial treatment failure (Fig.2; Appendix A2). Although LEV demonstrated overall greater treatment efficacy than BZ, it was considerably less effective against *T.circumcincta* and *Haemonchus placei* in farms 1 and 3 (Fig.2; Appendix A2). Despite this, the reduction maps portrayed that LEV generally outperformed BZ treatment especially against *H.contortus* which dominated the majority of farms (14/16). For instance, in farms 4, 6, 12, 13, 15 and 19, *H.contortus* was the predominant species, and in five of these six farms, LEV treatment achieved a greater reduction than BZ, demonstrating greater reduction against the species (Fig.2; Appendix A2). Yet, as observed through *T.circumcincta* and *H.placei,* LEV achieved fewer substantial reductions across other GIN species. This trend was evident in farms 1, 5, 7, 9, 10, 14 and 18, where *H.contortus* demonstrated a greater reduction than co-occurring GIN species following LEV treatment (Fig.2; Appendix A2).

3.3 Integration of ITS2 Nemabiome and FECRT uncovers Hidden Resistance and Susceptibility

To further assess the diagnostic value of combining molecular and traditional methods, ITS2 Nemabiome results were compared with corresponding FECRT outcomes (Table 2). The integration revealed several discrepancies between the results, exemplifying the value of species-specific reductions.

For BZ treatment, ITS2 Nemabiome integrated with FECRT revealed that farms 5, 11 and 19 produced different results to FECRT (Table 2). According to the FECRT results, 14 out of 15

(93%) farms were classified as resistant to BZ, however, the detection of susceptible GIN species by ITS2 Nemabiome indicated the presence of hidden susceptibility within mixed-species infections in these farms. It additionally demonstrated that BZ resistance is not as profound as FECRT claims.

Regarding LEV treatment, cases of hidden susceptibility and hidden resistance were identified. Although, FECRT found 8 out of 16 farms as resistant to LEV, half of these (50%) farms demonstrated hidden susceptibility through ITS2 Nemabiome (Table 2). Conversely, 37.5% of farms classified as susceptible by FECRT (farms 9, 10 and 15) displayed hidden resistance, reflecting the persistence of GIN subpopulations that remained undetected by FECRT. While Farm 14 yielded an “inconclusive” FECRT result and ITS2 Nemabiome yielded a result of “susceptibility”, this farm was not classified as hidden susceptibility due to the uncertainty of the FECRT outcome.

Overall, 10 out of 16 farms (63%) incurred additional diagnostic insight from the integration of ITS2 Nemabiome and FECRT.

4. Discussion

GIN infections in sheep have long relied on the FECRT for estimating parasite burdens and assessing treatment efficacy. However, PCR-based molecular tools are constantly evolving to easily identify a broader range of GIN species simultaneously, overcoming the limitations of conventional PCR’s species-specific primers (De Seram et al., 2023, Hoglund et al., 2023, Redman et al., 2019). This foundation offers the opportunity for the detection of emerging resistant GIN species, facilitating treatment strategies to be tailored to the farm predominant species and supporting the movement toward targeted parasite management. Consequently, this study demonstrates how integrating the quantitative reduction threshold estimates from

FECRT with the qualitative species composition data from ITS2 Nemabiome enhances the overall diagnostic output by linking treatment efficacy to individual species responses, rather than the collective GIN population. This integration reveals nuances such as the dynamic abundance of GIN species relative to treatment response that reflect the onset of anthelmintic resistance (Queiroz et al., 2020).

However, in order to successfully delay resistance, an understanding of the flock predominant GIN species and how the respective species individually respond to treatment is necessary. This enables the onset of informed treatment decisions where farmers can selectively use anthelmintics and adopt differing control strategies (e.g. Integrated Parasite Management) to reduce the parasitic burden from dominating species (Queiroz et al., 2020). Findings from the present study demonstrate how integrating ITS2 Nemabiome with FECRT can provide this standard of diagnostic insight. By scaling the ITS2 Nemabiome proportion reads to the faecal egg counts, *H.contortus* was determined as the predominant species across multiple farms. Additionally, applying FECRT's efficacy threshold revealed LEV to achieve greater reductions against GIN species, particularly against *H. contortus*, when compared to BZ. This finding resonated with previous studies that found LEV to be effective against *H. contortus* than other anthelmintics (Andrews, 2000, Ruffell, 2018, Tyrrell and LeJambre, 2010).

LEV's mechanisms of action were initially described in Ruffell et al. (2018), who demonstrated that high-level resistance in *H. contortus* is correlated with mutations in the nicotinic acetylcholine receptor subunit genes (target site of LEV) whereas low-level resistance in *H. contortus* is correlated with the overexpression of P-glycoproteins that induce LEV efflux. However, subsequent studies found that these markers were unreliable predictors of LEV resistance and that ≥ 16% of the S168T SNP in the *acr-8* gene is the most valid predictor of high-level LEV resistance (Francis et al., 2024). This exemplifies how the addition of ITS2 Nemabiome can strengthen the interpretation of species-specific resistance

patters by demonstrating which species persist after LEV treatment and hence, warrant targeted SNP analysis for a comprehensive assessment of anthelmintic resistance.

Although LEV was broadly effective against *H.contortus* and other GIN species, ITS2 Nemabiome integrated with FECRT additionally revealed how its diminishing efficacy may require judicious use of the anthelmintic. This was reflected in the post-treatment increase of other GIN species and was further exemplified by the detection of resistant species in FECRT susceptible farms. This implication may reflect anthelmintic preferences, as farmers often favour BZ due to its shorter withdrawal periods (time before the animal product can be sold after the administration of treatment) (Fischer et al., 2025). In this context, the value of integrating species-specific proportion data from ITS2 Nemabiome with FECRT's population-based efficacy threshold, highlights trends in management preferences. It also informs farmers of the importance of treatment rotation that delays anthelmintic resistance and the consequences of prioritising economic productivity over flock health (Fischer et al., 2025). This can encourage farmers to adjust their control strategies by rotating anthelmintic classes, adopting targeted selective treatment (only treating ill animals), quarantining and treating new animals, rotational grazing to disrupt the nematode life cycle and breeding for GIN resistance (Howell et al., 2025).

ITS2 Nemabiome complements FECRT by addressing its limitation in species-specific identification and reduction patterns, making it an effective addition for assessing mixed-species infections (Avramenko et al., 2015, Leathwick et al., 2025, Redman et al., 2019). However, an accurate quantification also underpins an enhanced diagnosis. ITS2 Nemabiome may offer greater sensitivity than FECRT due to the reliance on a conserved genetic region rather than morphological GIN eggs, but the accurate quantification of GIN species ultimately remains contingent on the FECRT itself (Avramenko et al., 2015; Hoglund et al., 2023). Since the ITS2 proportion reads are scaled to the faecal egg count values, any

inaccuracies in the egg counts will propagate through to the species-specific abundance values, skewing the final classification (resistant, susceptible or inconclusive) and interpretation of the results.

However, the factor of accuracy in faecal egg counts is confounded by biological fluctuations in GIN egg shedding, which can facilitate inherently false egg counts. Successful GIN egg production depends on the concurrent presence of both male and female species; therefore, an uneven sex ratio drives a reduced or absent egg population (Rinaldi et al., 2022). Fecundity and the length of the prepatent period also vary across GIN species, with *H. contortus,* displaying higher fecundity and short prepatency (Dobson et al., 2012; Pires et al., 2021), reflecting its predominance across 88% (14/16) of farms. Yet, more fecund and prepatent GIN species will inherently dominate the ITS2 Nemabiome proportion reads and suppress representation of others (Evans et al., 2023). This means that when the proportions are scaled to the faecal egg counts, less fecund and prepatent GIN species may be excluded from analysis as their pre-treatment egg counts will fall below W.A.A.V. P's > 150 EPG threshold (Kaplan et al., 2023). This implies that ITS2 Nemabiome cannot fully rectify FECRT's limitation of inaccurately estimating flock parasite burdens. Nevertheless, given that the quantification of true parasite burdens remain inherently elusive due to the variability in egg shedding, the integration of ITS2 Nemabiome with FECRT represents a robust and practical approach for assessing treatment efficacy in mixed-species infections.

While the amalgamation of FECRT's characteristics may aid in validating the interpretive power of GIN infections, W.A.A.V.P's > 150 EPG threshold constrains the inclusion of all GIN species detected by ITS2 Nemabiome. This aspect holds particular significance for targeted parasite control, as it echoes the importance of early detection of emerging species within a flock before they reach diagnostically resistant levels (Le Jambre, 2006). In the present study, multiple GIN species identified by ITS2 Nemabiome were excluded from

treatment efficacy analysis as a result of this pre-treatment threshold. Although it could be argued that species with relative abundance < 0.2% may hold negligible clinical relevance, this circumstance highlights a diagnostic paradox: PCR-based diagnostic tools offer greater sensitivity, capable of detecting all the species within a sample, yet their interpretive power is restricted by thresholds. In this light, the diagnostic value of ITS2 Nemabiome becomes constrained, particularly when only one GIN species such as *H. contortus* is included in treatment efficacy analysis, as observed across 38% (6/16) of farms.

Similarly, Fischer et al. (2025) excluded samples with pre-treatment egg count < 200 EPG (for pigs) to reduce false positives however, this approach introduced misclassifications of resistance and inconclusion, increasing uncertainty in both FECRT and ITS2 Nemabiome. Despite this, Leathwick et al. (2025) demonstrated that while the exclusion of low pre-treatment egg counts limits GIN species representation, ITS2 Nemabiome instead enables their inclusion due to its ability to sequence 2000-3000 L3. Its large sampling size permits the formation of tighter confidence intervals around efficacy estimates, suggesting that the inclusion of a pre-treatment threshold may be unnecessary if FECRT were to be coupled with ITS2 Nemabiome (Leathwick et al., 2025). This finding by Leathwick et al. (2025) raises a critical point for the value of integrating FECRT with ITS2 Nemabiome. Currently, the >150 EPG threshold ensures the statistical reliability of the FECRT 95% efficacy classification. However, ITS2 Nemabiome's high-throughput sequencing ensures the inclusion of less fecund and prepatent GIN species while maintaining precise efficacy estimates. This implies that, rather than incorporating FECRT's > 150 EPG threshold, the 95% efficacy threshold alone may be sufficient, enhancing the precision of FECRT and enabling ITS2 Nemabiome to capture full species composition and resistance patterns within a flock. For farmers, this would provide more informed diagnoses and treatment outcomes, detecting low abundant or

emerging GIN species, that may produce distinct host clinical signs not evident from prevalent species.

As next-generation sequencing molecular tools like ITS2 Nemabiome become increasingly employed for enhancing species-specific resolution, current efficacy thresholds may require reassessment for accurate interpretation (Guinda et al., 2025). The 95% efficacy threshold, defined by W.A.A.V.P for FECRT, was produced to assess GIN abundance at the population level, supported by prerequisites such as > 150 EPG pre-treatment egg count, sample size ≥ 5 animals and a 14-day period between anthelmintic administration and post-treatment sample collection (Kaplan et al., 2023). In contrast, ITS2 Nemabiome operates through relative species proportions. As the ITS2 proportion reads are scaled to the faecal egg counts, there is an implicit assumption that the relative proportion of sequence reads directly translates to the egg output of each GIN species rather than the entire GIN population. This may distort the interpretive power of the 95% threshold. For instance, an inconclusive result (reduction was 95%) was determined for one farm however, ITS2 Nemabiome reported this as a susceptible result. Accordingly, the inconclusion of FECRT's result compromises the validity of ITS2 Nemabiome's result and highlights the limitations of applying a fixed population-based threshold to species-specific data (Denwood et al., 2023).

Currently, no studies have investigated species-specific efficacy thresholds, and existing studies Queiroz et al. (2020), De Seram et al. (2023), Krücken et al. (2024) have yet to explore the interpretive challenges posed by the integration of ITS2 Nemabiome with FECRT. Future research should aim to establish threshold frameworks that account for biological variability in fecundity and anthelmintic response (Denwood et al., 2023; Krücken et al., 2024). Moreover, an understanding of the genetic markers for resistance through targeted SNP analysis is fundamental to the development of species-specific efficacy thresholds since different anthelmintics incur different mechanisms of action for GIN species

(Guinda et al., 2025). This aspect becomes particularly critical when the few GIN species surviving treatment in phenotypically susceptible populations are instead, a small subpopulation of phenotypically resistant GIN species (Guinda et al., 2025). Thus, addressing these gaps will be essential for actioning targeted parasite control, particularly as species-level diagnostic resolution becomes increasingly promoted for sustainable management strategies.

Overall, this study demonstrates that ITS2 Nemabiome enhances FECRT's diagnostic value by highlighting species-specific susceptibility and resistance in mixed-species infections. Across 63% (10/16) of studied farms, this integration revealed hidden susceptibility and resistance that would have not been attainable by FECRT, supporting more informed treatment decisions. However, in order to fully utilise the diagnostic aptitude of ITS2 Nemabiome, the development of species-specific efficacy thresholds would be required to enhance the accurate detection and interpretation of emerging GIN species. This assessment of emerging GIN species would be valuable for a profound understanding of anthelmintic resistance, particularly in targeted SNP analyses which validates thresholds for phenotypic resistance. Thus, these findings validate the application of ITS2 Nemabiome as a complementary tool to FECRT, enhancing diagnostic interpretation by capturing species-specific dynamics that underpin anthelmintic efficacy.

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581 **Tables and Figures:**

Species	Number of Reads
H.contortus	1020122
T.colubriformis	76289
T.circumcinta	388161
H.placei	2615
T.rugatus	1660
C.ovina	19
C.oncophora	58
O.venulosum	5050
T.axei	10160
T.vitrinus	2195

Table 1. Distribution of ITS2 amplicon sequencing reads

The read counts reflect the total number of sequences assigned to each species following quality filtering and taxonomic classification (n=62).

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FARM ID	FECRT Result (BZ)	ITS2 Nemabiome + FECRT Result (BZ)	Hidden Resistance?	Hidden Susceptibility?	FECRT Result (LEV)	ITS2 Nemabiome + FECRT Result (LEV)	Hidden Resistance?	Hidden Susceptibility?
FARM 1	n.d.	n.d.	n.d.	n.d.	Resistant	Mixed	☒	☑
FARM 3	Resistant	Resistant	☒	☒	Resistant	Mixed	☒	☑
FARM 4	Resistant	Resistant	☒	☒	Resistant	Resistant	☒	☒
FARM 5	Resistant	Mixed	☒	☑	Resistant	Mixed	☒	☒
FARM 6	Resistant	Resistant	☒	☒	Susceptible	Susceptible	☒	☒
FARM 7	Resistant	Resistant	☒	☒	Resistant	Mixed	☒	☑
FARM 8	Resistant	Resistant	☒	☒	Resistant	Resistant	☒	☒
FARM 9	Resistant	Resistant	☒	☒	Susceptible	Mixed	☑	☒
FARM 10	Resistant	Resistant	☒	☒	Susceptible	Mixed	☑	☒
FARM 11	Resistant	Mixed	☒	☑	Susceptible	Mixed	☒	☒
FARM 12	Resistant	Resistant	☒	☒	Susceptible	Susceptible	☒	☒
FARM 13	Resistant	Resistant	☒	☒	Susceptible	Susceptible	☒	☒
FARM 14	Resistant	Resistant	☒	☒	Inconclusive	Susceptible	☒	☒
FARM 15	Susceptible	Susceptible	☒	☒	Susceptible	Mixed	☑	☒
FARM 18	Resistant	Resistant	☒	☒	Resistant	Resistant	☒	☒
FARM 19	Resistant	Susceptible	☒	☑	Resistant	Mixed	☒	☑

Table 2. Comparison of Faecal Egg Count Reduction Test (FECRT) and ITS2 Nemabiome integrated with FECRT results for benzimidazole (BZ) and levamisole (LEV) across different farms.

A "Mixed" result signifies the presence of both resistant and susceptible species in a farm. "Hidden Resistance" indicates instances where ITS2 Nemabiome detected resistant species not apparent in FECRT, and "Hidden Susceptibility" indicates instances where ITS2 Nemabiome detected susceptible species not detected by FECRT. "n.d." denotes data not available.

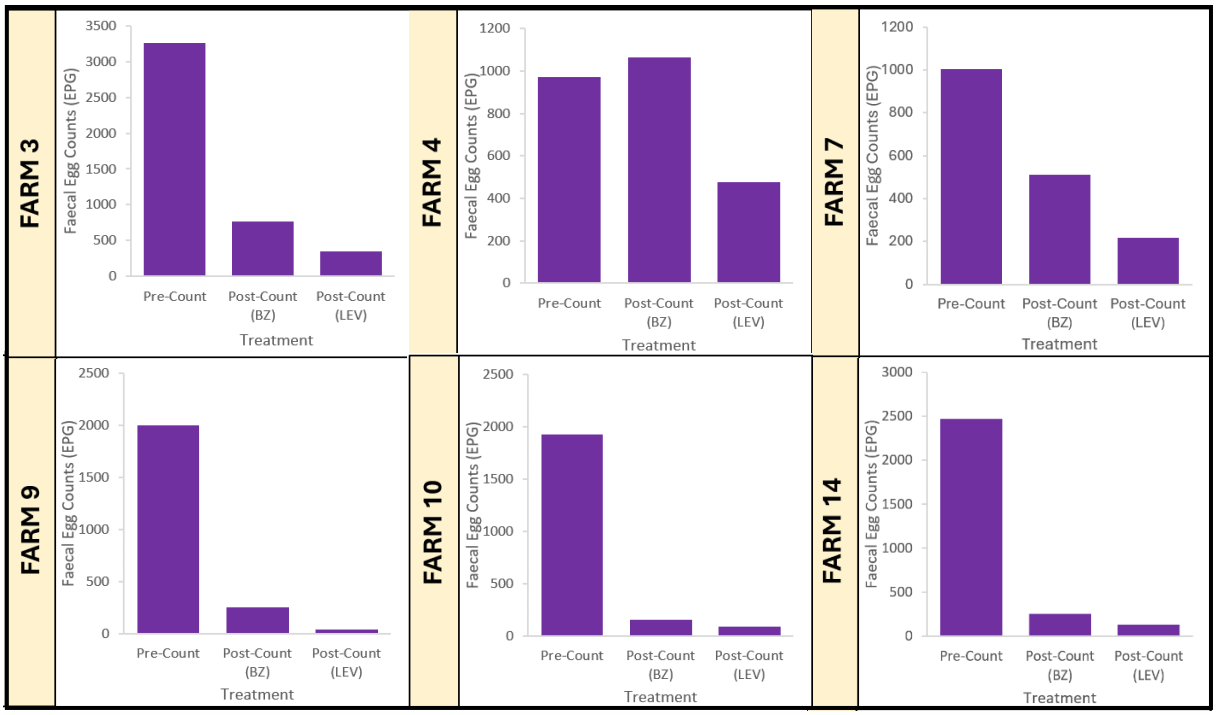

Figure 1. Faecal egg counts (EPG) of Pre-treatment, Post-treatment (BZ) and Post-Treatment (LEV).

Faecal egg counts were measured for six representative farms before (pre-treatment) and 14 days (post-treatment) after the administration of BZ and LEV.

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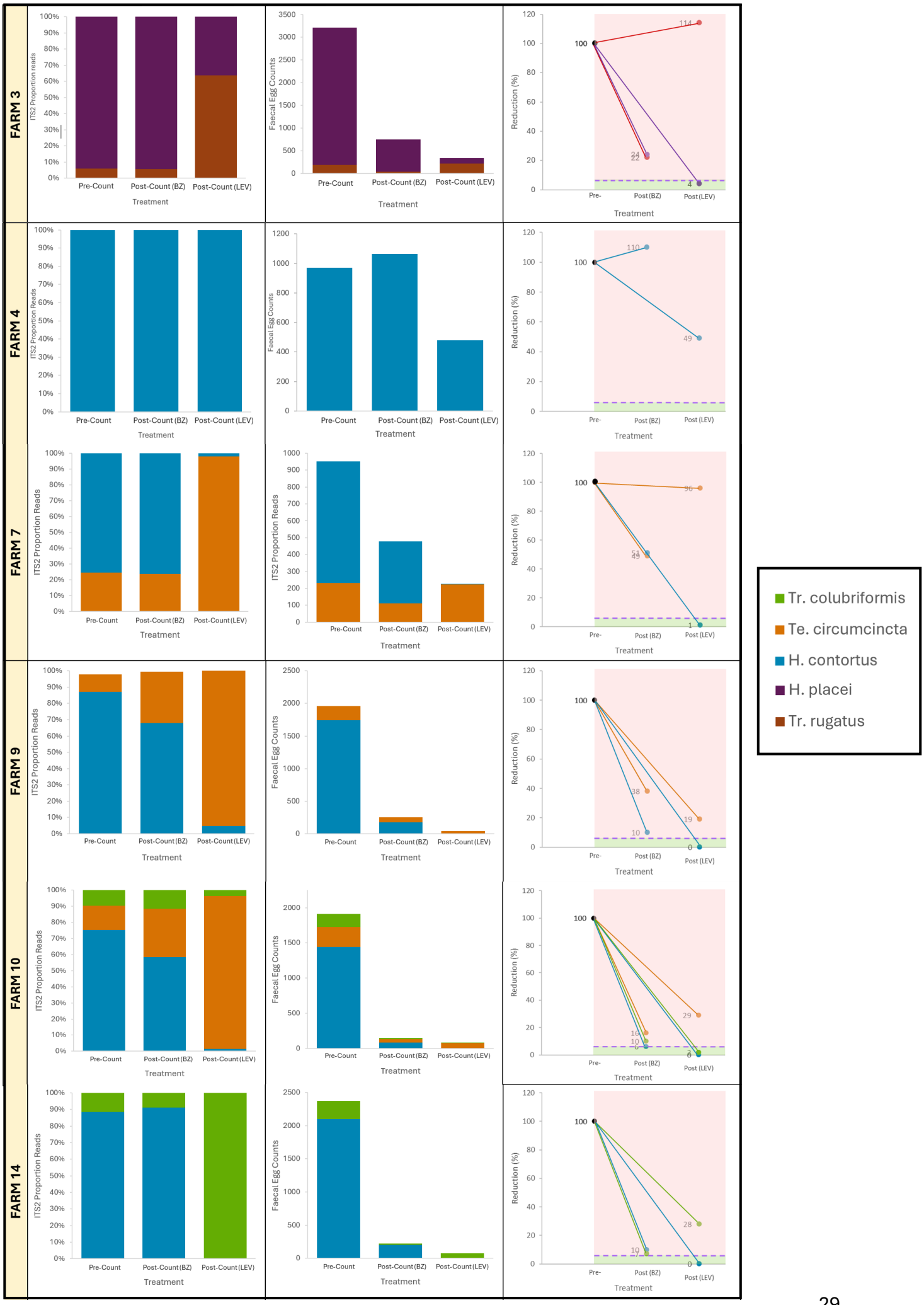
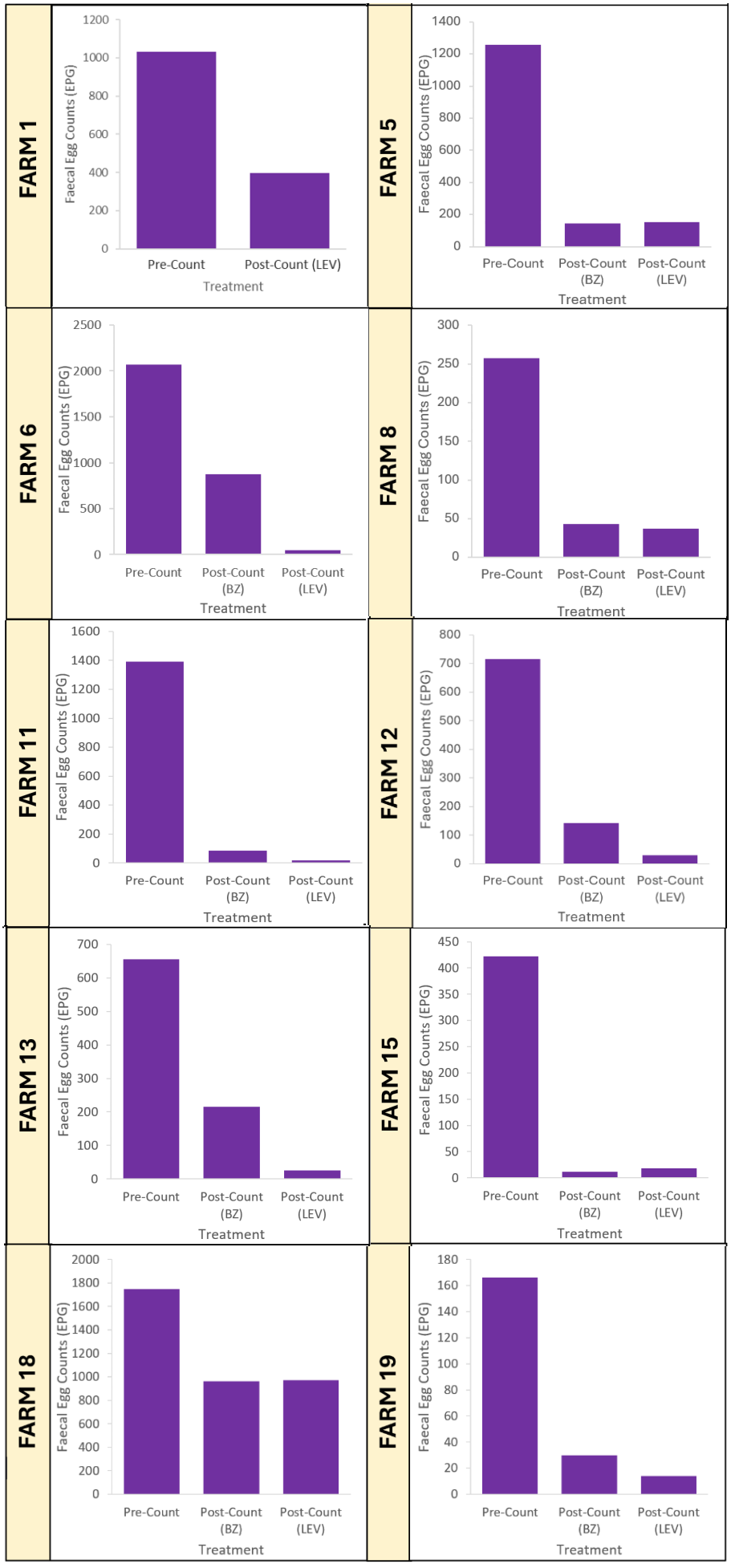
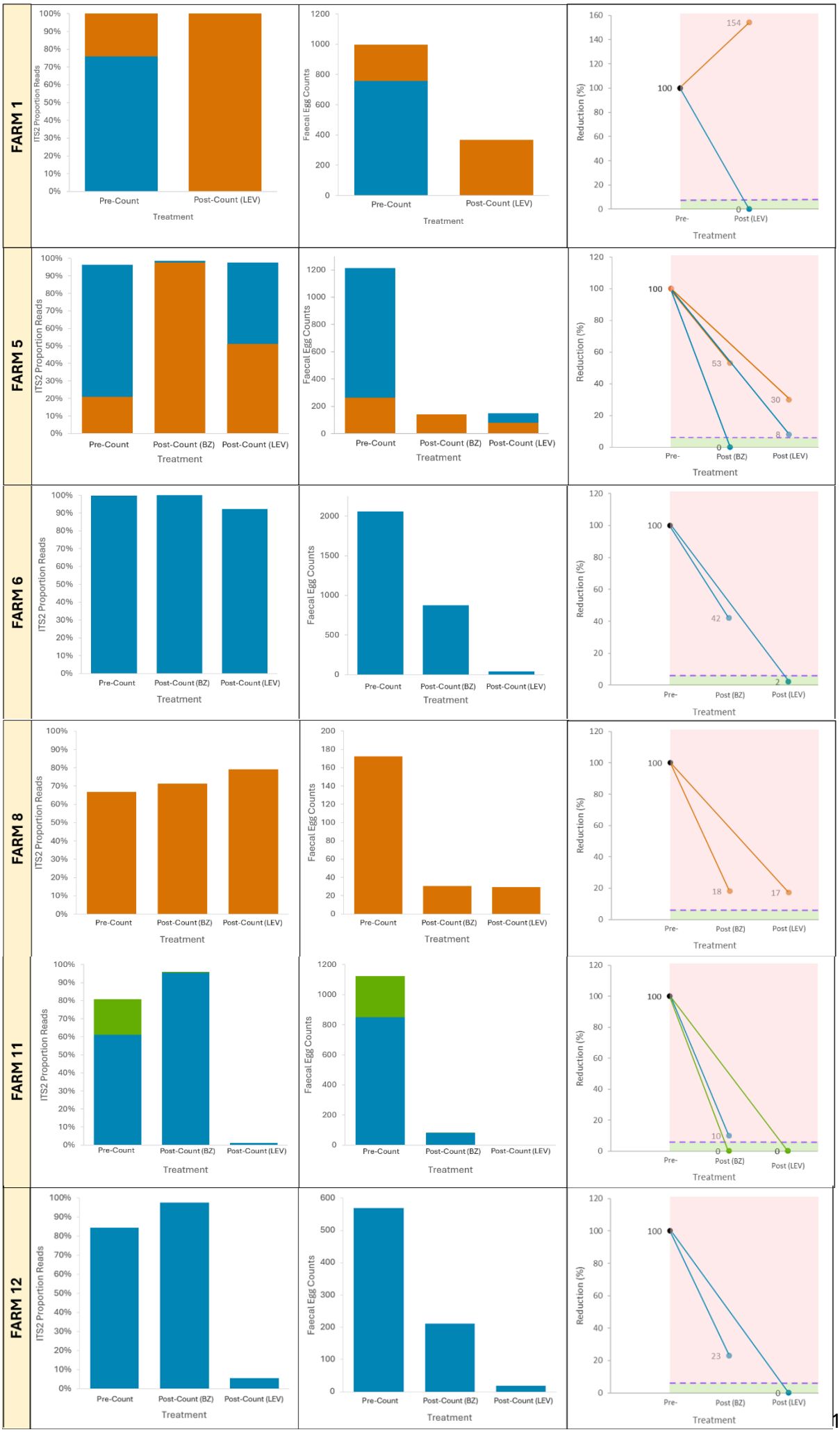


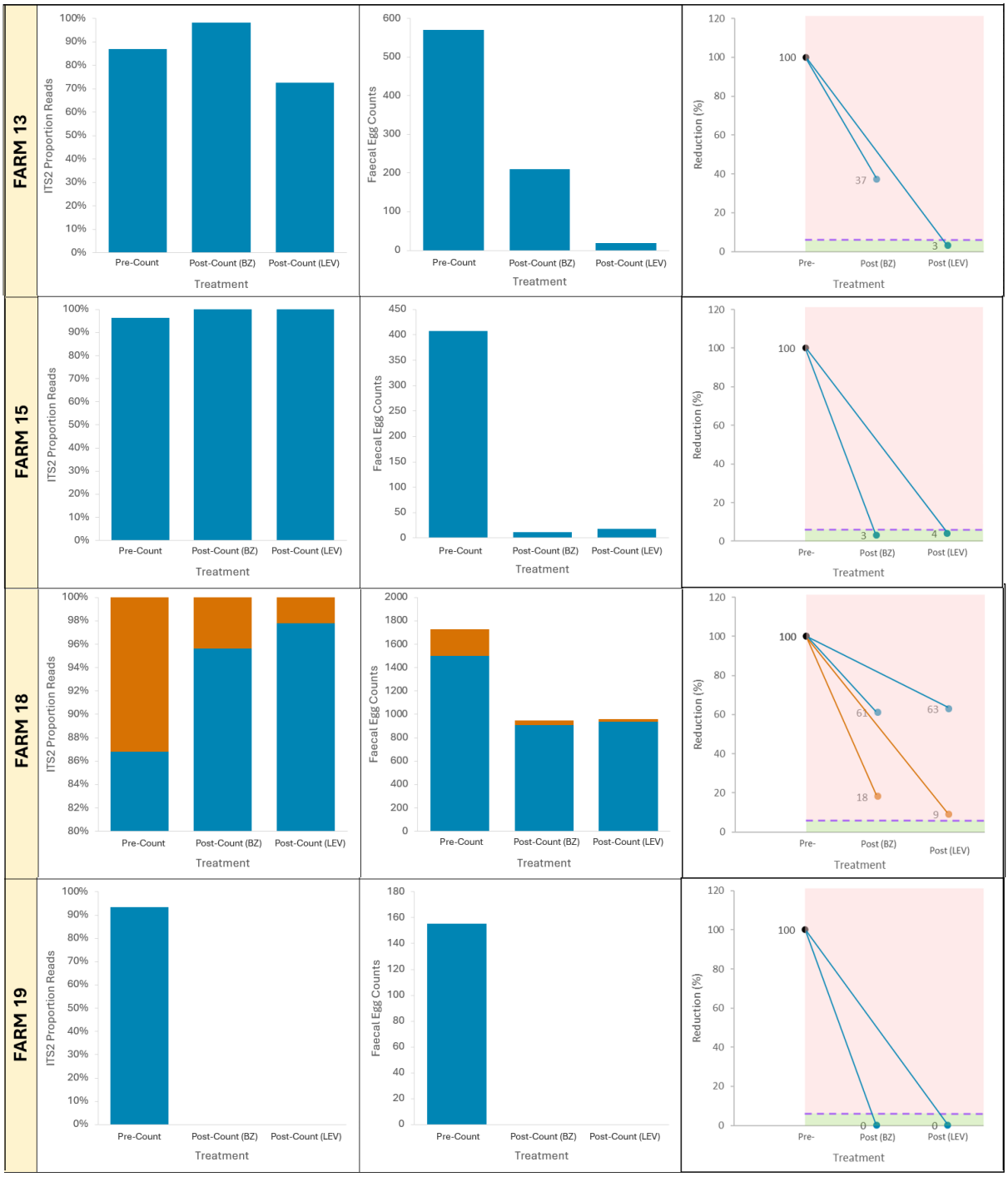
Figure 2. Integration of ITS2 Nemabiome and FECRT data across six farms.

Each panel of the farm displays the ITS2 proportion reads (left), ITS2 proportion reads scaled to the faecal egg counts (middle) and a species reduction map (right). The species reduction map represents the proportion of the pre-treatment population remaining post-treatment (pre-treatment assumed as 100%). The purple dashed line represents the efficacy threshold (5%), separating resistant (red zone; < 95%) and susceptible (green zone; > 95%) species.



Appendix Figure A1. Faecal egg counts (EPG) of Pre-treatment, Post-treatment (BZ) and Post-Treatment (LEV) across the remaining ten farms.


FARM 1
FARM 5
FARM 6
FARM 8
FARM 11
FARM 12
ITS2 Proportion Reads
Faecal Egg Counts
Reduction (%)
Treatment
Pre-Count
Post-Count (BZ)
Post-Count (LEV)
Pre-
Post (BZ)
Post (LEV)
154
100
0
53
30
8
42
2
18
17
10
23



Appendix Figure A2. Integration of ITS2 Nemabiome and FECRT data across the remaining ten farms.

Each panel of the farm displays the ITS2 proportion reads (left), ITS2 proportion reads scaled to the faecal egg counts (middle) and a species reduction map (right). The species reduction map represents the proportion of the pre-treatment population remaining post-treatment (pre-treatment assumed as 100%). The purple dashed line represents the efficacy threshold (5%), separating resistant (red zone; < 95%) and susceptible (green zone; > 95%) species.

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