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Comparative transcriptomics of naturally susceptible and resistant *Trypanosoma cruzi* strains in response to Benznidazole

Carlos Ospina [a], Tatiana Cáceres [a], Stivenn Gutiérrez [a], Luz Helena Patiño [a], Luis David Sáenz-Pérez [b], Karen Moreno Medina [b], Juan Carlos Villar [b], Juan David Ramírez [a,c,*]

[a] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), School of Sciences and Engineering, Universidad del Rosario, Bogotá, Colombia
[b] Fundación Cardioinfantil- Instituto de Cardiología, Bogotá, Colombia
[c] Center for Inter-Disciplinary Research, Department of Global, Environmental and Genomic Health Sciences, College of Public Health, University of South Florida, Tampa, FL, United States

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ABSTRACT

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi*, remains a major public health challenge due to limited treatment options, Benznidazole and Nifurtimox; which are associated with adverse effects and variable efficacy. The emergence of drug-resistant in *T. cruzi* strains, along with limited knowledge of the molecular mechanisms underlying resistance, hampers the development of more effective therapies. To explore these mechanisms, we performed a comparative transcriptomic analysis of two *T. cruzi* TcI strains: MG (naturally susceptible) and DA (naturally resistant) to Benznidazole. Parasites were cultured in LIT medium, and IC50 values were determined using the MTT assay. RNA was extracted and sequenced (RNA-seq), with reads aligned to a reference genome. Differential gene expressions were analyzed with DESeq2, functional enrichment through Gene Ontology (GO), and metabolic pathways were mapped via KAAS. The IC50 for Benznidazole in DA (28.92 μg/mL; 111.13 μM) was substantially higher than in MG (0.88 μg/mL; 3.39 μM), confirming differential susceptibility. DA showed 408 upregulated and 1515 downregulated genes, while MG had 153 upregulated and 866 downregulated ($Log_2FoldChange \geq 2$ or ≤ -2). GO analysis indicated divergent biological processes between strains: DA exhibited enrichment in electron transport and detoxification, while MG was enriched in DNA repair and energy metabolism. Metabolic mapping revealed significant differences in the pentose phosphate pathway, glycolysis/gluconeogenesis, and the tricarboxylic acid (TCA) cycle. Key genes potentially involved in resistance like prostaglandin F2α synthase, trypanothione synthase, thioredoxin, and prostaglandin F synthase were identified as candidate therapeutic targets. These findings suggest that Benznidazole resistance in *T. cruzi* involves multifactorial, strain-specific responses at the transcriptomic and metabolic levels. By analyzing naturally resistant and susceptible TcI strains of *T. cruzi* under identical experimental conditions, this study reveals strain-specific transcriptomic adaptations that have not been previously characterized in naturally resistant and susceptible populations. These findings expand our current understanding of intrinsic Benznidazole resistance in *T. cruzi*, moving beyond purely experimental models. Specifically, they highlight novel metabolic and redox pathways that could serve as therapeutic targets effective against diverse *T. cruzi* strains and Discrete Typing Units (DTUs).

1. Introduction

CD, caused by *T. cruzi*, remains a significant public health concern in the Americas, affecting 7–8 million people and placing an additional 25 million at risk, primarily in Latin America (Organización Mundial de la salud, 2023; Ramírez et al., 2010, 2015; Rassi et al., 2010). Although once geographically restricted, migration has expanded its reach globally (Olivera et al., 2019). The primary mode of transmission is via Triatominae insects, which spread the parasite through their feces. However, *T. cruzi* can also be transmitted through blood transfusion,

* Corresponding author. Center for Inter-Disciplinary Research, Department of Global, Environmental and Genomic Health Sciences, College of Public Health, University of South Florida, Tampa, FL, United States
E-mail address: jramirezgonzalez@usf.edu (J.D. Ramírez).

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congenital transmission, organ transplantation, laboratory accidents, and ingestion of contaminated food (Cantillo-Barraza et al., 2021; Hernández et al., 2016; Pérez-Molina and Molina, 2018; Ramírez et al., 2009, 2010).

The disease progresses through acute and chronic phases. The acute phase is frequently asymptomatic, though it may present with nonspecific signs. Following this stage, many patients enter the indeterminate form of chronic CD, which is characterized by the absence of clinical symptoms and normal findings on electrocardiographic and radiological evaluations, despite persistent infection. Over time, approximately 30–40 % of individuals in the indeterminate phase will progress to the determinate chronic form, developing severe cardiac or digestive complications (Pérez-Molina and Molina, 2018). Diagnosis and treatment remain challenging, as chronic manifestations are often recognized only after significant disease progression, necessitating complex and costly interventions (Bern et al., 2007; Rassi et al., 2010; Tarleton et al., 2014).

Trypanocidal treatment relies primarily on Benznidazole and Nifurtimox, which are most effective in the acute phase and younger patients (Fabbro et al., 2007; Rassi et al., 2010; Viotti et al., 2006). Benznidazole, the more commonly used drug, acts through nitroreduction, binding to parasite DNA and interacting with proteins and lipids (Coura, 2009). However, Benznidazole's effectiveness decreases in chronic cases and is significantly influenced by host-specific factors and parasite strain diversity, which together contribute to variable therapeutic outcomes (Clayton, 2010; Crespillo-Andújar et al., 2022; Lascano et al., 2022; Martín-Escolano et al., 2020). In pediatric populations from Bolivia, Argentina, and Brazil, the drug has been shown to induce serological clearance in 83–97 % of patients, providing a strong marker of therapeutic success (Crespillo-Andújar et al., 2022; DNDi, 2025). In contrast, observational studies conducted primarily in countries of the Southern Cone, including Argentina and Brazil, have suggested that Benznidazole may slow disease progression and reduce the risk of cardiomyopathy by approximately 50 % in adults with the indeterminate form of CD, though these findings have not been entirely consistent; however, despite these potential benefits, sustained parasitological cure rates remain low, highlighting the ongoing challenges in achieving lasting remission (Hasslocher-Moreno et al., 2021; Rassi et al., 2025).

WHO guidelines recommend treatment for children under 14 and adults under 50 with indeterminate CD, but whether to expand treatment to those with cardiomyopathy remains debated due to limited evidence from clinical trial data (Crespillo-Andújar et al., 2022; Hasslocher-Moreno et al., 2021; Meymandi et al., 2018; Viotti et al., 2014). A key limitation in current research is that most adult treatment data come from observational studies rather than clinical trials with clearly defined endpoints. To address this gap, a Phase III clinical trial was launched by DNDi in 2023 in Argentina to evaluate the efficacy of two-week and four-week Benznidazole regimens for adults in the chronic phase of the disease. This study aims to validate shorter treatment regimens and provide more robust evidence on therapeutic outcomes in patients without advanced disease (DNDi, 2025). Despite Benznidazole's long-standing use and safety profile, its tolerability is limited by a high incidence of adverse effects, including dermatological (29–50 %), gastrointestinal (5–15 %), and neurological symptoms (up to 33 %), sometimes necessitating treatment discontinuation (Calvet et al., 2020; Castro et al., 2014; Galván et al., 2019; Molina et al., 2015; Pinazo et al., 2010, 2014; Rassi et al., 2010). The demanding 60-day regimen and frequent monitoring further reduce adherence, particularly in resource limited settings, where discontinuation rates reach up to 20 %. Shorter treatment regimens may offer the potential for improved tolerability while maintaining comparable efficacy, potentially enhancing patient adherence and overall treatment success. (DNDi, 2025; Molina-Morant et al., 2020; Navarro et al., 2012).

Therapeutic efficacy for treating *T. cruzi* infections is significantly undermined by drug resistance (Campos et al., 2014; Muñoz-Calderón et al., 2012; Urbina, 2010). This resistance is not just acquired from long-term drug exposure; it is also an intrinsic trait found in naturally resistant strains, even in patients who have not been treated before (Campos et al., 2014; Filardi and Brener, 1987; Mejia et al., 2012). Beyond issues like poor patient adherence, drug resistance is predominantly linked to the parasite's genetic background, particularly its classification into discrete typing units (DTUs). For example, TcI strains are generally more resistant to common drugs like Benznidazole and Nifurtimox compared to TcII and other DTUs (de Azevedo et al., 2022; Filardi and Brener, 1987; Silvestrini et al., 2024). However, the high genetic diversity within each DTU and variations across different geographic regions make it essential to study drug response at an even finer level, specifically at the sub-DTU level. The existence of these naturally resistant strains may help explain the varied treatment outcomes observed in different endemic areas (Lima et al., 2023; Mejia et al., 2012).

Several molecular mechanisms underpin this resistance. Enhanced antioxidant defenses (e.g., superoxide dismutase), alterations in DNA repair pathways, and mutations in the TcNTR gene that impair Benznidazole activation have all been associated with reduced drug susceptibility (Campos et al., 2014; Machado-Silva et al., 2016). Resistant strains often overexpress detoxification enzymes such as glutathione transferases and superoxide dismutase, while also displaying broader metabolic reprogramming that favors survival under drug pressure (Jayawardhana et al., 2023; Mejia et al., 2012; Murta et al., 2024). Importantly, these features are not limited to experimentally selected strains but are increasingly documented in natural isolates from patients and vectors, reinforcing their epidemiological relevance (Campos et al., 2014; Lima et al., 2023).

Despite advances, the molecular basis of intrinsic resistance remains incompletely understood. Transcriptomic approaches have proven valuable for revealing differential expression patterns between susceptible and resistant strains (Maya and Morello, 2005; Temperton et al., 1998), yet most studies emphasize acquired resistance, leaving natural resistance mechanisms underexplored (Campos et al., 2014; Mejia et al., 2012). Although candidate biomarkers have been proposed, no validated diagnostic tools exist to detect naturally resistant strains before treatment, limiting their clinical applicability (Bahia et al., 2014).

Given the growing concern over resistance in *T. cruzi*, particularly in naturally resistant strains that compromise therapeutic outcomes, it is critical to better understand the molecular adaptations that allow the parasite to evade trypanocidal action. Therefore, in this study, we performed a comparative transcriptomic analysis of a naturally susceptible strain (MG) and a naturally resistant strain (DA) of *T. cruzi* exposed to Benznidazole. Unlike previous studies that have primarily examined laboratory selected resistant clones, our work focuses on naturally resistant and susceptible TcI strains isolated from Colombian hosts. This comparative transcriptomic approach provides an integrative analysis of gene expression, metabolic pathways, and redox mechanisms associated with natural Benznidazole resistance within a clinically relevant DTU, offering a new perspective on the evolutionary basis of resistance in *T. cruzi*.

2. Materials and methods

2.1. Biological material

The strains DA (MHOM/CO/01/DA) and MG (MHOM/CO/04/MG), belonging to the *T. cruzi* DTU TcI, were used, characterized by their resistance and sensitivity to Benznidazole, respectively (Cruz et al., 2015). These strains were thawed from the cryobank of the Microbial Research Group of Universidad del Rosario (GIMUR). After thawing, they were maintained in culture in LIT medium, supplemented with 10 % fetal bovine serum (FBS), and incubated at 26 °C, with subcultures every 5 days.

2.2. Calculation of Benznidazole IC50

To determine the IC50 value of Benznidazole in the DA and MG strains, the parasites were cultured in LIT medium for 5 days, and a count was performed using a Neubauer chamber. Subsequently, 1×10^6 parasites/mL were seeded into 24-well culture plates and exposed to serial dilutions of Benznidazole, ranging from 50 μg/mL (192.13 μM) to 0.19 μg/mL (0.73 μM). As a positive control, two wells with 1×10^6 parasites/mL in LIT medium were included, and as a negative control, another two wells with 1×10^6 parasites/mL in LIT medium and hydrogen peroxide to induce cell death. The plate was incubated at 37 °C for 72 h.

Following incubation, the MTT colorimetric assay from Abcam (https://www.abcam.com/en-us/technical-resources/protocols/mtt-assay) was performed to measure cell viability, and the absorbance results were analyzed using GraphPad Prism 9 software. IC50 value was calculated with these data.

2.3. RNA sequencing

After calculating the IC50 concentration, triplicate cultures of the DA and MG strains were performed in LIT medium for 72 h at 26 °C. For each culture, the corresponding IC50 concentration of Benznidazole for each strain was added, and untreated controls were included for comparison. After incubation, total RNA was extracted from each of the cultures, including the controls, using the Qiagen RNeasy Plus Mini Kit. The extracted RNA was sequenced on the Illumina NovaSeq 6000 platform using Pair-end 150 bp RNA-Seq technology, generating a total of 21.63 GB of data, with a minimum of 20 million reads per sample. The data obtained was stored on the institutional server of Universidad del Rosario for detailed bioinformatic analysis.

2.4. Bioinformatic pre-processing

Using the data stored, a transcriptomic analysis that included several key steps was carried out. First, quality control and adapter trimming were performed using the Trimmomatic program (http://www.usadellab.org/cms/?page=trimmomatic), to remove adapters and low-quality bases. For this, the parameters SLIDINGWINDOW:4:20, MINLEN:100, and AVGQUAL:20 were used, ensuring the high quality of the reads for subsequent analysis. Subsequently, the processed reads were aligned to the "*Trypanosoma cruzi* BrazilA4″ reference genome (https://tritrypdb.org/tritrypdb/app/search?q=Trypanosoma+cruzi+Brazil+A4), using the STAR software (https://github.com/alexdobin/STAR), configured with specific parameters to maximize alignment accuracy and reduce multiple mapping. In particular, the two-pass Mode Basic alignment mode was activated, which improves accuracy by detecting new junctions in the first pass and applying that information in the second. Additionally, the outFilterMultimapNmax 1 parameter was set to limit the maximum number of loci where a read can be mapped, thus minimizing multiple mapping. Finally, gene expression quantification was performed using the BAM files generated during alignment, with HTSeq (https://github.com/htseq/htseq) in "intersection-nonempty" mode, which counts only the reads that overlap with the genomic annotations.

2.5. Differential gene expression analysis

A differential gene expression analysis of the DA and MG strains was performed using the DESeq2 package (https://github.com/thelovelab/DESeq2) in the R statistical software (Version 4.4.1). To achieve this, we loaded the gene count data obtained from the previous HTSeq analysis and created a DESeqDataSet object. Subsequently, we filtered this data to remove genes exhibiting low counts in at least one sample group. The differential analysis was carried out between the C1 (Control) and C2 (Strain exposed to Benznidazole) conditions for both strains, using a negative binomial generalized linear model implemented in DESeq2, with a design that included the condition as an explanatory variable. The results were filtered to include only differentially expressed genes with an adjusted p-value (padj) less than 0.05 and a Log_2FoldChange ≥ 2 or ≤ -2. For the visualization and validation of the results, various graphs were generated as follows: principal component analyses (PCAs) using ggplot2 (https://ggplot2.tidyverse.org/), volcano plots using the EnhancedVolcano package (https://github.com/kevinblighe/EnhancedVolcano), Venn diagrams using the InteractiVenn online tool (https://www.interactivenn.net/), and heatmaps using the pheatmap package (https://github.com/raivokolde/pheatmap).

2.6. Functional enrichment analysis (GO) and metabolic pathways

A functional enrichment analysis was performed using the Gene Ontology (GO) database hosted on TriTrypDB (https://tritrypdb.org/tritrypdb/app), to identify biological processes (BP), cellular components (CC), and molecular functions (MF) enriched in the differentially expressed genes. The results were visually represented using enrichment bubble plots. Additionally, the KAAS (KEGG Automatic Annotation Server) online tool (https://www.genome.jp/kaas-bin/kaas_main) was used to assign the sequences of the upregulated and downregulated genes of the DA and MG strains in the context of specific *T. cruzi* metabolic pathways.

2.7. RT-qPCR and gene expression

2.7.1. Primer design

Based on the differential gene expression analysis between the resistant *T. cruzi* DA strain and the susceptible MG strain, fifteen candidate genes were selected for validation by RT-qPCR using the ΔCt method. Selection criteria included genes consistently upregulated in both strains upon exposure to Benznidazole, indicating a potential role in the parasite's drug response, as well as evidence from the literature linking several of these genes to resistance mechanisms in *T. cruzi* or related trypanosomatids. To ensure specificity and interpretability of the assays, only single copy genes were considered, while members of highly redundant multigene families, hypothetical proteins without reliable functional annotation, and genes associated with membrane or kinetoplast processes were excluded. The nucleotide sequences of the selected genes were retrieved in FASTA format from the TriTrypDB database (Supplementary Table 1).

Primer design was performed using the Primer-BLAST tool, applying the following criteria: specificity against the RefSeq mRNA database and the *T. cruzi* genome, absence of secondary structures (such as dimers or hairpins), and a maximum difference of 5 °C between the melting temperatures (Tm) of the forward and reverse primers. One primer pair per gene was selected based on these requirements. Primer specificity was subsequently validated using BLAST analysis. Primer pairs that failed to meet the criteria were discarded, and the design process was repeated with alternative sequences. The housekeeping gene GAPDH was selected as the internal reference due to its stable expression under various physiological conditions and its well-established role as a constitutive gene in *T. cruzi*. The primers and RT-qPCR conditions for this gene were adopted from Alves et al. (2018).

2.7.2. cDNA synthesis by reverse transcription

cDNA synthesis was performed using the LunaScript® RT Super Mix Kit (New England Biolabs) in a final volume of 10 μL, following the manufacturer's protocol. Each reaction contained 2 μL of LunaScript® enzyme mix and 8 μL of total RNA. The RNA was extracted from three biological replicates of *T. cruzi* strains DA and MG after exposure to their respective IC50 concentrations of Benznidazole. In parallel, RNA from three biological replicates of the corresponding untreated control samples for each strain was also used. All reverse transcription reactions were carried out using the QuantStudio™ Real-Time PCR System

(Applied Biosystems).

2.7.3. RT-qPCR

Relative gene expression analysis in *T. cruzi* DA and MG strains was performed using real-time quantitative PCR (RT-qPCR) with gene-specific primers targeting the selected candidate genes (Supplementary Table 2). Reactions were prepared using SYBR Green 2 × Master Mix (Applied Biosystems) in a final volume of 15 μL per well, in 96-well plates. Each reaction contained 1 μL of cDNA (50 ng/μL), 0.1 μM of each primer, and nuclease-free water. For each strain, three biological replicates were analyzed, each run-in technical duplicate. Additionally, positive control (genomic DNA from *T. cruzi* TcI) and negative control were included, both in technical duplicates.

Thermal cycling was carried out on the QuantStudio™ Real-Time PCR System (Applied Biosystems) using the following program: an initial denaturation step at 95 °C for 3 min, followed by enzyme activation at 95 °C for 10 min. Amplification was then performed over 40 cycles consisting of denaturation at 95 °C for 15 s and primer annealing/extension at 55–68 °C for 30 s. The annealing temperature was optimized for each gene using the average melting temperature (Tm) of the forward and reverse primers. The amplification profile for the housekeeping gene GAPDH, along with the number of cycles and time conditions used for all reactions, was based on the protocol described by Alves et al. (2018).

2.7.4. Quantification of relative gene expression

Relative gene expression of the selected genes in *T. cruzi* DA and MG strains was quantified using the ΔCt method. For this purpose, the mean Ct values from each biological replicate were calculated for every gene, including the reference gene GAPDH, following the RT-qPCR described previously. These values were organized in a spreadsheet using Microsoft Excel, and the following formula was applied:

$$\Delta Ct = \text{Average CT}_{\text{biological replication of the strain treated with the target gene}} - \text{Average Ct}_{\text{GAPDH (reference gene)}}$$

Subsequently, changes in gene expression between experimental and control conditions were determined by calculating ΔΔCt and $2^{-\Delta\Delta Ct}$, using the following equations:

$$\Delta\Delta Ct = \Delta Ct - \text{Average } \Delta Ct_{\text{biological replication of the strain untreated (Control) with the target gene}}$$

$$\textit{Relative expression} = 2^{-\Delta\Delta Ct}$$

Once the relative gene expression values for the different genes were calculated, the standard deviation (SD) and standard error (SE) were determined. Subsequently, statistical comparisons between control and treated groups were performed using Student's *t*-tests to evaluate significant differences in gene expression. The data were then exported to the statistical software RStudio, where they were analyzed and visualized using the "tidyverse" package.

3. Results

3.1. IC50 calculation and RNA sequencing

The IC50 calculation, defined as the Benznidazole concentration required to inhibit 50 % of parasite growth, revealed that the DA strain had an IC50 of approximately 28.92 μg/mL (111.13 μM), while the MG strain showed an IC50 of 0.88 μg/mL (3.39 μM) (Fig. S1). This indicates that the MG strain is susceptible to Benznidazole compared to the DA strain.

After calculating the IC50 concentration for each strain, they were exposed *in vitro* to these drug concentrations, and total RNA was extracted and subsequently sequenced. The quality and quantity of the extracted RNA were satisfactory in all samples, with yields exceeding 200 ng/μL and an A260/A280 ratio close to 2.0, confirming RNA purity. Sequencing was performed using the Illumina paired end 150 bp platform, yielding a minimum of 20 million reads per sample. The data exhibited high accuracy, with an average Phred quality score exceeding 30, reflecting the overall reliability and fidelity of the sequencing output.

3.2. Differential gene expression analysis

To assess the global variation in gene expression, a principal component analysis (PCA) was first conducted for each strain, comparing control and Benznidazole-treated samples. The PCA plots capture most of the variation in the dataset: 84.1 % for the DA strain (Fig. 1A) and 82 % for the MG strain (Fig. 1B), based on the first two principal components. Specifically, the DA strain shows 72.3 % and 11.8 % variance along PC1 (X-axis) and PC2 (Y-axis), respectively, while the MG strain shows 69.4 % and 12.6 %. In both strains, the PCA revealed a clear separation between control (burgundy) and treated (olive green) samples, indicating distinct transcriptional changes in response to Benznidazole. The spread of samples along PC1 suggests that this component is sufficient to differentiate treated from untreated conditions, irrespective of the strain's susceptibility.

Subsequently, using the DESeq2 package in R, 13,725 differentially expressed genes were identified in the DA strain and 13,701 in the MG strain, both with an adjusted P-value $\leq$0.05. Among these genes, 6940 upregulated and 6785 downregulated genes were identified in the DA strain, while 6892 upregulated and 6809 downregulated genes were found in the MG strain.

After performing PCA and differential gene expression analyses, a Venn diagram and various Volcano plots were generated, representing differentially expressed genes, filtering for genes with a Log_2FoldChange $\geq$ 2 and $\leq$ −2. The DA strain showed 408 upregulated and 1515 downregulated genes (Fig. 1C and E), while the MG strain showed 153 upregulated and 866 downregulated genes (Fig. 1D and E). These results suggest a strain-specific transcriptomic response upon Benznidazole exposure. However, shared genes between both strains were also identified (Fig. 1E), indicating that certain cellular processes respond to the drug in a conserved manner. Precisely, it was observed that 99 genes exhibited upregulation and 718 genes showed downregulation in both strains.

3.3. Functional annotation of genes

Successively, functional annotation of up and downregulated genes (with Log_2FoldChange $\leq$ 2 and $\geq$ 2, respectively) for the DA and MG strains (Fig. 1) was performed using the TriTrypDB database. A total of 1314 genes for the DA strain and 723 genes for the MG strain were identified. Additionally, 609 and 296 differentially regulated genes were assigned as hypothetical proteins without predicted function in the DA and MG strains, respectively. Finally, to provide a more focused perspective on differential gene expressions in the DA and MG strains, a heatmap was constructed with the 20 genes with the highest and 20 with the lowest Log_2FoldChange (Fig. 2).

Among the most prominent upregulated genes were those encoding putative proteins like kinesins, retrotransposon hot spot (RHS) proteins, transialidases, and small GTP-binding proteins RAB6 in the DA strain (Fig. 2A). For the MG strain, genes encoding proteins like kinesins, retrotransposon hot spot (RHS) proteins, zinc finger proteins, and phosphatase 2C-like proteins were also identified (Fig. 2B) (Supplementary Tables 3 and 4).

Furthermore, among the most relevant downregulated genes were those encoding pteridine reductase 2 (ptr2), elongation factor 1-alpha (EF-1α), surface proteases GP63, and CMGC/DYRK protein kinases in the DA strain (Fig. 2A); while in the MG strain, genes encoding surface proteases GP63, CMGC/DYRK protein kinases, aspartyl-tRNA synthetase, and D-isomer-specific 2-hydroxyacid dehydrogenase were

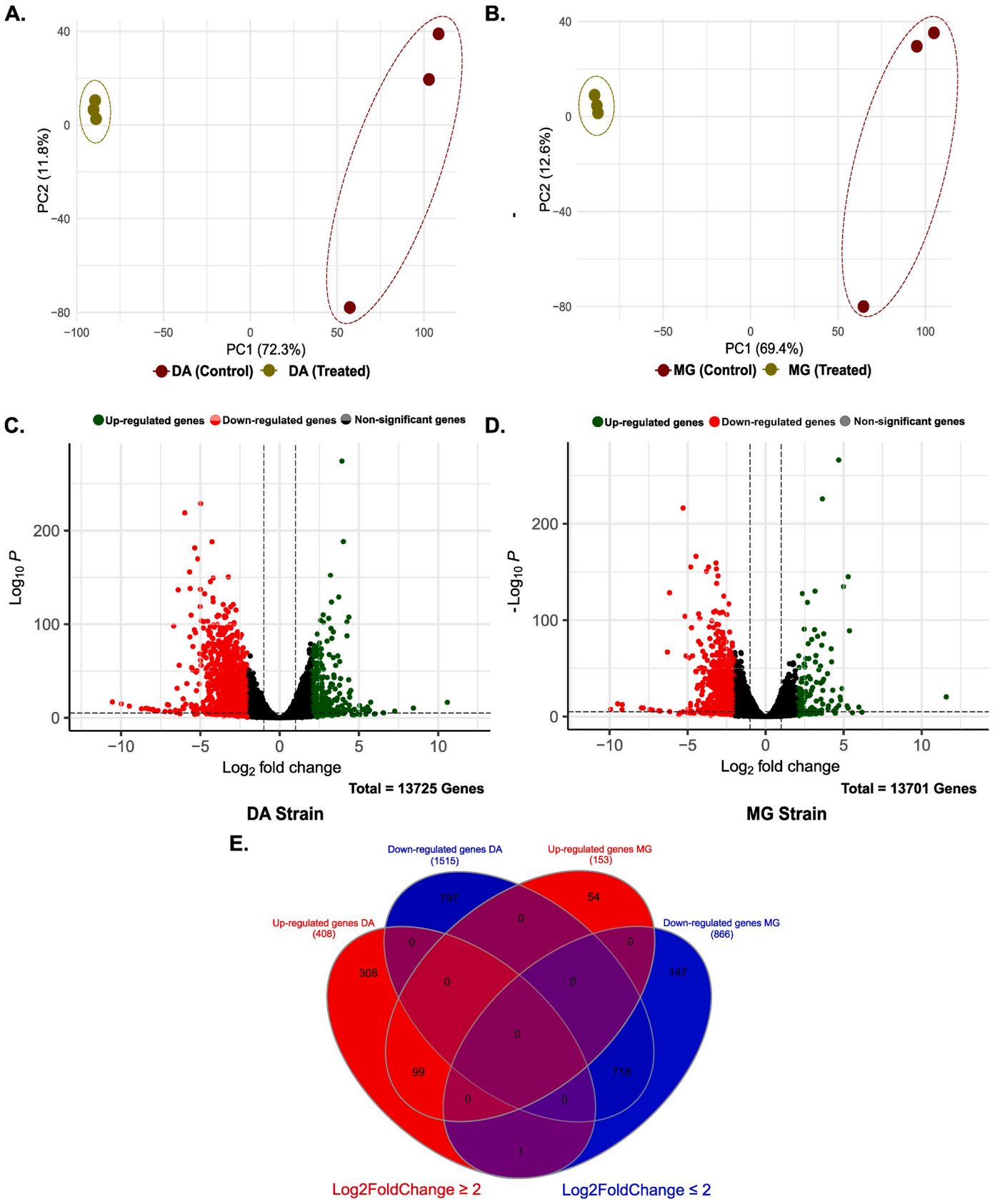

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Fig. 1. Gene expression analysis in DA and MG strains after Benznidazole treatment. (**A**) Principal Component Analysis (PCA) of the DA strain under control and post-treatment conditions. The spatial distribution of points in the principal component space reveals a clear separation between treated and untreated samples, indicating significant alterations in gene expression following Benznidazole exposure. (**B**) Principal Component Analysis (PCA) of the MG strain, comparing control and post-treatment conditions. Like the DA strain, a well-defined segregation between sample groups is observed, suggesting a distinct transcriptional response to Benznidazole. (**C**) Volcano plot for the DA strain, representing the distribution of 13,725 differentially expressed genes. Red points indicate significantly downregulated genes ($Log_2FoldChange \leq 2$), green points correspond to upregulated genes ($Log_2FoldChange \geq 2$), and black points represent genes with no significant expression changes. (**D**) Volcano plot for the MG strain, displaying 13,701 differentially expressed genes, following the same color representation as in the DA strain. Notable differences in the quantity and magnitude of regulated genes highlight a strain-specific response to Benznidazole, further underscoring the genetic variability in *T. cruzi* and its influence on drug susceptibility. (**E**) Venn diagram displaying the total number of differentially expressed genes in both strains, distinguishing between upregulated ($Log_2FoldChange \geq 2$) and downregulated ($Log_2FoldChange \leq 2$) genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prominent (Fig. 2B). Differences in expression patterns between both strains suggest that the Benznidazole response involves modulating specific metabolic pathways, and genes responsible for these differences may be directly associated with drug resistance mechanisms in these strains.

3.4. Functional enrichment (GO) of genes

The GO analysis identified 126 upregulated and 53 downregulated terms in the DA strain, and 40 upregulated and 91 downregulated terms in the MG strain. Notably, for the MG strain, no GO terms were retrieved in the cellular component category due to the limited number of upregulated genes ($Log_2FoldChange \geq 2$) falling below the statistical significance threshold for enrichment analysis (P-value cutoff = 0,05). An enrichment graph (Fig. 3) was generated to display the top 30 GO terms with the highest number of associated genes in both strains, along with the percentage of differentially expressed genes within each category. Fig. 3A and B displays the upregulated and downregulated GO terms for the DA strain, respectively, while Fig. 3C and D presents the corresponding GO terms for the MG strain, covering all three GO domains: biological process, molecular function, and cellular component. Overall, GO analysis revealed that the differentially expressed genes in both strains are involved in a wide range of essential biological functions. Upregulated genes were predominantly associated with pathogenesis, catalytic activity, and cellular organization, while downregulated genes were mainly linked to metabolic processes, cell adhesion, and ribosomal functions.

3.5. Metabolic pathways analysis

We identified three metabolic pathways with the highest number of upregulated genes in both strains (Fig. S2)): the pentose phosphate pathway (PPP), glycolysis/gluconeogenesis, and the citrate cycle (TCA). In the pentose phosphate pathway (Fig. S2A), 6-phosphogluconolactonase, deoxyribose-phosphate aldolase, and a phosphomannomutase-like protein were detected in the DA strain, whereas only the latter two were found in the MG strain. In glycolysis/gluconeogenesis (Fig. 2B), the DA strain showed upregulation of phosphomannomutase-like protein and phosphoglycerate kinase, while the MG strain also displayed pyruvate phosphate dikinase and dihydrolipoyl dehydrogenase. Finally, in the citrate cycle (Fig. S2C), both strains presented two upregulated genes, albeit with differences: DA expressed isocitrate dehydrogenase [NADP] and cytosolic malate dehydrogenase, whereas MG showed cytosolic malate dehydrogenase and dihydrolipoyl dehydrogenase. Pathways with downregulated genes were not included in this analysis, since most pathways contained no such genes, and those that did only harbored a single gene whose function was not related to processes potentially involved in Benznidazole resistance. These findings highlight variations in the upregulated metabolic responses of the DA and MG strains, which may contribute to differences in their metabolic adaptation and response to treatment.

3.6. RT-qPCR and quantification of relative gene expression

Based on the differential expression analysis conducted in the DA and MG strains of *T. cruzi* exposed to their respective Benznidazole IC50 values, 15 candidate genes were selected to evaluate their relative expression using RT-qPCR, quantified through the $2^{-}\Delta\Delta Ct$ method. The results are summarized in Fig. 4. In the resistant DA strain, a strong and statistically significant overexpression was detected for several genes, including E1-like ubiquitin-activating enzyme-putative, Phosphomannomutase-like protein-putative, Cytidine triphosphate synthase-putative, Casein kinase II subunit beta, Glycogen synthase kinase-3 alpha-putative, Aldo-keto reductase-putative, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase-putative, and Seryl-tRNA synthetase (Fig. 4A; Supplementary Table 5). In contrast, the susceptible MG strain showed no significant differences in most of the analyzed genes, with expression levels comparable to or slightly lower than those of untreated controls (Fig. 4B; Supplementary Table 5). This pattern suggests that the MG strain exhibits a limited transcriptional response to Benznidazole exposure, whereas the DA strain activates a robust transcriptional program involving multiple metabolic pathways.

4. Discussion

Despite being identified more than a century ago, CD remains a neglected tropical disease, largely due to difficulties in early diagnosis and the limited efficacy of available therapies (Lima et al., 2023; Organización Mundial de la salud, 2023). Currently, the only drugs approved for clinical use are Benznidazole and Nifurtimox. Although these nitroheterocyclic compounds constitute the main therapeutic tools against *T. cruzi*, their use is hindered by frequent adverse effects, poor tolerance, and reduced effectiveness during the chronic phase of the disease (Kelly and Wilkinson, 2013; Sales et al., 2017). The challenges of treatment are further exacerbated by the emergence of drug-resistant strains of *T. cruzi*, which employ diverse molecular mechanisms to circumvent drug action, complicating parasite clearance and aggravating the disease burden (Kelly and Wilkinson, 2013; Sales et al., 2017).

Most transcriptomic studies investigating Benznidazole resistance have focused on experimentally induced models, which may not capture the complex adaptations that occur in naturally circulating strains. One of the central mechanisms proposed for Benznidazole resistance involves a reduction in the generation of toxic free radicals, normally produced by nitroreduction within the parasite (Molina et al., 2015). In addition, alterations in drug transporter activity and upregulation of DNA repair pathways have been implicated in resistance (Lima et al., 2023; Mejia et al., 2012). However, while several candidate genes and pathways have been identified, the molecular basis of resistance remains incompletely defined. This limitation is largely due to the predominance of *in vitro* selected resistance models, which do not fully reflect the evolutionary and ecological complexity of naturally resistant populations (Lima et al., 2023; Mejía-Jaramillo et al., 2011, 2025; Murta et al., 2024). Addressing this gap requires a shift toward the study of naturally resistant and susceptible strains, which may hold the key to understanding clinically relevant resistance mechanisms.

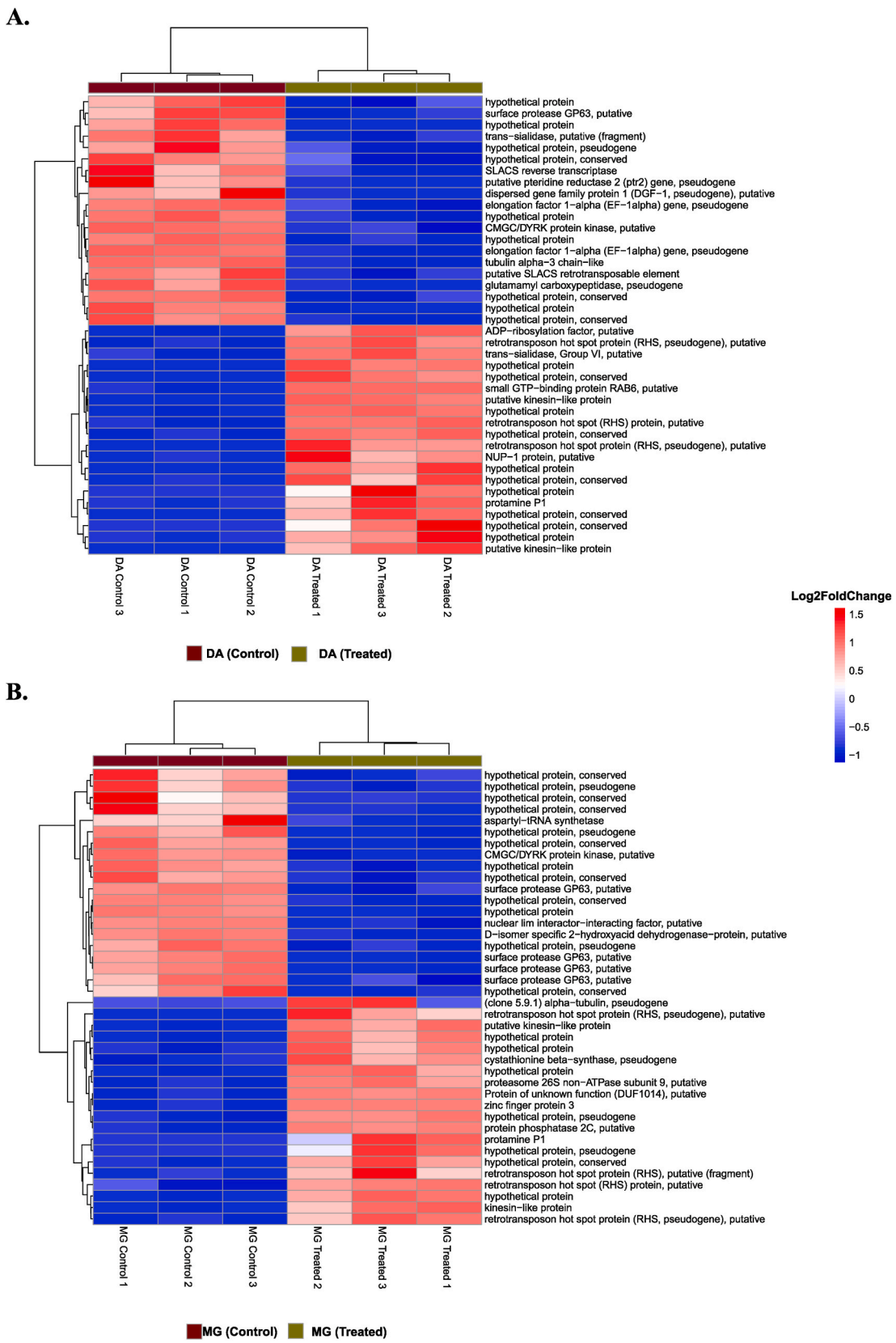

Fig. 2. Heatmap of the 20 genes with the highest differential expression (Log2FoldChange) in DA and MG TCI strains treated with Benznidazole. The heatmap visualizes expression levels of the 20 genes showing the greatest differences in expression between DA (**A**) and MG (**B**) TCI strains. Colors represent the change in gene expression (Log2FoldChange), where red indicates increased expression and blue indicates decreased expression. Rows correspond to different genes, and columns to different replicates for both DA and MG strains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

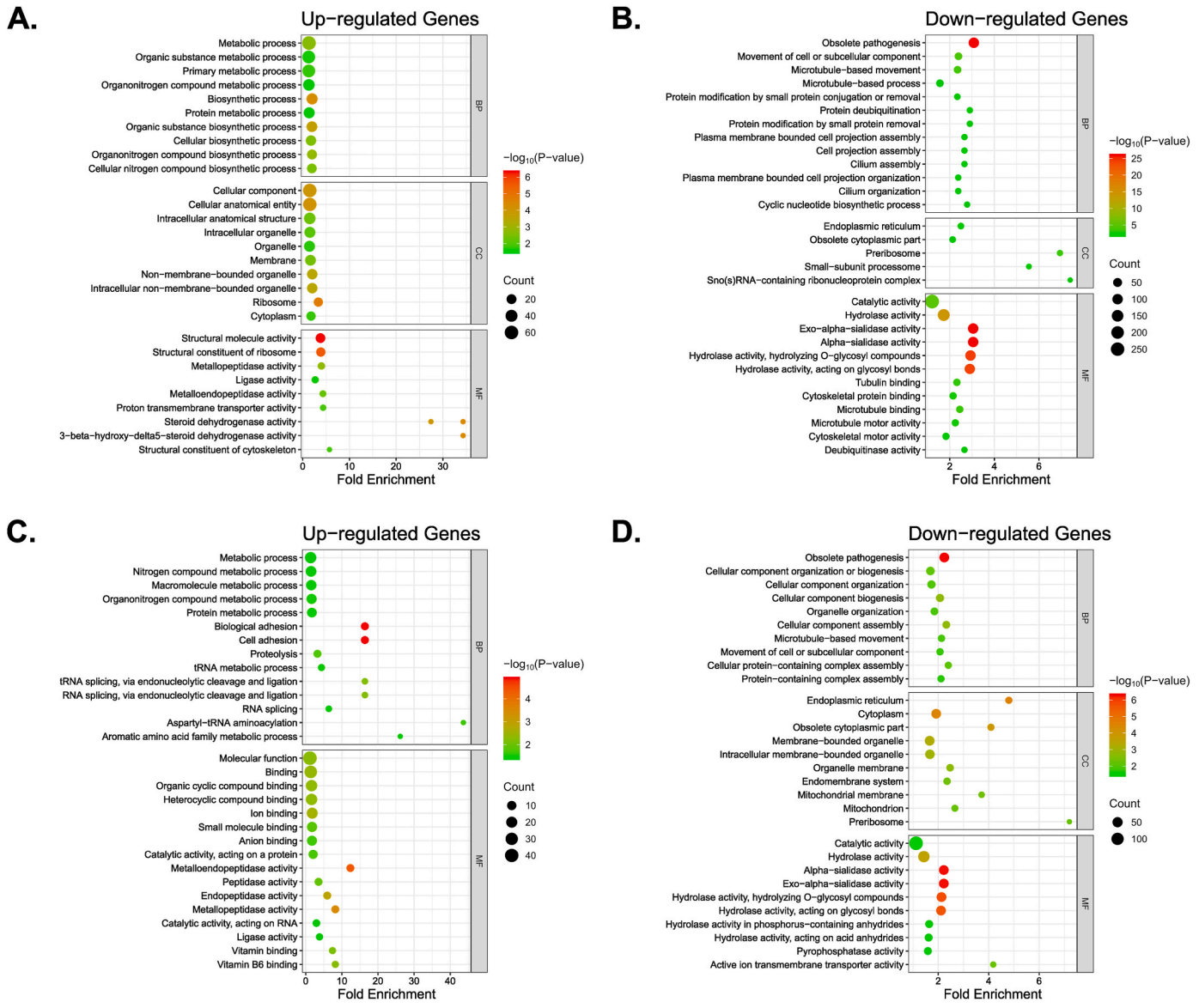

Fig. 3. Gene ontology enrichment analysis in DA and MG strains. Gene ontology (GO) enrichment analysis was performed to identify over-represented biological processes (BP), cellular components (CC), and molecular functions (MF) in differentially expressed genes of DA and MG strains. Graphs show the most significant GO terms, with point size corresponding to the number of annotated genes and color intensity reflecting the adjusted P-value, indicating statistical significance. The x-axis represents the frequency of annotated genes for each GO term, while the y-axis classifies GO terms by significance. Panels are organized as follows: **A** and **B**, positively and negatively regulated genes in the DA strain, respectively; **C** and **D**, positively and negatively regulated genes in the MG strain, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Our study bridges this gap by performing a comparative transcriptomic analysis of two *T. cruzi* TcI strains with contrasting susceptibility to Benznidazole: DA (naturally resistant) and MG (naturally susceptible). By investigating transcriptional variation under identical experimental conditions, we reveal strain specific regulatory networks and adaptive strategies that are obscured in laboratory induced models. This perspective situates resistance within a physiologically and evolutionary relevant context, providing novel insights into the intrinsic mechanisms that shape drug responsiveness in *T. cruzi* (Genois et al., 2014; Lima et al., 2023).

The striking differences in gene expression observed between DA and MG are not mere artifacts of drug exposure but instead reflect deeply embedded regulatory programs shaped by long-term selective pressures throughout the parasite's life cycle. Stressors such as temperature fluctuations between insect vectors and mammalian hosts, oxidative stress from the immune system, and the need for dynamic modulation of surface proteins and DNA repair pathways have all been linked to resistance mechanisms (Campos et al., 2014; Lima et al., 2023; Murta et al., 2008). Notably, our analysis highlights the enrichment of genes associated with amino acid metabolism, DNA repair, redox homeostasis, and protein translation, indicating survival strategies that go beyond general stress responses (Lima et al., 2023; Santi and Murta, 2022). The upregulation of energy metabolism pathways in the resistant DA strain is particularly noteworthy, as similar metabolic rewiring has been associated with parasite persistence in patients with chronic chagasic cardiomyopathy (Cunha-Neto et al., 2005). These observations point to metabolic flexibility as a defining trait of naturally resistant strains, enabling them to endure the oxidative stress generated by Benznidazole.

Resistance, therefore, emerges as a multifaceted phenomenon involving coordinated modifications across diverse cellular pathways. In the DA strain, enhanced expression of DNA repair genes and antioxidant enzymes demonstrates an intrinsic ability to mitigate drug induced

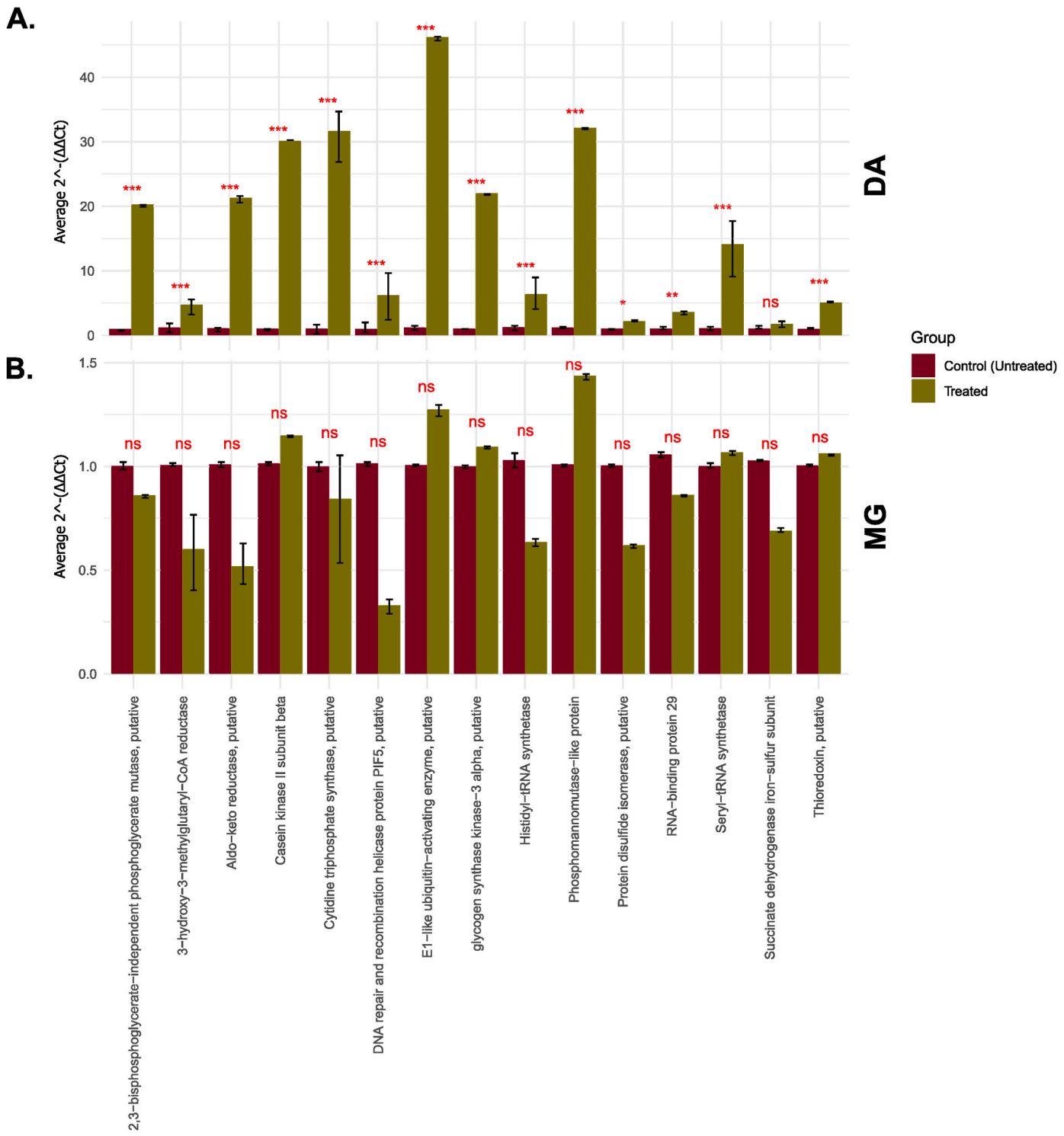

Fig. 4. Analysis of differential gene expression of candidate genes in susceptible (MG) and resistant (DA) *T. cruzi* strains in response to Benznidazole. The figure presents the quantification of the relative gene expression of 15 candidate genes in the DA (resistant) and MG (susceptible) strains of *T. cruzi* following exposure to Benznidazole. Differential expression was determined by RT-qPCR and normalized using the $2^{-\Delta\Delta Ct}$ method. Panel **A** shows gene expression levels in the DA strain, comparing untreated samples (control, burgundy bars) with Benznidazole-treated samples (olive green bars). Similarly, Panel **B** illustrates gene expression levels in the MG strain under the same experimental conditions. Bars represent mean values ± standard error (SE). Statistical significance between treated and untreated groups for each gene was evaluated using Student's *t*-tests. Asterisks denote significant differences: $p < 0.05$ (*), $p < 0.01$ (**), whereas "ns" indicates non-significant differences ($p \geq 0.05$). Visual comparison of bar heights, together with the indicated levels of significance, highlights the contrasting transcriptional responses between the resistant and susceptible strains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genotoxicity (Gupta et al., 2018; Lima et al., 2023; Reis-Cunha et al., 2017). These adaptations are not transient responses to stress but rather pre-existing features of the strain's regulatory architecture. Functional validation of these genes through CRISPR/Cas9 based gene editing will be critical for establishing causality, as such approaches have already proven effective in dissecting resistance pathways in related parasites (Lander and Chiurillo, 2019). For example, targeting DNA repair enzymes or thiol-based antioxidant systems in resistant strains may clarify their role in drug sensitivity. The recent demonstration that alterations in nitroreductase I (NTRI), a key enzyme for drug activation, confer resistance underscores the value of functional genomic approaches (Mejía-Jaramillo et al., 2025).

Our findings align with prior studies, such as those of García-Huertas et al. (2017a), who identified more than 130 differentially expressed genes in a naturally resistant strain, many involved in metabolism, catalysis, and redox regulation (Coura and Borges-Pereira, 2012; García-Huertas et al., 2017a; Lima et al., 2023; Vela et al., 2021; Zingales and Macedo, 2023). In the DA strain, genes like prostaglandin F2α synthase and trypanothione synthetase, both associated with oxidative stress management are highly expressed (Ariyanayagam and Fairlamb, 2001; García-Huertas et al., 2017a, 2017b; Mejía-Jaramillo et al., 2025). Thioredoxin, also upregulated, plays a critical role in maintaining redox balance and detoxifying reactive oxygen species (Couto et al., 2016; Lima et al., 2023). Collectively, these elements form a robust antioxidant defense, enabling resistant strains to survive the oxidative burden of Benznidazole therapy. Nevertheless, paradoxical findings were observed: some stress related genes, such as aldo-keto reductase and heat shock proteins, were downregulated in resistant strains, challenging canonical models of stress adaptation (González et al., 2017; Jamabo et al., 2022; Mejía-Jaramillo et al., 2025). These findings highlight the strain specific and context dependent nature of resistance, where multiple compensatory mechanisms may substitute for downregulated pathways.

The MG strain, in contrast, reveals an alternative resistance blueprint. Downregulation of kinesins and motor proteins suggests impaired intracellular transport and replication capacity under stress conditions (Douglas et al., 2020; Zingales and Macedo, 2023). Additionally, suppression of redox-related genes such as glutaredoxin and thioredoxin indicates reliance on other strategies, such as DNA repair and metabolic adjustments, to endure genotoxic insults (Couto et al., 2016; Lima et al., 2023). These differences between DA and MG underscore the genetic and regulatory diversity within *T. cruzi* populations and the complexity of resistance phenotypes.

A unique feature of *T. cruzi* transcriptomics is the pervasive expression of pseudogenes (Fig. 2) (Supplementary Tables 3 and 4). In both DA and MG, many differentially expressed genes were annotated as pseudogenes, reflecting the highly repetitive genome architecture of the parasite (Clayton, 2019). Families such as trans-sialidases, mucins, and MASPs contain numerous truncated members that may still produce functional transcripts (El-Sayed et al., 2005; Herreros-Cabello et al., 2020). The co-transcription of pseudogenes within polycistronic units complicates annotation, and ongoing reclassification efforts have revealed that some pseudogenes may encode functional proteins (Borujeni and Salavati, 2024; Reis-Cunha et al., 2022). This genomic plasticity contributes to the parasite's adaptability and may play a role in drug resistance (Abrahim et al., 2022; Callejas-Hernández et al., 2018).

The polygenic and multilayered nature of Benznidazole resistance is further supported by gene ontology analyses. In the DA strain, significant enrichment of oxidative phosphorylation, electron transport, and nitrogen metabolism pathways indicates a profound metabolic reconfiguration designed to support survival under oxidative stress (Fig. 3A) (Docampo and Moreno, 1996; Scarpelli et al., 2021). By contrast, the MG strain favors enrichment of DNA repair and genome stability processes, suggesting an alternative adaptation strategy (Fig. 3C) (Rassi et al., 2010). These patterns are consistent with other studies reporting broad metabolic and biosynthetic alterations in resistant strains (Lima et al., 2023). Complementary analyses also reveal upregulation of glycolysis, the TCA cycle, and the pentose phosphate pathway in both DA and MG (Fig. S2) (Lima et al., 2023; Murta et al., 2024; Sales et al., 2017). These metabolic shifts generate NADPH and ATP, bolstering antioxidant defenses and energy supplies, thereby enhancing resilience to drug induced stress (Lima et al., 2023; Loureiro et al., 2017; Márquez et al., 2014; Murta et al., 2024; Sales et al., 2017).

Validation using RT-qPCR confirms these observations. In the resistant DA strain, detoxification enzymes, DNA repair genes, and energy metabolism factors such as succinate dehydrogenase are significantly upregulated. Antioxidant systems, including thioredoxin, and ubiquitin pathway genes, show strong induction, pointing to an adaptive strategy that integrates redox defense, protein quality control, and energy metabolism to counteract Benznidazole cytotoxicity (Couto et al., 2016; Gupta et al., 2018; Kazemi-Rad et al., 2013; Lima et al., 2023). In contrast, the MG strain exhibits minimal transcriptional induction, with some genes even downregulated. This limited adaptive response likely explains its greater susceptibility to the drug (Lima et al., 2023). Notably, discrepancies between RNA-seq and qPCR results in MG highlight the importance of integrating global transcriptomic data with targeted assays to capture the complexity of drug responses.

Nevertheless, several limitations temper the conclusions of current studies. Analyses based on only two strains cannot capture the full spectrum of genetic diversity across *T. cruzi* DTUs. In addition, experiments were conducted at a single drug concentration and time point, restricting the ability to evaluate dose dependent responses and does not represent the relevant therapeutic ranges in a clinical context, as has been discussed in the scientific literature (Frade et al., 2020). The use of repository strains, rather than clinical isolates, limits direct clinical applicability, while the absence of whole genome sequencing hampers discrimination between stable genomic changes and transient transcriptional responses (Lander and Chiurillo, 2019). Future research must expand to multiple strains across DTUs, incorporate clinical isolates, and integrate genomic, transcriptomic, and proteomic data over time and across life stages to achieve a comprehensive understanding of resistance mechanisms.

This study represents an approach toward elucidating the molecular mechanisms underlying the differential response of *T. cruzi* to Benznidazole. The identification of distinct gene expression profiles and metabolic adaptations provides novel evidence that intrinsic resistance involves constitutive regulatory programs rather than solely drug-induced responses, thereby redefining current paradigms of Benznidazole resistance in *T. cruzi*. These findings demonstrate that naturally resistant strains constitute a critical framework for understanding the evolution and persistence of drug resistance. Unlike artificially induced models, naturally resistant populations harbor deeply entrenched adaptations shaped by long-term evolutionary and ecological pressures. Resistance in *T. cruzi* emerges as a polygenic and multifactorial phenomenon, resulting from the coordinated interplay of antioxidant defenses, DNA repair systems, metabolic reprogramming, and protein quality-control pathways. Within this complex network, candidate genes such as prostaglandin F2α synthase, trypanothione synthetase, thioredoxin, and other redox-related enzymes stand out as promising therapeutic targets and potential biomarkers for resistance (García-Huertas et al., 2017a; González et al., 2017; Lima et al., 2023; Mejía-Jaramillo et al., 2025). Given the regional predominance of discrete typing units such as TcI in Colombia and across Latin America (Hoyos Sanchez et al., 2024), the molecular characterization of these resistance determinants can inform geographically tailored treatment strategies and public health interventions. Ultimately, the integration of transcriptomic data with complementary genomic and proteomic approaches will be essential for unraveling the complex adaptive landscape of *T. cruzi*. Such integrative omics and precision medicine strategies hold great promise for the rational design of targeted therapies, enabling interventions that are adapted to the molecular

characteristics of both the parasite and its host, and thereby improving therapeutic outcomes in CD.

5. Conclusions

This study offers valuable insights into the transcriptomic architecture underlying natural Benznidazole resistance in *T. cruzi*, revealing that drug resistance is a complex, multigenic phenomenon shaped by diverse and strain-specific adaptive responses. By comparing two naturally distinct strains, one resistant (DA) and one susceptible (MG), we were able to characterize differential expression patterns across key metabolic, redox, and DNA repair pathways, many of which appear to be constitutively regulated rather than solely induced by drug exposure. These findings suggest that resistance in naturally circulating strains may stem from long-term evolutionary pressures rather than short-term selective events, as is often the case in *in vitro* models. This distinction is critical for the identification of clinically relevant resistance mechanisms and may help explain the limited success of current chemotherapies in field settings.

Our results further emphasize the importance of integrating transcriptomic data with functional assays and genome-wide analyses to validate the role of candidate genes such as prostaglandin F2α synthase, thioredoxin, and trypanothione synthetase. These genes not only emerge as potential biomarkers of resistance but also represent promising targets for the development of adjunctive or combination therapies aimed at overcoming current pharmacological limitations. Moreover, understanding the molecular underpinnings of resistance in dominant DTUs such as TcI, prevalent in Colombia and much of Latin America, has direct implications for improving clinical management and shaping public health strategies tailored to regional epidemiological contexts. Ultimately, this study contributes to a growing body of evidence that positions comparative transcriptomics as a powerful tool in the fight against CD, offering a roadmap toward precision medicine and more effective, targeted interventions.

CRediT authorship contribution statement

Carlos Ospina: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tatiana Cáceres:** Writing – review & editing, Visualization, Resources, Investigation, Formal analysis, Data curation. **Stivenn Gutiérrez:** Writing – review & editing, Methodology, Investigation, Data curation. **Luz Helena Patiño:** Writing – review & editing, Validation, Project administration, Methodology, Investigation, Formal analysis. **Luis David Sáenz-Pérez:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Karen Moreno Medina:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Juan Carlos Villar:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Juan David Ramírez:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2025.100623.

Data availability

All transcriptomic sequences from the DA and MG strains have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB89709.

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