# SYSTEMS BIOLOGY OF HOSTS, PARASITES AND VECTORS

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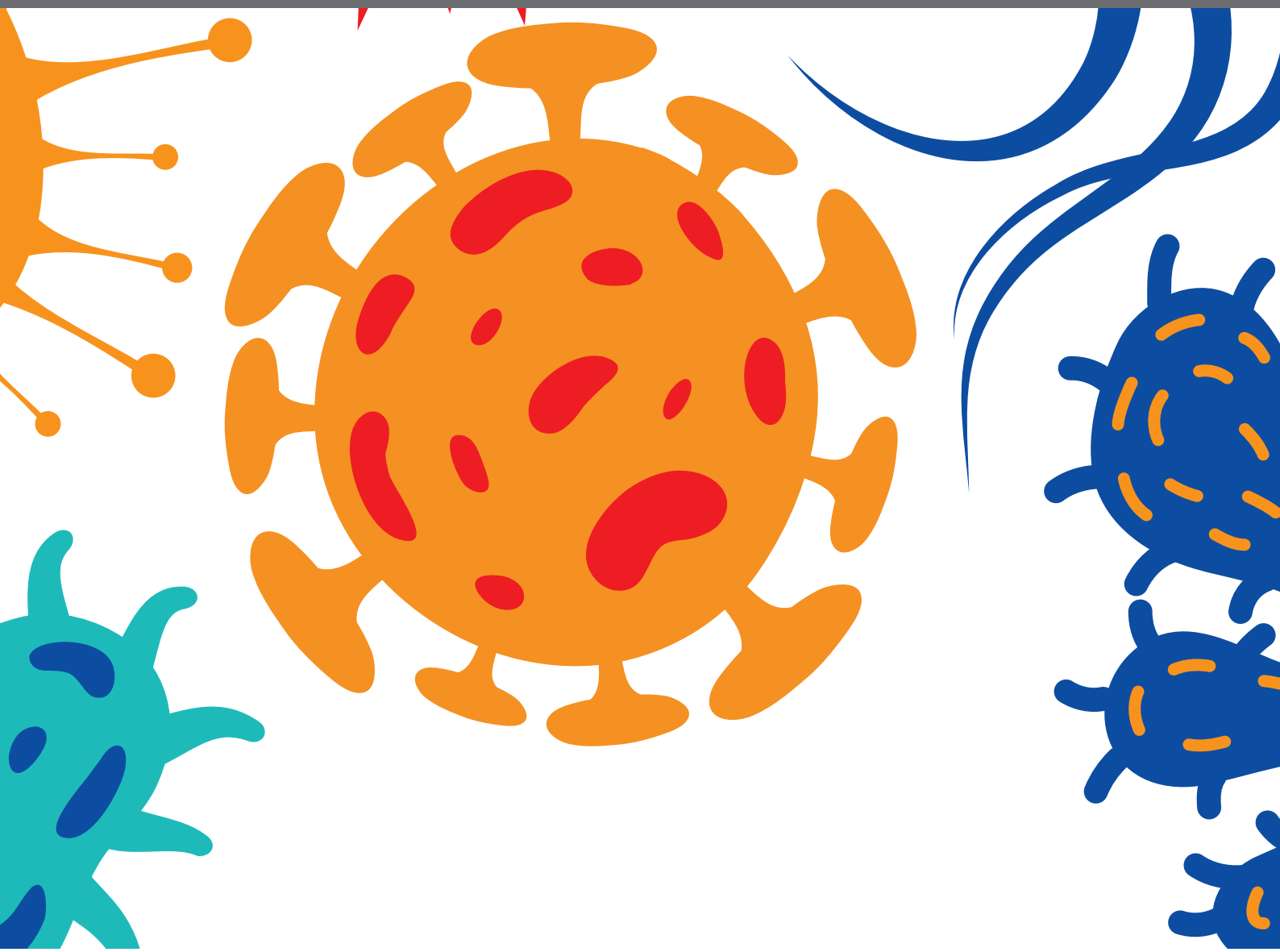

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# SYSTEMS BIOLOGY OF HOSTS, PARASITES AND VECTORS

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EDITORIAL
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# Editorial: Systems Biology of Hosts, Parasites and Vectors

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**Keywords: genome, transcriptome, proteome, microbiome, vector interaction, control, drug resistance, evolution**

## Editorial on the Research Topic

## Systems Biology of Hosts, Parasites and Vectors

Parasites cause significant morbidity and mortality in humans, livestock, and companion animals, imposing major challenges for public and veterinary health, and the food industry, worldwide. Parasitic diseases are influenced by a wide range of factors that add complexity to the understanding of the host-parasite-vector triad. A thorough knowledge of parasite biology, and of the interactions between these pathogens and their vertebrate and/or invertebrate hosts is key to discover and develop novel and sustainable strategies to reduce infection burden and assist elimination.

Systems Biology has emerged to provide an integrated overview of biological systems on the "post-genomic era" and predict how they operate to produce a determined phenotype (Ideker et al., 2001; Chuang et al., 2010). Omics technologies coupled to computational biology have been applied to study different aspects of parasites (Bourgard et al., 2018; Maruyama et al., 2019b; Garcia et al., 2020), host responses (Saric et al., 2010; Gardinassi et al., 2016; Gardinassi et al., 2018; Hargrave et al., 2019; Maruyama et al., 2019a), interaction with vectors (Srivastava et al., 2016; Inbar et al., 2017) and the host microbiome (Jenkins et al., 2018; Cortés et al., 2020; Jenkins et al., 2021). Considering these concepts and philosophy, this Research Topic was formulated to communicate high-quality work in this area of investigation and associated disciplines, which gathered original research, review, perspective and hypothesis and theory articles.

The first published article of this Research Topic tackled a technical issue related to the zoonotic protozoan parasite, Giardia *lamblia*. Heller et al. applied shotgun mass spectrometry to uncover that transfection of the reference strain WBC6 with glucuronidase A (GusA) from *Escherichia coli* affects the cellular proteomic profile and induces antigenic variation. Results from this study have strong implications for the interpretation of experiments involving transfected *Giardia* and related controls, because comparisons with un-transfected trophozoites may lead to spurious results.

Other protozoan parasites including *Leishmania* and Plasmodium were also addressed. Horácio et al. provided a perspective of how systems biology approaches have been used and can contribute to future discoveries in drug resistance of *Leishmania* parasites. Current therapeutics and how networks of biochemical pathways can be involved in drug resistance were explored, with the authors indicating antioxidant defense enzymes as biomarkers of resistance and drug targets.

The development of new therapeutic venues or vaccines to prevent disease can be explored with systems biological approaches, including immunopeptidomics. In this Research Topic, Juanes-Velasco et al. reviewed advances and strategies used to identify immunogenic peptides presented on human leukocyte antigen (HLA) molecules and recognized by T cells. The review is broad and provides an informative overview of immunopeptidomics in infectious diseases and cancer.

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We highlight the discussion of the potential application of this technology to identify *Plasmodium falciparum* antigens to compose improved vaccines against malaria.

Other strategies have focused on the identification of targets blocking transmission of *Plasmodium* to their vectors (Gonçalves and Hunziker, 2016). In This Research Topic, Niu et al. have analyzed transcriptomics data of *P. falciparum* to study genes coding for proteins that interact with the vector midgut. The authors identified 6 proteins interacting with vector midgut lysate, with further experiments suggesting that Pfs16 might interact with the vector midgut, and this might represent a target to block transmission.

Addressing aspects of helminth parasites, Fontenla et al. used bioinformatics approaches to analyze and compare small non-coding RNA pathways in Platyhelminthes. The authors observed that Piwi proteins, which interact with and regulate piRNA, are conserved in free-living but absent in parasitic flatworms, which could potentially influence the divergence to parasitism in flatworms.

Genomic diversification and evolution of helminths have led to the emergence of defense strategies, some of which favor parasitism, such as enzymatic antioxidant systems (Chiumiento and Bruschi, 2009). In this Research Topic, Dorey et al. discussed genomic, transcriptomic, and proteomic analyses contributing to the emerging concept of non-antioxidant roles of thioredoxin-1 and peroxiredoxin-1 of the fluke *Fasciola hepatica*. The authors propose these proteins operate mostly *via* immunomodulatory mechanisms rather than the stress-inducible thiol-dependent cascade.

A Hypothesis and Theory article by da Costa et al. discusses carcinogenic- helminthiasis, chemical carcinogenesis, and therapeutic strategies to control helminths and associated pathology. The authors focused on carcinogenic potential of urogenital schistosomiasis and suggested that selected parasite metabolites could interact with host DNA and inhibit repair. Many other factors can be implicated in this process, that systems biology tools might contribute to discover.

Animal models of helminthic diseases are critical to understand both parasite development in hosts and evasion mechanisms, as well as the dynamics of the mammalian immune response to these parasites. In this Research Topic, Montaño et al. compared and discussed the proteomes of *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus bakeri* and *Trichuris muris*, all important models of human helminthiases, and argue that these parasites represent key sources of potential vaccine candidates and novel immunomodulatory molecules.

In this Research Topic, Rosa et al. analyzed the gut microbiome of humans infected with *Trichuris trichiura* and compared it with that of mice infected with *T. muris*. Besides demonstrating the applicability of this mouse model to study whipworm infection, the authors identified bacterial taxa whose dynamics were associated with infection in mice and humans, as well as with anthelmintic treatment, and suggested that the genera *Escherichia* and *Blautia* might represent promising targets for future mechanistic studies of host-parasite-microbiome interactions.

The impact of *Calicophoron daubneyi*, a parasite of ruminant livestock over the rumen microbiome was investigated by Allen et al.; the authors described 378 proteins in extracellular vesicles (EVs) released by *C. daubneyi via* proteomic analyses. Furthermore, parasite EVs modulated total bacteria concentrations in rumen, suggesting a role for parasite EVs in worm-microbiome interactions and a possible role of these vesicles in the establishment and maintenance of fluke infections.

Overall, the articles included in this Research Topic covered intriguing questions related to protozoans and helminths, and their interaction with vectors, hosts and associated microbiome, control strategies, drug resistance, as well as diversification and evolution. These studies contribute to a better understanding of parasites and the pathological processes they cause, while stimulating further research in the field.

## AUTHOR CONTRIBUTIONS

LG wrote the editorial draft. SM and CC revised the editorial. All authors approved the final version of the manuscript.

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# Transfection With Plasmid Causing Stable Expression of a Foreign Gene Affects General Proteome Pattern in *Giardia lamblia* Trophozoites

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*Giardia lamblia* is an important causative agent of persistent diarrhea in humans, domestic animals, and cattle. Basic research is usually performed with the strain WBC6 and includes genetic manipulations such as transfections. Here, we investigate how transfection with a plasmid causing stable expression of a foreign gene affects the whole proteome pattern. Using shotgun mass spectrometry, we compare the proteomes of untransfected trophozoites to trophozoites transfected with *Escherichia coli* glucuronidase A (GusA). Besides GusA, which is detected in the transfected trophozoites only, the proteomes of untransfected and transfected trophozoites differ by 132 differentially expressed proteins. In particular, transfection induces antigenic variation. Since transfection causing stable expression affects the proteome pattern, transfection experiments should take into account this effect. Due to a unique peptide panel, GusA is an example for a suitable internal standard for experiments involving transfected cells. Data are available *via* ProteomeXchange with identifier PXD022565.

**Keywords: model organisms, reverse genetics, untargeted proteomics, experimental proteomics, controls and standards**

## INTRODUCTION

The diplomonadid *Giardia lamblia* (syn. *G. duodenalis*, *G. intestinalis*), is an early diverging, anaerobic eukaryote (Müller and Müller, 2016; Cernikova et al., 2018) causing persistent diarrhea, especially in regions with low hygienic standards (Hemphill et al., 2019). After stomach passage, ingested cysts transform into trophozoites colonizing the duodenum. Hosts in good physical condition face the strongest symptoms of giardiasis 1 week post infection, and recover within two to three weeks. Rarely, the infection becomes chronical causing severe damage of the intestinal epithelium, which may result in the development of irritable bowel syndrome (Allain et al., 2017; Litleskare et al., 2018). Giardiasis can be regarded as a zoonosis since it occurs in humans as well as in other mammals (Thompson, 2004).

The largest part of laboratory research is based on the strain WBC6, cloned from an isolate obtained from a patient (with the initials W.B.) suffering from chronical giardiasis (Nash et al., 1985; Campbell and Faubert, 1994). WBC6 is amenable to reverse genetics *via* transfection with

double-stranded RNA (Furfine and Wang, 1990) or DNA (Singer et al., 1998; Sun et al., 1998), respectively. The genome of WBC6 (Morrison et al., 2007) and of other strains have been sequenced so far (see www.giardiadb.org). One unique feature of the *Giardia* genome is the presence of more than hundred open reading frames encoding cysteine-rich surface proteins, the so-called "variant-specific surface proteins" (VSPs), the "cysteine-rich proteins", and the "high cysteine membrane proteins" (HCMPs). In a recently published chromosome-scale reference genome, 133 genes encoding for VSPs, as well as other highly repetitive genes such as 184 never-in-mitosis gene a (NIMA) related kinases and 305 ankyrin-repeat proteins (formerly annotated as proteins 21.1), are distributed over all five chromosomes (Xu et al., 2020). VSPs are considered as the predominant surface antigens of ***G. lamblia*** trophozoites (Adam et al., 2010). According to a generally admitted hypothesis, one single trophozoite expresses only one VSP at any one time (Nash et al., 2001). The switching from one VSP to another is called "antigenic variation" (Nash, 2002). Antigenic variation depends on epigenetic and post-transcriptional mechanisms, as evidenced by various studies (Kulakova et al., 2006; Prucca et al., 2008) and reviewed elsewhere (Prucca et al., 2011; Lagunas-Rangel and Bermudez-Cruz, 2018).

Shotgun mass spectrometry proteome studies have revealed that WBC6 trophozoite populations and trophozoite populations from other strains express an impressive number of VSPs at the same time (Emery et al., 2014; Emery, Lacey et al., 2015a; Emery et al., 2015b). Moreover, strain-dependent antigenic variation occurs upon drug pressure, concomitantly to large changes in expression patterns of other proteins (Emery et al., 2018; Müller et al., 2019).

As for many other model organisms, reverse genetics based on transfection with suitable plasmids or RNA viruses leading to transient or stable overexpression of genes of interest constitute an important research tool for *Giardia* (Davis-Hayman and Nash, 2002). In current protocols, transfectants are selected *via* a resistance marker encoded by the transfected plasmid, in particular neomycin phosphotransferase (Sun et al., 1998) and puromycin acetyltransferase (Singer et al., 1998; Jiménez-García et al., 2008). Since exposure to drugs and selection of resistance causes marked changes in gene expression patterns, it is logical to ask which side effects the transfection procedure has on gene expression in the transfected cells, independently of the transfected gene. In one of the first studies addressing this topic on the transcriptional level, neomycin and puromycin selection affected the expression patterns of various genes (Su et al., 2007).

Based on these findings, we contend that stable transfection of *G. lamblia* based on puromycin selection induces changes in proteome patterns to the same extent as differences amongst strains from different genotypes and differences between drug resistant and susceptible strains of the same background. Using shotgun mass spectrometry, we compare the proteomes of *G. lamblia* WBC6 trophozoites transfected with a plasmid containing *Escherichia coli* glucuronidase A (GusA) and *Streptomyces alboniger* puromycin_N-acetyltransferase (Pac) as a resistance marker (Singer et al., 1998) with untransfected trophozoites. The GusA transfection plasmid has been used by our group as a standard control in previous experiments (Nillius et al., 2011; Müller et al., 2013) involving transgenic *Giardia*. GusA-transfected trophozoites are not affected in growth (Müller et al., 2009) and have a metabolomics profile similar to untransfected trophozoites (Müller et al., 2020b). Here, we investigate the impact of transfection on the proteome pattern with a major focus on antigenic variation. Furthermore, we ask the question whether the transfected transgenes, i.e. the resistance marker Pac, GusA, or both can be used to estimate the expression level of the transgene in comparison to "housekeeping" proteins.

# MATERIALS AND METHODS

## Chemicals

If not otherwise stated, all biochemical reagents were from Sigma (St Louis, MO, USA). Puromycin was obtained from Invivogen (Toulouse, France).

## Axenic Culture, Harvest, and Storage of *Giardia lamblia* Trophozoites

Trophozoites from *G. lamblia* WBC6 were grown under anaerobic conditions in 10 ml culture tubes (Nunc, Roskilde, Denmark) on modified TYI-S-33 medium as previously described (Clark and Diamond, 2002). Subcultures were performed by inoculating 100 µl of cells from a confluent culture detached by incubation on ice for 15 min to a new tube containing 10 ml culture medium (Müller et al., 2006). Trophozoites were harvested by incubation on ice for 15 min followed by centrifugation (300×g, 10 min, 4°C).

## Transfection of *Giardia lamblia* Trophozoites

Transfection with the plasmid pPAC-V-GusA (Müller et al., 2009) and selection of transgenic trophozoites were performed as previously described (Yee and Nash, 1995; Singer et al., 1998). Briefly, prior to transfection, 1 µg of pPAC-V-GusA (in 20 µl digest mix) were linearized by digestion with SwaI (New England Biolabs, Ipswitch, MA) according to the instructions by the manufacturer. Then, $10^7$ trophozoites from a confluent culture were mixed with the digested plasmid DNA and incubated on ice for 5 min. Electroporation of trophozoites with linearized plasmid DNA was done in a 0.4 cm cuvette using an ECM 600 (BTX, San Diego, CA) at setting 350 V, 1000 µF, and 720 Ω. Electroporated trophozoites were immediately transferred to 10 ml medium in a plastic tube and incubated overnight at 37°C before puromycin was added to a final concentration of 100 µM. After 4 days, the medium was replaced by fresh medium containing 100 µM puromycin and drug-resistant cells visible after about 8 days post transfection were grown to confluence and then passaged once in the same medium. Before shotgun mass spectrometry analysis, the untransfected cultures were routinely passaged two times, the GusA-transfected cultures in

absence of puromycin. Pellets were washed three times with ice-cold PBS, counted, and stored at −80°C for subsequent proteomic analysis or for enzymatic assays, respectively.

## Proteomics

Cell pellets were lysed in 100 µL 8M urea/100 mM Tris/HCl pH8/cOmplete™ protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland) by incubation for 15 min at room temperature followed by 15 min in an ultrasonic water bath. Protein concentration was determined by BCA assay, followed by reduction and alkylation of proteins with 10 mM DTT for 30 min at 37°C and 50 mM iodoacetamide for 30 min at 37°C in the dark. Proteins were precipitated at −20°C by addition of 5 vol cold acetone and incubation at −20°C overnight. All liquid was carefully removed and the pellet dried in ambient air for 15 min before reconstitution of proteins to a concentration of 1mg/ml in 8 M urea, 50 mM Tris-HCl pH 8.0. An aliquot corresponding to 10 µg protein was digested by trypsin (1:50 trypsin/protein ratio) for 6 hours at 37°C after dilution of urea concentration to 1.6M with 20 mM Tris-HCl pH 8.0 and 2 mM $CaCl_2$. The digests were acidified with TFA (1%) and analyzed by LC-MS/MS. Three repetitive injections of an aliquot corresponding to 500 ng protein digest were analyzed on an EASY-nLC 1000 coupled to a QExactive HF mass spectrometer (ThermoFisher, Reinach, Switzerland). Peptides were trapped on an Acclaim PepMap100 C18 pre-column (3µm, 100 Å, 300 µm x 5 mm, ThermoFisher, Reinach, Switzerland) and separated by backflush on a C18 column (3µm, 100 Å, 75µm x 15 cm, Nikkyo Technos, Tokyo, Japan) by applying a 60 min gradient of 5% acetonitrile to 40% in water, 0.1% formic acid, at a flow rate of 400 nl/min. Peptides of m/z 400–1,400 were detected with resolution of 60,000 applying an automatic gain control (AGC) target of 1E06 and a maximum ion injection time of 50 ms. A top fifteen data dependent method for precursor ion fragmentation with a stepped 27% normalized collision energy was applied with the following settings: precursor isolation width of 1.6 m/z, resolution 15,000, AGC of 1E05 with a minimum target of 1E03, maximum ion time of 110 ms, charge exclusion of unassigned and 1+ ions, peptide match on, and dynamic exclusion for 20 s, respectively. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD022565.

## Statistics

The MS data for each strain consisted of three biological replicates, with three technical replicates each. All MS data were processed by MaxQuant (version 1.6.14.0) with matching between runs for the same strain activated, but not between different strains, in order to avoid over-interpretation of the data. Fragment spectra were interpreted against a recent *Giardia* protein sequence database in FASTA format (GiardiaDB-47_GintestinalisAssemblage AWB_AnnotatedProteins), supplemented by the two protein sequences of GusA and Pac from Swissprot database (accession numbers P05804 and P13249). The trypsin cleavage rule allowed amide bond cleavage after lysine and arginine but not if a proline follows and up to three missed cleavage sites, fixed carbamidomethylation modification of cysteine residues, variable oxidation of methionine and acetylation of protein N-termini. Precursor and fragment mass tolerances were set to 10 and 20 ppm, respectively. Peptide spectrum matches, peptide and protein group identifications were filtered to a 1% false discovery rate (FDR) based on reversed database sequence matches, and a minimum of two razor or unique peptides were required to accept a protein group identification.

Protein identifications considered as contaminations (e.g. trypsin) as well as proteins identified only by site were removed for statistical validation. The normalized label-free quantification (LFQ) protein group intensities as calculated by MaxQuant and a top3 approach were used for relative proteome quantifications. For top3, peptide intensities were median-normalized to the global median, then missing peptide imputation was done per sample by drawing values from a Gaussian distribution of width 0.3x sample standard deviation centered at the sample distribution mean minus 1.8x sample standard deviation if there were at least two peptide intensities in a group, otherwise a Maximum Likelihood Estimation (MLE) method was applied, followed by summation of the three most intense peptides per protein group to a surrogate of the real protein abundance, named iTop3. For LFQ values, we imputed with the same left-censored method as for peptides if there was a single missing protein LFQ value in the three biological replicates. Any remaining missing values were again imputed by the MLE method. In order to perform statistical tests, missing iTop3 or LFQ values were further imputed at the protein level using the MLE method, and the final imputed LFQ or iTop3 values were called iLFQ or iiTop3, respectively.

Differential expression tests were performed by applying the (Welch-) Student's t-test (unequal variance). Log2-fold changes and adjusted p-values (FDR-controlled Benjamini and Hochberg correction) were reported. Furthermore, a check was performed by repeating the imputation cycle and significance testing 20 times. Protein groups with a persistent reporting of differential expression were considered as true differentially expressed between the two groups. A log2-fold change of at least one and a corrected p-value of $\leqq 0.05$ were required to be considered as significant. Statistical testing and imputation were made using a set of freely available R package tools running under R studio.

# RESULTS

## Proteome Parameters of *Giardia lamblia* Trophozoites

Shotgun mass spectrometry of the proteomes of untransfected *G. lamblia* WBC6 trophozoites (WT) and of trophozoites transfected with a plasmid containing the *S. alboniger* puromycin-N-acetlytransferase (Pac) as a resistance marker and the *E. coli* glucuronidase A gene (Gus A) resulted in the identification of 20'091 unique peptides matching to 1'705 proteins. Moreover, 12 unique peptides matching to GusA were consistently detected in the transfected trophozoites, but not in the WT. The identified peptides are depicted in **Figure 1**. Unique peptides matching to Pac could not be detected.

The expression levels of GusA in the transfected trophozoites were compared to four housekeeping proteins expressed at equal LFQ and iTop3 levels in both transfected and untransfected strains, namely pyruvate-ferredoxin oxidoreductase, arginine deiminase, thioredoxin reductase, and glutamate dehydrogenase. The levels of GusA were one to two magnitudes (in base 10) lower than the levels of these proteins (**Table 1**).

## Transfection Induces Significant Changes in *Giardia lamblia* Trophozoite Proteomes

Overall analysis of the data by principal component analysis (PCA) revealed that the proteomes from WT and GusA trophozoites were distinctly separated along the principal component 1. As expected, technical replicates clustered together, whereas biological replicates were separated by principal component 2 for both groups (**Figures 2A, B**). Differences in the proteome patterns of both strains were confirmed by Volcano plot analysis (**Figure 2 C**).

MVRPVETPTREIK **KLDGLWAFSLDRENCGIDQR** WWESALQ
ESR **AIAVPGSFNDQFADADIR** NYAGNVWYQREVFIPKGWA
GQRIVLRFDAVTHYGK **VWVNNQEVMEHQGGYTPFEADVTP**
**YVIAGK** SVRITVCVNNELNWQTIPPGMVITDENGKKK **QSY**
**FHDFFNYAGIHR** SVMLYTTPNTWVDDITVVTHVAQDCNHA
SVDWQVVANGDVSVELRDADQQVVATGQGTSGTLQVVNPH
LWQPGEGYLYELCVTAK **SQTECDIYPLR** VGIRSVAVK **GEQ**
**FLINHKPFYFTGFGR HEDADLR** GK **GFDNVLMVHDHALMDW**
**IGANSYR** TSHYPYAEEMLDWADEHGIVVIDETAAVGFNLS
LGIGFEAGNKPK **ELYSEEAVNGETQQAHLQAIK** ELIARDK
NHPSVVMWSIANEPDTRPQGAR **EYFAPLAEATR** KLDPTRP
ITCVNVMFCDAHTDTISDLFDVLCLNRYYGWYVQSGDLET
AEKVLEKELLAWQEKLHQPIIITEYGVDTLAGLHSMYTDM
WSEEYQCAWLDMYHRVFDR **VSAVVGEQVWNFADFATSQGI**
**LR** VGGNKKGIFTRDRKPKSAAFLLQKRWTGMNFGEKPQQG
GKQ

**FIGURE 1** | Primary sequence of the transgene *E. coli* glucuronidase A with the identified peptides highlighted in bold red letters. The underlined sequence is a separate peptide generated from the longer peptide sequence with a missed trypsin cleavage site at position twelve. Otherwise, trypsin cleavage sites are indicated by a space. Each line consists of 40 amino acids.

## Differentially Expressed Proteins

Further analysis of the dataset revealed that the proteomes of WT and GusA contained 132 differentially expressed proteins, namely 39 with higher levels in WT and 93 with higher levels in GusA trophozoites, when the evaluation was done by both iLFQ and iiTop3 algorithms as shown in **Figure 3**.

Besides 70 differentially expressed (DE) hypothetical proteins, the two categories with the highest numbers of DE proteins, namely 17, comprised surface antigens and proteins involved in intermediary metabolism, followed by 14 proteins involved in gene expression, cell cycle and development and 14 proteins involved in cytoskeleton, flagella, adhesion and organelle transport (**Table 2**). The complete list of the accession numbers of the DE proteins is given as **Supplementary Table S1**.

## Antigenic Variation

As mentioned above, surface antigens constituted one of the two categories with the highest amount of DE proteins, namely 17. In particular, 14 variant-specific surface proteins (VSP) had significantly higher levels in GusA-transfected trophozoites and one VSP in wildtype trophozoites. Conversely, only two high cysteine membrane proteins (HCMP), namely 115066 and 25816, had significantly higher levels in WT than in GusA trophozoites. The VSP 89315 was the predominant differential surface antigen with significantly higher levels in wildtype trophozoites. VSP14586 was the predominant DE VSP in GusA trophozoites (**Figure 4**).

The expression levels of the major surface antigens, namely the VSPs 188, 88, 8, and the HCMP with the ORF number 15317, however, were not significantly affected (**Table 3**).

# DISCUSSION

The present dataset confirms our initial hypothesis that the transfection of *G. lamblia* by plasmids followed by an antibiotic-based selection of transfected trophozoites affects the whole genome expression pattern thereby confirming and extending previous findings (Su et al., 2007). Furthermore, it induces antigenic variation like other drug-based selections (Emery et al., 2018; Müller et al., 2019) or in strains from different genetic backgrounds (Emery et al., 2014; Emery et al., 2015a; Müller et al., 2020a). For instance, we have found 17 DE surface antigens

**TABLE 1** | Major housekeeping proteins and transgenes in untransfected *Giardia lamblia* WBC6 wildtype (WT) and *Escherichia coli* glucuronidase A (GusA)-transfected trophozoites.

| Protein | Accession No. | LFQ | | iTop3 | |
|---|---|---|---|---|---|
| | | WT | GusA | WT | GusA |
| Pyruvate ferredoxin oxidoreductase | 17063 | 10,464 ± 407 | 8,096 ± 70 | 249 ± 36 | 196 ± 17 |
| Arginine deiminase | 112103 | 31,263 ± 450 | 39,025 ± 1,129 | 1,110 ± 431 | 1,550 ± 289 |
| Thioredoxin-reductase | 9827 | 1,725 ± 556 | 1,804 ± 458 | 108 ± 42 | 144 ± 33 |
| Glutamate dehydrogenase | 21942 | 10,315 ± 1015 | 7,159 ± 826 | 309 ± 39 | 216 ± 39 |
| Glucuronidase A | P05804 | nd | 128 ± 7 | nd | 13 ± 2 |
| Puromycin-N acetyltransferase | P13249 | nd | nd | nd | Nd |

*Mean values (± SD) of LFQ, and iTop3 levels (x10$^6$) are given for three biological replicates. The accession numbers of the transgenes are from the Uniprot database, the others from GiardiaDB. nd, not detected.*

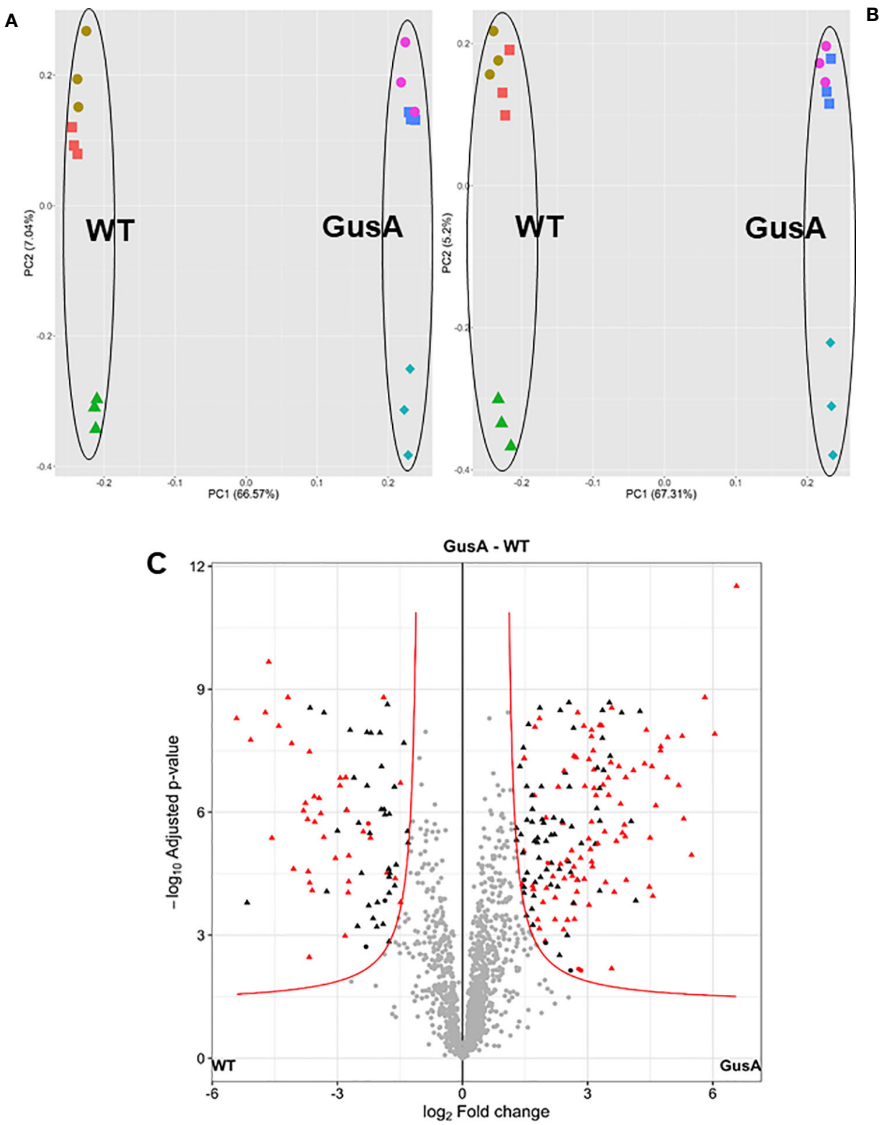


**FIGURE 2** | Principal component analysis plots **(A, B)** and volcano plot **(C)** of proteome data set from *G. lamblia* trophozoites transfected with a plasmid containing *E. coli* glucuronidase A (GusA) and of not transfected, control trophozoites (WT). The principal component analysis plots show for each strain, all technical and biological (red square, brown circle, green triangle for WT; pink circle, blue square, turquoise diamond for GusA) replicates based on iiTop3 data **(A)** or iLFQ data **(B)**. The volcano plot **(C)** is based on iiTop3 data. Differential proteins identified by both iiTop3 and iLFQ algorithms are depicted in red.

between WT and Gus (one permutation) and 89 between WT and nitro drug resistant C4 cells grown either in the presence of metronidazole or nitazoxanide (three permutations), as shown in a previous study (Müller et al., 2019; in particular **Supplementary Table S2** therein).

Since antigenic variation is caused by epigenetic (Lagunas-Rangel and Bermudez-Cruz, 2018) and post-transcriptional (Prucca et al., 2008) mechanisms, the degree of variation could be used as a tool to estimate the impact of a given treatment, in our case transfection, on these mechanisms. As generally admitted, trophozoites express only one VSP on their surface at the same time (Nash et al., 2001). Thus, the fact that the number of differentially expressed VSPs is higher in the transfected than in the untransfected trophozoite population is indicative for a higher degree of diversification most likely caused by the selection process. Moreover, the transfection and the subsequent selection procedure may influence metabolism, cell organization and motility, as indicated by a number of differentials in these categories. In fact, we observe only small metabolic differences between WT and GusA transfected strains, as published in a previous study (Müller et al., 2020b).

It is surprising that 12 unique peptides can be attributed to the GusA protein thereby allowing an easy identification, but no peptides to the puromycin resistance marker Pac protein, which

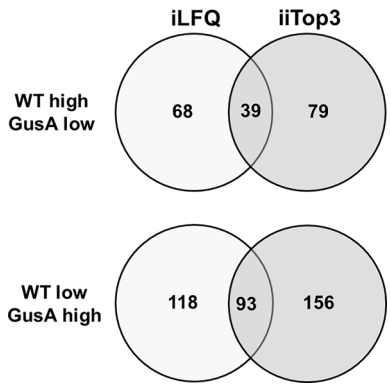


**FIGURE 3** | Venn diagram depicting the number of differentially expressed proteins in transfected and untransfected *G. lamblia* trophozoites. Untransfected trophozoites (WT) and trophozoites transfected with *E. coli* glucuronidase A were subjected to MS shotgun analysis as described in Materials and Methods. The differential proteins were determined *via* the iLFQ, the iiTop3 algorithm or *via* both.

**TABLE 2** | Overview of differentially expressed proteins in untransfected *G. lamblia* wildtype (WT) and *E. coli* glucuronidase A-transfected trophozoites (GusA).

| (Hypothetical) function | Higher in WT | Higher in GusA |
|---|---|---|
| Surface antigens: (a) Variant-specific surface proteins | 1 | 14 |
| (b) High cysteine membrane proteins | 2 | 0 |
| 21.1 proteins (ankyrin repeat proteins without kinase domain) | 1 | 3 |
| Gene expression, cell cycle, development | 3 | 11 |
| Intermediary metabolism | 5 | 12 |
| Transport of micromolecules | 0 | 1 |
| Cytoskeleton, flagella, adhesion and organelle transport | 2 | 5 |
| Chaperones | 0 | 2 |
| Hypothetical | 25 | 45 |
| Total | 39 | 93 |

*The proteins were identified by MS shotgun. Only proteins, which were differential by both iiTop3 and iLFQ algorithms were considered. The complete dataset is available online, the GiardiaDB open reading frame numbers and annotations of the differentials as* ***Supplementary Table S4****.*

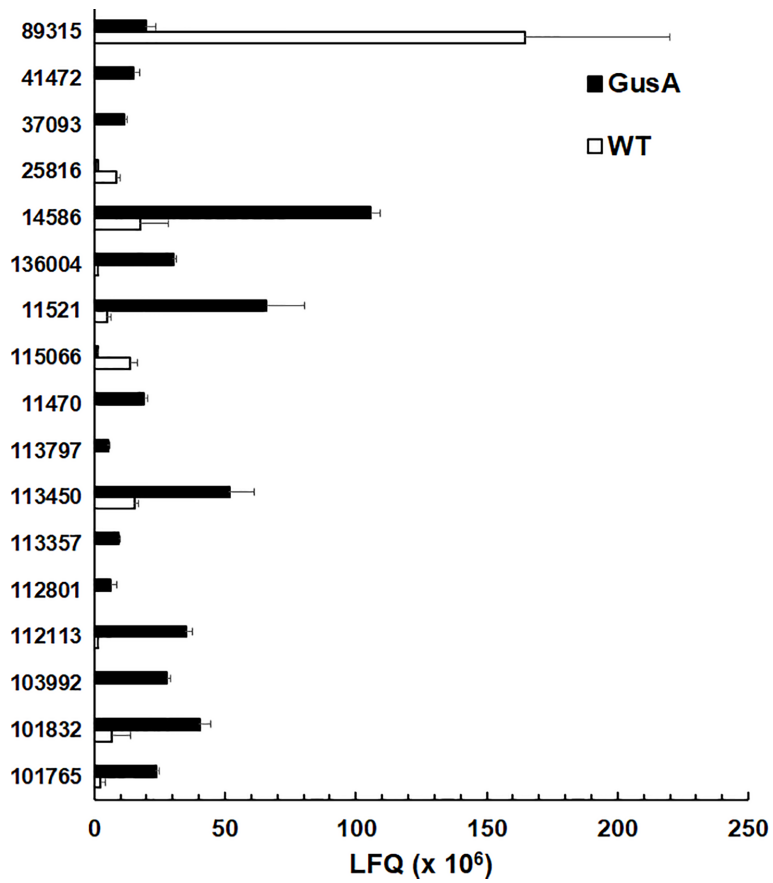


**FIGURE 4** | Quantitative assessments of differential surface antigens. Untransfected *G. lamblia* WBC6 trophozoites (WT; white bars) and *E. coli* glucuronidase A-transfected trophozoites (GusA; black bars) were subjected to MS shotgun analysis as described in *Materials and Methods* section. For all proteins, mean values ± one standard deviation for LFQ intensities ($x10^6$) in three biological replicates are shown. The proteins are termed by their respective accession numbers in the GiardiaDB. 25816 and 113416 are high cysteine membrane proteins.

**TABLE 3 |** Major surface antigens without significant differences in expression levels between untransfected *G. lamblia* WBC6 wildtype (WT) and *E. coli* glucuronidase A (GusA)-transfected trophozoites.

| Accession N° | Annotation | LFQ | | iTop3 | |
|---|---|---|---|---|---|
| | | WT | GusA | WT | GusA |
| GL50803_101074 | VSP with INR; VSP-88 | 1,315 ± 172 | 1,740 ± 148 | 746 ± 162 | 975 ± 185 |
| GL50803_112207 | VSP | 102 ± 44 | 35 ± 2 | 168 ± 88 | 24 ± 2 |
| GL50803_137612 | VSP | 195 ± 28 | 352 ± 27 | 119 ± 10 | 218 ± 24 |
| GL50803_137613 | VSP with INR; VSP-188 | 3,368 ± 217 | 3,170 ± 96 | 3,104 ± 96 | 2,728 ± 237 |
| GL50803_137617 | VSP | 102 ± 3 | 123 ± 4 | 66 ± 3 | 79 ± 3 |
| GL50803_137618 | VSP with INR; VSP-8 | 979 ± 173 | 637 ± 54 | 619 ± 157 | 386 ± 37 |
| GL50803_15317 | HCMP group 1 | 835 ± 110 | 401 ± 23 | 495 ± 102 | 330 ± 29 |
| GL50803_221693 | (hypothetical), VSPA6 | 124 ± 60 | 48 ± 4 | 179 ± 75 | 42 ± 3 |
| GL50803_33279 | VSP | 181 ± 12 | 118 ± 9 | 276 ± 6 | 150 ± 4 |

*Mean values (± SD) of LFQ and iTop3 levels (x$10^6$) are given for three biological replicates. Only surface antigens with LFQ levels above $10^8$ in at least one strain are shown. The accession numbers of the GiardiaDB are given. HCMP, high cysteine membrane protein; VSP, variant-specific surface protein.*

has allowed to select the transfectants. The expression of both proteins is controlled by the same promoter, namely the strong promoter of the glutamate dehydrogenase (Singer et al., 1998). The lack of unique Pac peptides could be due to a high similarity to peptides derived from *Giardia* proteins—what is, however, very unlikely—or to a rapid post-transcriptional silencing.

Consequently, each experiment investigating the role of a given transgene should contain an irrelevant transfection (e.g. with GusA) as a control. Untransfected WT trophozoites do not reflect the impact of the transfection and selection procedures on gene expression. So far, it is unclear how other genetic manipulations such as RNA virus-mediated transfection (Janssen et al., 2015) or CRISPR/Cas9 (Lin et al., 2019; McInally et al., 2019) affect gene expression in *G. lamblia*. Proteome studies should be performed with trophozoites transfected by these methods and investigate the impact on epigenetic and post-transcriptional regulation of gene expression by estimating the degree of antigenic variation. Further, more detailed studies comparing either different selection methods, or different control genes, or both, would be useful for helping researchers to determine methods that induce minimal proteomic perturbation. Indeed, the ideal vector control will induce minimal proteomic changes relative to wild-type untransfected cells. By including a reference transgene such as Gus A, expressing a clearly detectable, unique peptide may help to establish an internal standard, e.g. for comparative studies in different genetic backgrounds.

## DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD022565.

## AUTHOR CONTRIBUTIONS

MH and SB analyzed the trophozoite proteomes. NM and JM conceptualized the study, constructed and cultured the strains, and evaluated the results. JM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.602756/full#supplementary-material

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# Whipworm-Associated Intestinal Microbiome Members Consistent Across Both Human and Mouse Hosts

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The human whipworm *Trichuris trichiura* infects 289 million people worldwide, resulting in substantial morbidity. Whipworm infections are difficult to treat due to low cure rates and high reinfection rates. Interactions between whipworm and its host's intestinal microbiome present a potential novel target for infection control or prevention but are very complicated and are identified using inconsistent methodology and sample types across the literature, limiting their potential usefulness. Here, we used a combined 16S rRNA gene OTU analysis approach (QIIME2) for samples from humans and mice infected with whipworm (*T. trichiura* and *T. muris*, respectively) to identify for the first time, bacterial taxa that were consistently associated with whipworm infection spanning host species and infection status using four independent comparisons (baseline infected vs uninfected and before vs after deworming for both humans and mice). Using these four comparisons, we identified significant positive associations for seven taxa including *Escherichia*, which has been identified to induce whipworm egg hatching, and *Bacteroides*, which has previously been identified as a major component of the whipworm internal microbiome. We additionally identified significant negative associations for five taxa including four members of the order *Clostridiales*, two from the family *Lachnospiraceae*, including *Blautia* which was previously identified as positively associated with whipworm in independent human and mouse studies. Using this approach, bacterial taxa of interest for future association and mechanistic studies were identified, and several were validated by RT-qPCR. We demonstrate the applicability of a mouse animal model for comparison to human whipworm infections with respect to whipworm-induced intestinal microbiome disruption and subsequent restoration following deworming. Overall, the novel cross-species analysis approach utilized here provides a valuable research tool for studies of the interaction between whipworm infection and the host intestinal microbiome.

**Keywords: microbiome, intestinal microbiota, helminth, whipworm, animal model**

## INTRODUCTION

The intestinal microbiota has a significant impact on human physiology, and it has been demonstrated to modulate immune function, growth (Subramanian et al., 2014; Lim et al., 2015; Subramanian et al., 2015), metabolism, and overall health (Guinane and Cotter, 2013). Soil transmitted helminths (STH) residing in the host intestine can directly affect the immune system (Else, 2005; Altmann, 2009; Elliott and Weinstock, 2012) and may indirectly influence it by affecting the intestinal microbiota and the mucosa. Children are often exposed to infections (especially large roundworm and whipworm) prior to the stabilization of their microbiota (at around 3 years of age (Yatsunenko et al., 2012)), resulting in disruption of normal microbial community development and long-term dysbiosis and malnourishment due to reduced absorption of nutrients (Glendinning et al., 2014). Therefore, as a modifiable part of human "pan-genome", it may be possible to prudently manipulate an at-risk host microbiome through simple and inexpensive nutritional strategies (e.g. *via* inclusion of local fermented food) that supplement therapeutic use of anthelmintics. However, the role of commensal bacteria in STH infections is not well understood, although it is recognized that active cross-kingdom talk occurs.

Among the three most prevalent STHs (hookworm, large roundworm and whipworm), whipworm (*Trichuris trichiura*) infects over 289 million people mainly in Sub-Saharan Africa, India, China, East Asia, and South America, resulting in an estimated 212,700 years living with disability (YLD) (GBD, 2018). Whipworm eggs hatch to L1 stage in the large intestine (caecum and/or proximal colon), after which they penetrate the epithelium and form multicellular epithelial 'tunnels', which are poorly understood (Else et al., 2020). From there, they molt to adult stage, but extend their posterior end into the lumen while keeping the anterior end embedded in the epithelial layer (Else et al., 2020). Whipworm infections are correlated with impairments in growth and cognitive development in children (preschool and school-age), impairments in the health and well-being of women and increases in the risk of adverse maternal and neonatal outcomes (Nokes and Bundy, 1994). To reduce morbidity due to infections (Brooker et al., 2015), the US Agency for International Development and the World Health Organization (WHO) support mass drug administration (MDA) to treat at least 75% of all at-risk children by the year 2020 and to reduce maternal anemia by 50% by 2025 (World Health Organization, 2014). However, it is not expected that these measures will lead to sustainable control or elimination of whipworm infection, since prevalence remains high due to very high rates of reinfection post-treatment [82% (Jia et al., 2012)] and low and variable cure rates [~50% (World Health Organization, 2008)] especially after treatment with a single anthelmintic drug. Furthermore, anthelmintic drug resistance is a potential future risk for STH control programs, since benzimidazole resistance is already widespread in veterinary helminths (specifically against albendazole and mebendazole, which represent the primary drugs used for MDAs) (Kaplan, 2004). There is an urgent need to develop sustainable and integrated helminth control strategies as alternatives (or complementary) to MDA, especially in developing economies unable to support the necessary infrastructure and sanitation interventions.

There are a limited number of published papers studying human microbiome members associated with whipworm infections and these papers report disparate results. One study from Ecuador failed to identify significant associations between STHs (*T. trichiura* and *Ascaris lumbricoides*) and the fecal microbiome (Cooper et al., 2013); a study on Malaysian subjects identified several bacterial taxa associated with inflammation (Ramanan et al., 2016), and another study from Malaysia identified microbiome taxa (and blood markers) associated with whipworm infections (Lee et al., 2019). A study from Sri Lanka identified several taxa significantly associated with hookworm, whipworm, or roundworm infection (Jenkins et al., 2017). Animal models have also proven useful for studying whipworm infections, and previous studies have utilized mice models to study microbiome interactions with the mouse whipworm (*T. muris*) (Holm et al., 2015; Houlden et al., 2015; White et al., 2018; Schachter et al., 2020).

Unfortunately, translational research on the role of commensal bacteria in whipworm infections has been impeded by knowledge gaps in cross-kingdom molecular interactions across host species. For example, antibiotic treatment protects against the establishment of *T. muris* in mice, and bacteria are thought to provide molecules necessary for hatching of *T. muris* ova (Hayes et al., 2010). Conversely, bacteria and their products can enhance the mucosal barrier to protect from invading parasites, and support host repair mechanisms to reduce pathology, which collectively suggests the existence of active parasite-host-microbial interactions (Hayes et al., 2010; Dawson et al., 2020). In pigs, *T. suis* infection affects the abundance of ~13% of bacteria in the proximal colon in a worm burden-dependent manner (Wu et al., 2012). In a previous study, we analyzed microbiome assemblages during moderate and heavy STH infections in Indonesia and Liberia and for the first time identified specific members of the intestinal microbiome that discriminate between STH-infected and non-infected states across very diverse geographical regions using multiple statistical methods (Rosa et al., 2018). We also detected microbiome-encoded biological functions potentially associated with STH survival strategies. However, there has been little research to date that integrates datasets across species.

While previous results provide insight into cross-kingdom interactions, key mechanistic studies in both humans and model organisms are necessary to produce translational discoveries. We therefore urgently need a comprehensive understanding of common bacterial taxa that modulate or are altered by STH infection in humans as well as in relevant animal models. Here, we compare the microbiome of samples from humans with exclusive whipworm infections (Rosa et al., 2018) to newly generated samples from mice infected with whipworm. We use

**TABLE 1** | Gender and age metadata summary for human samples used in each comparison.

| Statistic | | Primary comparisons | | | Control comparisons | |
|---|---|---|---|---|---|---|
| | | Baseline Uninfected | Baseline Infected | Deworming | Uninfected, no treatment | Uninfected, anthelmintic treatment |
| Gender | Female | 24 | 14 | 8 | 10 | 9 |
| (# of individuals) | Male | 23 | 6 | 3 | 6 | 7 |
| Age (years) | Lowest | 4.2 | 4.7 | 5.1 | 5.0 | 4.9 |
| | Highest | 55.4 | 57.9 | 57.9 | 50.4 | 50.4 |
| | Average | 29.4 | 19.5 | 24.2 | 34.6 | 28.6 |

a 16S rRNA gene analysis pipeline to both datasets simultaneously to identify and compare bacterial taxa members consistently associated with whipworm infection (at baseline and following deworming) spanning human and mouse host species. A subset of taxa was validated by real-time quantitative PCR (RT-qPCR). This not only highlights the importance of these specific bacterial taxa but also highlights the usefulness of the mouse whipworm infection model for future mechanistic studies, holding potential for translational applications in humans.

# MATERIALS AND METHODS

## Sample Sources

For the mouse experiments, mixed sex C57BL/6N/STAT6KO mice were inoculated per os with 250 infective *T. muris* eggs. Mice were derived from breeding pairs of B6.129S2(c)-Stat6 (tm1Gru)/J purchased from Jackson Laboratories. After 35 days, stool samples were collected from 18 infected and 18 uninfected mice ("baseline" infection samples). Six of these infected mice were treated per os with a combination of mebendazole (Fluka, City, Country) used at 80 mg/kg body weight (solution prepared at 50 mg/ml in 10% Tween 20 + $dH_2O$) and ivermectin (used at 4 mg/kg body weight; Vetrimec 1%; MWI Animal Health, City, Country) to deworm them. Fecal samples were collected from these mice after an additional 19 days, on day 54 ("dewormed" samples). Control mice which were (i) uninfected and received no anthelmintic treatment, (ii) uninfected and received anthelmintic treatment, and (iii) infected and received no anthelmintic treatment (N=6 each) were also included in the dataset.

Human stool samples from Indonesia were retrieved for analysis from our previous study (Rosa et al., 2018) and collected as previously described (Wiria et al., 2010) (sequenced on the Genome Sequencer Titanium FLX [Roche Diagnostics, Indianapolis, Indiana] to an average of 6,000 reads per sample). Samples were collected in Flores island Indonesia between 2008 and 2010, and patients received albendazole treatment every 3 months from 2008 to 2010 (Rosa et al., 2018). Only the subset of these samples that were uninfected or exclusively *T. trichiura* infected were used for the "baseline" dataset (positive by the formol ether concentration method, with no other *Ascaris*, *Necator* or *Ancylostoma* infection as determined by RT-qPCR and egg counting (Rosa et al., 2018), and no evidence of protozoan parasites observed by the formalin ether concentration test; N = 20 infected and N = 47 uninfected). Only samples from individuals treated with albendazole (400 mg) who cleared the infection over the course of a 2 year treatment (every 3 months; eight total treatments) were used in the "dewormed" cohort (N = 11). This repeated treatment regime is necessary to effectively clear *Trichuris* infection (Vlaminck et al., 2020). The formol ether method is semi-quantitative, so before treatment, samples had moderate to high infection intensities. Additional human samples from this dataset were included as control samples for (i) uninfected and untreated (N=16) and (ii) uninfected and albendazole-treated (N=16). Gender and age metadata for individuals from each sample cohort are summarized in **Table 1**.

## 16S rRNA Gene Sample Sequencing and Analysis

The V1-V2 hypervariable region of the 16S rRNA gene was amplified by PCR (30 cycles) using forward and reverse primers "AGAGTTTGATCMTGGCTCAG" and "CTGCTGCCTY CCGTA" (respectively). Samples were assessed post amplification on the LabChip GXII (Caliper Life Sciences, Hopkington, Massachusets). 5nM dilutions of amplified samples were prepared and pooled for qPCR. PCR products were purified and sequenced on the MiSeq Genome Sequencer (Illumina, San Diego, California). Illumina paired reads were assembled using FLASH (v1.2.7) (Magoc and Salzberg, 2011) and low quality and chimeric sequences were removed using the UCHIME *de novo* tool (Edgar et al., 2011) from inside the Mothur package (v 1.37.5-64) (Schloss et al., 2009). The processed V1-V2 amplicons were clustered into OTUs (99% similarity for strain-level clustering) and classified using the developer's QIIME2 (Bolyen et al., 2019) docker container (qiime2/core:2018.8), using a classifier based on SILVA (release 132) (Quast et al., 2013), a comprehensive database that provides accurate annotations (Balvociute and Huson, 2017). 16S rRNA gene read sequences can be downloaded from SRA (BioProject PRJNA679627). Read counts were normalized per sample by dividing the number of reads associated with each OTU by the total number of reads assigned to any OTU. The taxonomic identifications used throughout the manuscript are the ones provided by SILVA (Quast et al., 2013). Complete OTU taxonomy, counts and relative abundance values per sample are provided in **Supplementary Table S1A–C**. The human and mouse samples generated during this study were statistically

compared in four comparisons (**Figure 1A**), each of which compared infected to uninfected individuals. For each host species, samples were compared at "baseline" (different individuals at the same timepoint, either infected only with whipworm, or not infected with any of the three main STH species) or they were compared before and after deworming with anthelmintic treatment (the same individuals at different time points). Control comparisons were performed between the first timepoint (corresponding to infected individuals at baseline) and the second timepoint (corresponding to dewormed individuals following anthelmintic treatment) and included uninfected individuals receiving no treatment (both species), uninfected individuals receiving anthelmintic treatment (both species) and infected individuals receiving no treatment (mouse only) (**Supplementary Figure S1**).

LEfSe (Linear discriminant analysis Effect Size) (Segata et al., 2011) was used for differential taxa abundance testing, using default recommended settings according to the author's instructions, at an adjusted $P \leq 0.05$ for significance and requiring an LDA effect size of at least 2 in order to identify differentially abundant taxa. LEfSe's algorithm performs class comparison tests, validates for biological consistency, and considers the hierarchy of the taxonomy to perform tests at all taxonomic levels. We have previously used this statistical approach to identify common helminth-associated taxa between human cohorts (for any of the three main STH species) (Rosa et al., 2018), and it has also been used to identify intestinal bacteria associated with pathogenic infections (Villarino et al., 2016), to identify intestinal biomarkers for disease (Segata et al., 2011), and to track microbiome recovery following disease (Rooks et al., 2014). Complete LEfSe statistics for each comparison are provided in **Supplementary Table S1D**.

Shannon index diversity values were calculated for each sample using the normalized read counts across all taxa using the "diversity" function in the "vegan" library in R (version 2.5), and Bray-Curtis dissimilarity values were calculated using the "vegdist" function. Multidimensional scaling (MDS) was performed using the same Bray-Curtis dissimilarity values, using the R function "cmdscale" (R version 4.0.2). Clustering with t-SNE was also performed using the "Rtsne" package in R (version 0.15) using the same Bray-Curtis dissimilarity values, in addition to Non-metric Multidimensional Scaling (NMDS) using the "bestnmds" function in the "LabDSV" package (version 2.0; http://ecology.msu.montana.edu/labdsv/R/). One-way analysis of variance (ANOVA) testing was performed with a Tukey HSD post-hoc test, for both the Shannon diversity and Bray-Curtis dissimilarity comparisons.

## RT-qPCR Analysis

Relative abundance of selected infection-associated taxa was measured by real-time quantitative PCR (RT-RT-qPCR) for the mouse samples and for a subset of the human samples from the 16S rRNA gene analysis for which sufficient template DNA was still available (nine individuals before and after deworming). All consumables were purchased from ThermoFisher Scientific (Waltham, MA, USA), unless otherwise specified. Reactions for *Blautia* were performed in a 25 µl set-up per sample using Power SYBR Green Master Mix, and a final concentration of 300 nM forward and reverse primers with 2 µl of stool DNA extract was run through 40 amplification cycles according to the standard master mix manufacturer's two-step protocol. *Lachnospiracea*, *Prevotella*, and *Collinsella* were detected and quantified using Taq Man Fast Advanced Mastermix in a 20 µl set-up. Both primers were used in a final reaction concentration of 400 nM and the concentration of the probe was 125 nM. Two microliters of stool DNA extract was used per reaction. All reactions were performed using a QuantStudio 6 Flex Real-Time PCR System. The cycling conditions were as follows: A pre-read stage of 30 s at 60°C, followed by 20 s at 95°C. The 40 cycles amplification consisted of a 1 s denaturation step at 95°C followed by 20 s annealing and extension at 60°C. The Post-Read stage is 30 s at 60°C. Each DNA sample was tested in duplicate against genus-specific and universal bacterial 16S rRNA gene primers (400 nM) (**Supplementary Table S2**). Cycle threshold (CT) values were calculated by taking the average CT for each sample and primer set. Relative fold change in bacterial abundance was calculated using the $2^{-\Delta\Delta CT}$ method using the 16S rRNA gene CT values as a calibrator. P values were calculated from the Δ CT values comparing before and after deworming samples using a two-tailed T test with unequal variance (Schmittgen and Livak, 2008).

# RESULTS AND DISCUSSION

Previous studies of associations among microbiome and whipworm infections have been performed in a single host species. To support translational discoveries, a comprehensive identification of common bacterial taxa protective from (and supportive of) infection across hosts is needed. Therefore, we studied microbiome responses to whipworm infection in a murine model and compared it to a human cohort from Indonesia from our previous study (Rosa et al., 2018). From the human dataset, we re-analyzed only those samples which were infected with *T. trichiura* and no other nematode species (N = 20 infected and N = 47 uninfected at baseline; N = 11 infected and dewormed with albendazole), so that we could provide the closest possible comparison to the *T. muris*-infected C57BL/6N/STAT6KO mice analyzed (N = 18 infected and N = 18 uninfected at baseline; N = 6 infected and dewormed). This mouse strain provides a relatively high and uniform worm burden throughout the treatment groups, and modulation of STAT6 is a predictor of resistance to gastrointestinal nematodes including *Trichuris* species in humans, pigs and "rewilded" mice that are affected by changes in the intestinal microbiome (Moller et al., 2007; Leung et al., 2018; Dawson et al., 2020). The human and mouse samples were analyzed using same pipeline and then statistically compared in four comparisons (**Figure 1A**). For each host species, samples were compared at "baseline" (different individuals at the same timepoint, either infected only with whipworm, or not infected with any helminth) or they were compared before and after

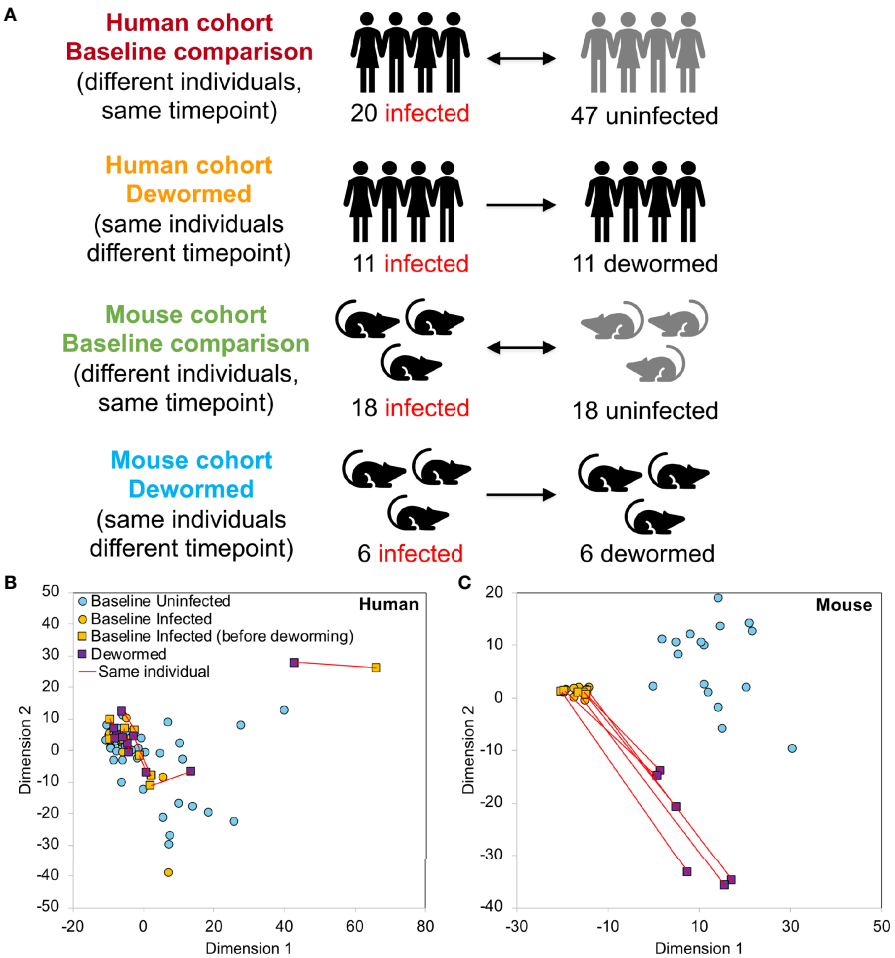



**FIGURE 1** | Overview of 16S rRNA gene sample sets. **(A)** Sample cohorts used for differential intestinal microbiome analysis comparing samples from *Trichuris trichiura*-infected and uninfected humans and *T. muris*-infected and uninfected mice before and after deworming. At "baseline", the microbiomes of different individuals were compared and in the "dewormed" comparisons, the microbiomes of same individuals before and after curing of the infection were compared. MDS-based clustering of samples based on their normalized microbiome abundance profiles are shown for human **(B)** and mouse **(C)**. Red lines connect samples from the same individuals before and after deworming. Normalization was performed by dividing OTU read counts by the total number of read counts assigned to OTUs in each sample.

curing whipworm infection (the same individuals at different timepoints).

Control comparisons across the deworming timepoints were also included (**Supplementary Figure S1**) for uninfected humans receiving no anthelmintic treatment (N=16), uninfected humans receiving albendazole treatment (N= 16), infected mice receiving no anthelmintic treatment (N=6), uninfected mice receiving no anthelmintic treatment (N=6), and uninfected mice receiving mebendazole/ivermectin treatment (N= 6).

## Microbiome Profile Overview and Analysis

MDS-based clustering of samples showed that while the overall human microbiome profiles did not have any observable correlation with whipworm infection status (**Figure 1B**), the mouse samples clustered separately by infection status (**Figure 1C**), with the dewormed samples clustering between the infected and uninfected samples. This pattern in microbiome profiles was expected since human samples have high variability due to differences in genders, ages, village locations, diets, and histories of infection status, while the mice were in a laboratory-controlled environment that ensured consistent microbiomes and therefore more consistent responses to infection. Similar clustering patterns were also observed with both NMDS and tSNE-based clustering (**Supplementary Figure S2A**), and human data did not cluster according to either gender or age (**Supplementary Figure S2B**). Control sample cohorts showed similar clustering profiles, with infected mouse samples clustering away from uninfected samples, regardless of timepoint or anthelmintic treatment (**Supplementary Figure S3**). Overall microbiome profiles in each sample for both human and mouse cohorts are summarized at the class level in **Supplementary Figure S4**.

The Shannon diversity index (**Figure 2A**) represents the within-sample or "alpha" diversity, reflecting the number and the evenness of the distribution of microbiome members. While

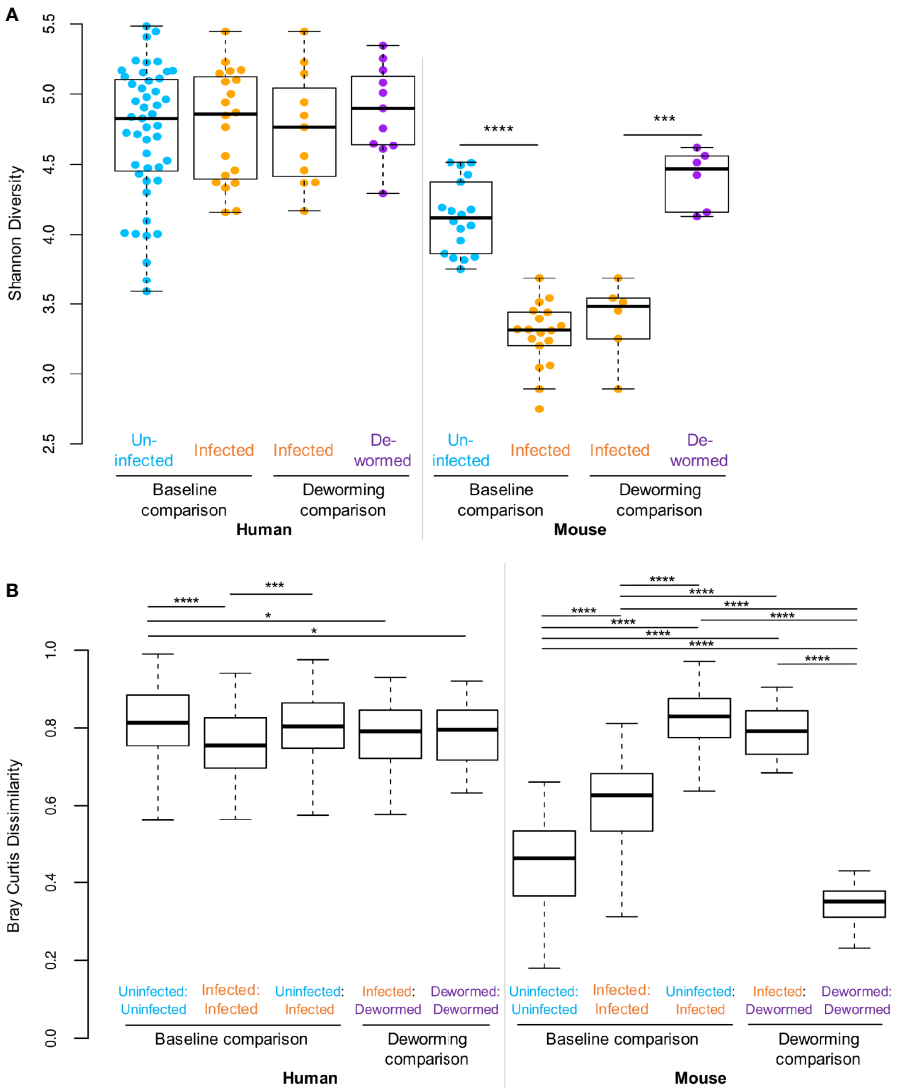



**FIGURE 2** | Statistical comparisons of diversity measures. **(A)** Shannon (alpha) diversity values for all samples in each sample set. **(B)** Bray-Curtis dissimilarity (beta diversity) values either between samples from the same sets, or between sample sets. *$P \leq 0.05$, ***$P \leq 0.001$, ****$P \leq 0.0001$, according to ANOVA with Tukey HSD *post-hoc* test. F-statistic values for human and mouse were 13.8 and 464.6, respectively ($P < 10^{-5}$ in both cases).

there were no significant differences between infection groups in the human cohort, the within-sample diversity was significantly lower ($P < 10^{-10}$, two-tailed T-test, unequal variance) in the whipworm-infected mice at baseline compared to uninfected mice, and this diversity was increased back to the same level as the uninfected samples following deworming ($P < 10^{-10}$). Bray-Curtis dissimilarity (**Figure 2B**) was used to quantify the between-sample differences in the overall microbiome profiles. The samples from infected humans had microbiome profiles that were significantly more similar to each other compared to samples from uninfected humans, or compared to dewormed subjects ($P < 10^{-10}$). However, in the more controlled laboratory setting, uninfected samples had significantly lower baseline diversity that was disrupted by whipworm infection, resulting in higher average dissimilarity between infected samples and uninfected samples, compared to within-infected and within-uninfected sample groups ($P < 10^{-10}$). Following deworming, the between-sample differences were very low in the mice, having lower diversity than between infected samples ($P < 10^{-10}$), and even lower diversity than between baseline uninfected samples ($P = 1 \times 10^{-5}$). These results suggest that whipworm-associated disruptions in the microbiomes resolved in similar ways in the dewormed samples. Relative abundance values per OTU per sample can be accessed in **Supplementary Table S1C**.

Using LEfSe (Segata et al., 2011), we identified significant differentially abundant microbiome taxa at all taxonomic levels in each of the four comparisons (baseline infected vs uninfected, and infected vs dewormed, in both humans and mice; **Figure 1A**). The number of significant differentially abundant OTUs is shown in **Table 2** and **Supplementary**

**TABLE 2 |** Summary of sample groups used for each statistical comparison, and the counts of the number of significant OTUs and taxa at any taxonomic level, based on LEfSe results.

| Comparison group | | Species | Samples in Group 1 | | Samples in Group 2 | | # OTUs significantly higher | | Total # taxa significantly higher | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Status | N | Status | N | Group 1 | Group 2 | Group 1 | Group 2 |
| Four primary comparisons | Baseline Comparison | Human | Uninfected | 47 | Infected | 20 | 43 | 88 | 58 | 116 |
| | | Mouse | Uninfected | 18 | Infected | 18 | 257 | 107 | 373 | 169 |
| | Deworming Comparison (paired) | Human | Infected | 11 | Dewormed | 11 | 23 | 18 | 39 | 18 |
| | | Mouse | Infected | 6 | Dewormed | 6 | 69 | 309 | 125 | 395 |
| Control comparisons (all paired) | Uninfected, no treatment | Human | Before | 16 | After | 16 | 1 | 0 | 5 | 0 |
| | | Mouse | Before | 6 | After | 6 | 20 | 47 | 27 | 71 |
| | Uninfected, anthelmintic treated | Human | Before | 16 | After | 16 | 0 | 0 | 0 | 0 |
| | | Mouse | Before | 6 | After | 6 | 41 | 56 | 59 | 90 |
| | Infected, untreated | Mouse | Before | 6 | After | 6 | 5 | 20 | 9 | 27 |

**Figure S5A**. Although none of the significant differentially abundant OTUs overlapped between the two host species, these OTUs represented variable 16S rRNA gene sequences from many overlapping species, genera, families, orders, classes, and phyla (**Supplementary Figure S5B–G; Supplementary Table S1D**). For the sake of simplifying the analysis of these comprehensive taxonomic results, we focused on the genera-level annotations of the significant OTUs. Genera were identified that were either significantly differentially abundant at the entire genera level, as well as those that were not significant but were represented by one or more OTUs that were significant in the comparison (**Figure 3A**). The four comparisons shown in **Figure 3A** correspond to the comparisons shown in **Figure 1A**, and the black-circled genera represent genera with at least one significant OTU in each of the four comparisons while the grey-circled genera represent genera with at least one significant OTU in each of the comparisons, excluding the dewormed human cohort, since this utilized a smaller sample set (N = 11) with a high noise background and weaker statistical signal. Each of these genera is discussed in detail below.

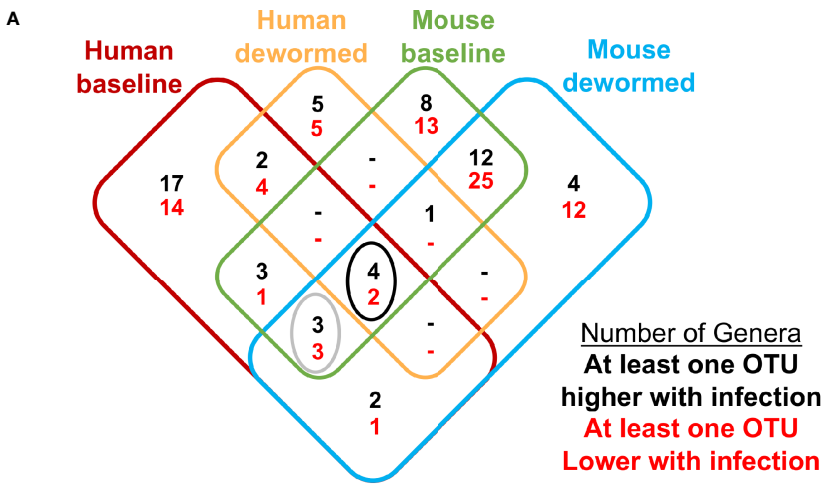
We have also included control LEfSe comparisons including (i) uninfected, untreated (humans and mice), (ii) uninfected, treated (humans and mice), and (iii) infected untreated (mice only) (**Supplementary Figure S1**). Overall results for these are shown in **Table 2**, complete LEfSe results for these five comparisons are shown in **Supplementary Table S1D**, and any overlaps between OTUs in control comparisons and the genera from the four primary comparisons will be highlighted in the text below.

## Microbiome Taxa Associated With Infection Across Human and Mice

Based on the results from the LEfSe analysis, four bacterial genera were represented by OTUs that were significantly more abundant in the samples of infected humans and mice compared to uninfected individuals, and which were less abundant following deworming (positively associated with infection). These included:

(i) *Escherichia/Shigella* (**Figure 4**) was represented by a single OTU in mice (species *Escherichia coli* Xuzhou21), but several OTUs in human. In a previous mouse whipworm infection study, *Escherichia* was one of just four genera significantly associated with infection (200-egg infection in Swiss Webster outbred mice, 45 days post-infection) (Schachter et al., 2020), and in another, *Escherichia* was only detectable following infection (20-egg infection, C57BL/6 mice, 35 days post-infection) (Holm et al., 2015). Here, we observed that it not only increased with infection in both mice and humans (at both the OTU and genera level), but also decreased following deworming in mice (OTU and genera levels) and humans (OTU level). In control uninfected anthelminthic-treated mice over the same time period, this genus and the same OTU significantly increased over time (the opposite of the pattern observed with deworming). This finding is particularly interesting since *E. coli* (spanning many different strains) can induce *T. muris* egg hatching *in vitro* (Vejzagic et al., 2015), so known associations exist between this bacterial genus and whipworm.

(ii) *Prevotella* 2 (**Figure 5**) and (iii) *Prevotella* 9 (**Figure 6**) were higher in whipworm-infected compared to uninfected individuals. For both *Prevotella* 2 and *Prevotella* 9, the entire genus was significantly associated with infection in both comparisons in mice, and for *Prevotella* 2, the baseline comparison in the humans was also significant for the entire genera. In both genera, there were several representative OTUs in each of the mouse comparisons which had zero detection in the sample from uninfected subjects but high detection in the samples from infected subjects, indicating a potential presence/absence relationship with whipworm infection (although low levels may be present below the detection limit of the experiment). In control samples, three OTUs and the entire genus for *Prevotella* 2 were significantly increased in uninfected treated mice over the testing time period (the opposite of the pattern observed in infected samples over the time period). In the same control comparison, one OTU for *Prevotella* 9 was significantly increased over the time period, and the entire genus was increased (but not signficantly). *Prevotella* was identified as




**B**

| Genus | Figure | Positively associated with whipworm infection: Human 16S Baseline # OTUs | Baseline Genus | De-wormed # OTUs | De-wormed Genus | Mouse 16S Baseline # OTUs | Baseline Genus | De-wormed # OTUs | De-wormed Genus | Negatively associated with whipworm infection: Human 16S Baseline # OTUs | Baseline Genus | De-wormed # OTUs | De-wormed Genus | Mouse 16S Baseline # OTUs | Baseline Genus | De-wormed # OTUs | De-wormed Genus |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Escherichia/Shigella* | 4 | 8 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | | | | | | | |
| *Prevotella* 2 | 5 | 2 | 1 | 1 | | 6 | 1 | 3 | 1 | | | | | | | | |
| *Prevotella* 9* | 6 | 11 | | 2 | | 6 | 1 | 3 | 1 | 4 | | | | 1 | | 2 | |
| *Streptococcus* | 7 | 5 | | 1 | | 1 | | 1 | 1 | 1 | | | | | | | |
| *Bacteroides* | 8 | 2 | | | | 5 | 1 | 6 | 1 | | | | | | | | |
| *Subdoligranulum* | 9 | 7 | | | | 1 | 1 | 1 | 1 | | | 2 | | | | | |
| *Collinsella* | 10 | 4 | | | | 1 | 1 | 1 | 1 | 2 | | 1 | | | | | |
| *Blautia* | 11 | 4 | | | | | | | | 4 | | 3 | | 5 | 1 | 9 | 1 |
| *Clostridium sensu stricto* 1 | S7 | | | | | 14 | 1 | 3 | 1 | 1 | | 1 | | 1 | | 2 | |
| *Lachnospiraceae* NK4A136 group | 12 | | | | | 1 | | 1 | | 1 | | | | 25 | 1 | 23 | 1 |
| *Ruminococcaceae UCG* 014 | S8 | 1 | | | | 1 | 1 | | 1 | 1 | | | | 5 | | 2 | |

**FIGURE 3** | Overview of differential taxa abundance results. **(A)** Venn diagram of differentially abundant genera counts across each comparison (corresponding to each of the uninfected vs infected comparisons outlined in **Figure 1A**), requiring at least one OTU belonging to the genera to be signficantly differentially abundant. Black circle = differentially abundant genera in each of the four comparisons, gray circle = differentially abundant genera in the human baseline, mouse baseline, and mouse dewormed comparisons, but not in the human dewormed comparison. **(B)** Genera from the circled regions of panel **(A)**, indicating the genera name, the corresponding figure number displaying full OTU abundance data, the number of OTUs significantly associated with whipworm infection, and whether the entire genus was significantly associated with infection. Lighter and darker shades of green and red are used to indicate lower/higher significant OTU counts (respectively) for each genus. **Prevotella* 9 was also one of the three genera downregulated in three comparisons (human baseline, mouse baseline and mouse dewormed comparisons, but not in the human dewormed comparison).

significantly more abundant in a previous mouse infection study with the small intestinal parasitic nematode *H. polygyrus bakeri* (200 iL3 infection in C57BL/6 mice, 14 days post-infection) (Rausch et al., 2013), but has also been observed to decrease in whipworm-infected C57BL/6 mice (41 days post-infection) (Houlden et al., 2015). However, unlike the current study which used a short-term high-infection model, this previous study (Houlden et al., 2015) used a chronic low-burden infection model, which may have caused some of the disagreement for this particular genus. In humans, *Prevotella* was significantly increased in helminth-infected (*Ascaris* and/or whipworm) individuals from Colombia (Toro-Londono et al., 2019). Prevotella represents one of the two enterotypes of the human intestinal microbiome (the other being *Bacteroides*) (Cheng and Ning, 2019), and it often dominates other genera in abundance, so significant shifts in its abundance may reflect overall disturbances in microbiome profiles induced by the whipworm.

(iv) *Streptococcus* (**Figure 7**) was represented by at least one significant OTU that was detected in samples from infected but not uninfected subjects in each of the four comparisons. In a previous study of whipworm-infected children from Ecuador, 10 of 50 infected children had microbiomes

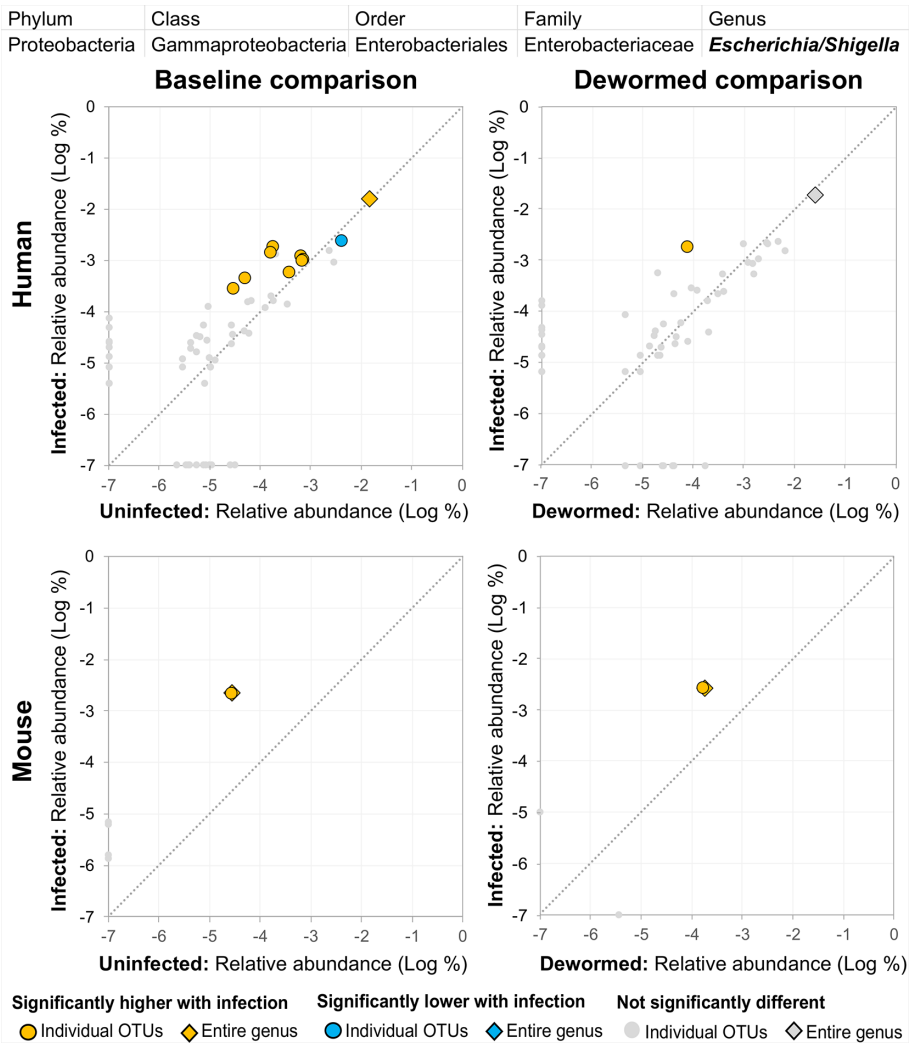



**FIGURE 4** | The relative abundance (%) of all *Escherichia/Shigella* (genus) OTUs in each differential comparison among samples from whipworm-infected and uninfected humans and mice. Significant differentially abundant OTUs in each comparison (as identified by LEfSe) are colored in orange (higher with infection) and blue (lower with infection). The sum of all OTUs in the genus is represented with a diamond symbol. This is one of four genera with at least one OTU significantly higher in infection in all four comparisons.

dominated by unusually high *Streptococcus*, which is not typically dominant in the intestines of healthy individuals (Cooper et al., 2013).

Three bacterial genera were represented by OTUs that were significantly more abundant in the intestines of infected humans and mice compared to uninfected individuals, and that were less abundant following deworming (positively associated with infection) in mice, but not in humans. These included:

(i) *Bacteroides* (**Figure 8**), for which many OTUs and the entire genus were significantly more abundant in the mice. In a previous mouse whipworm infection experiment, uninfected mice had undetectable levels of *Bacteroides*, but infected mice exhibited large amounts (200-egg infection in Swiss Webster outbred mice, 45 days post-infection) (Schachter et al., 2020), and *Bacteroides* is one of the major components of the *T. muris* microbiome (White et al., 2018). Here, we also identified two *Bacteroides* OTUs that were also significantly higher in whipworm-infected humans. Just one of the *Bacteroides* OTUs significant in the mice was also significant in one of the control comparisons (infected, untreated mice).

(ii) *Subdoligranulum* (**Figure 9**) was represented almost entirely by a single highly abundant OTU in infected but not uninfected mouse samples at baseline or after deworming, while seven relatively highly abundant OTUs were associated with infection at baseline in human. We previously associated *Subdoligranulum* with eventual self-clearing of helminth

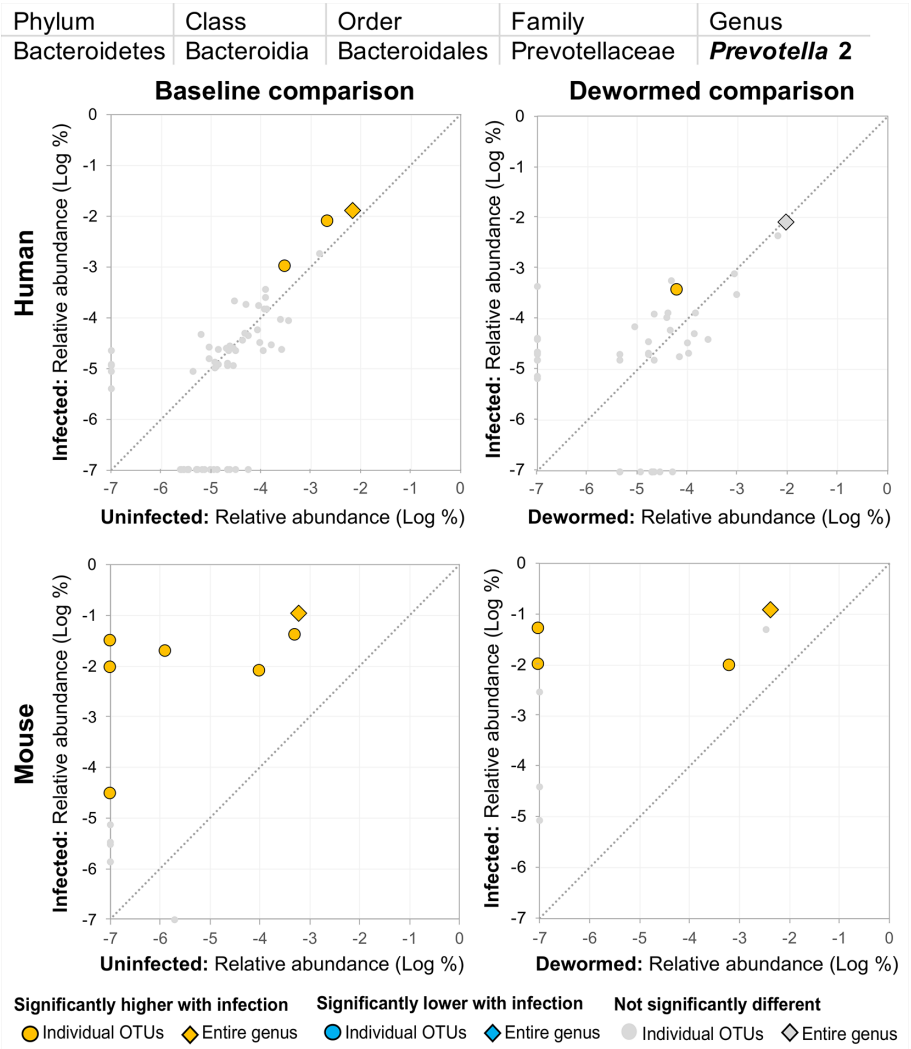



**FIGURE 5** | The relative abundance (%) of all *Prevotella* 2 (genus) OTUs in each differential comparison among samples from whipworm-infected and uninfected humans and mice. Significant differentially abundant OTUs in each comparison (as identified by LEfSe) are colored in orange (higher with infection) and blue (lower with infection). The sum of all OTUs in the genus is represented with a diamond symbol. This is one of four genera with at least one OTU significantly higher in infection in all four comparisons.

infections in samples from an Indonesia cohort (considering all three STH infections) (Rosa et al., 2018), but not otherwise associated with parasitic infections.

(iii) *Collinsella* (**Figure 10**) was significantly higher in both comparisons in mice, along with one significant OTU which was only detectable in samples from infected humans. We had also previously identified *Collinsella* as being associated with general helminth infection (inclusive of whipworm, *Ascaris*, and hookworm) in a human cohort from Liberia (Rosa et al., 2018), but it has not been associated with whipworm infection in other studies.

Overall, we consistently identified seven bacterial genera with representative OTUs that are significantly associated with whipworm infection, most of which have been identified in previous literature, and also demonstrated genera-level significant associations in both human and mouse host species (*Escherichia* and *Prevotella* 2 at baseline in humans, and all genera at both baseline and following deworming in mice, with the single exception of *Streptococcus* at baseline; **Figure 3B**). Most notably, we identified *Escherichia* as being positively associated in all four comparisons (baseline infected vs uninfected, and infected vs dewormed, in both human and mice), a genus which has known for functional interactions with whipworm.

## Microbiome Taxa Negatively Associated With Infection

Two bacterial genera were represented by OTUs that were significantly less abundant in the samples of infected humans

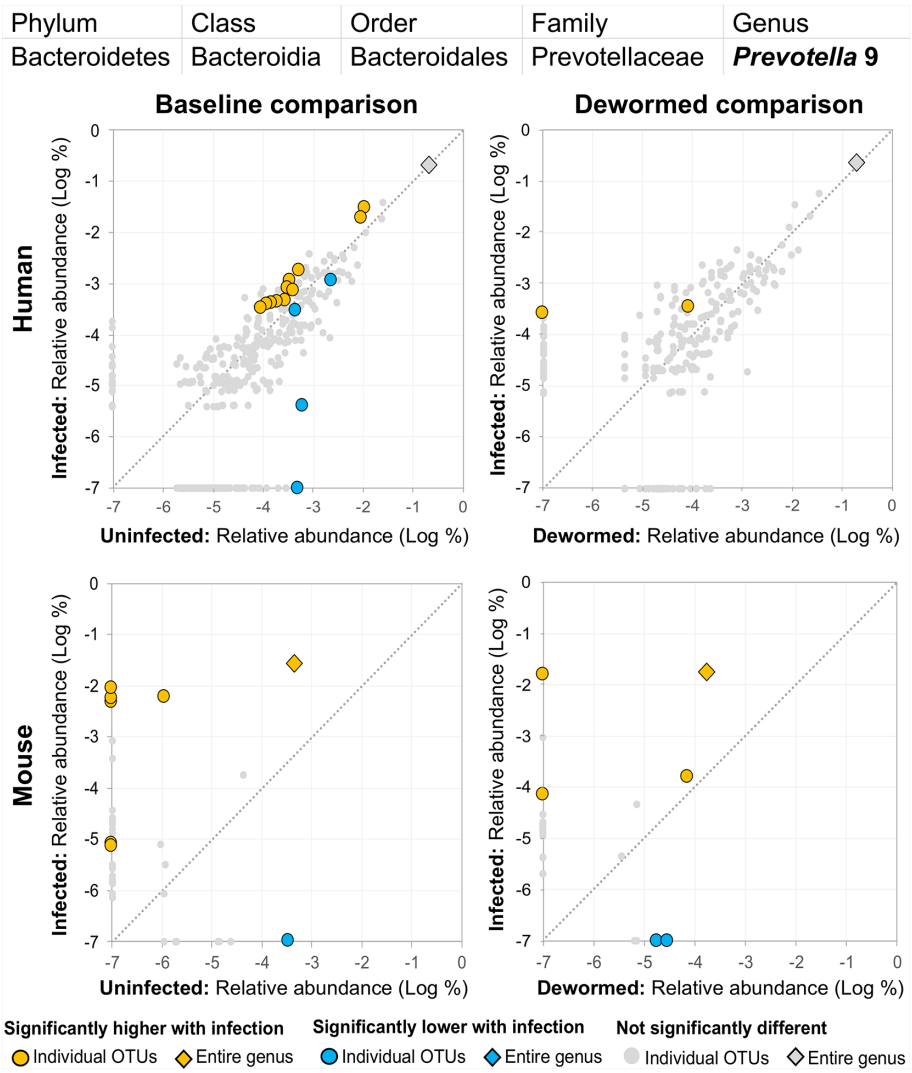



**FIGURE 6** | The relative abundance (%) of all *Prevotella* 9 (genus) OTUs in each differential comparison among samples from whipworm-infected and uninfected humans and mice. Significant differentially abundant OTUs in each comparison (as identified by LEfSe) are colored in orange (higher with infection) and blue (lower with infection). The sum of all OTUs in the genus is represented with a diamond symbol. This is one of four genera with at least one OTU significantly higher in infection in all four comparisons and is also one of three genera with at least one OTU significantly lower in infection in three of four comparisons (because different OTUs were both higher and lower in the same comparisons).

and mice, and which were more abundant following deworming (negatively associated with infection):

(i) *Blautia* (**Figure 11**), a member of the *Lachnospiraceae* family, was previously identified in a study of whipworm (*T. suis*) infection in pigs as one of the intestinal microbiome genera most significantly lower during infection compared to uninfected controls (2,000 eggs per pig, 21 days post-infection) (Li et al., 2012). Our previous study also identified *Lachnospiraceae* as negatively associated with the three major STH infections, in human cohorts from both Liberia and Indonesia (Rosa et al., 2018). Another previous study of humans from India noted a significant negative association with *Lachnospiraceae* OTU and whipworm infection (Huwe et al., 2019). We confirmed a significant negative association with whipworm infection by RT-qPCR in mice ($P = 0.005$; **Supplementary Figure S6**). *Blautia* in the human intestinal microbiome has been shown to be associated with visceral fat accumulation in adults (Ozato et al., 2019) and with reduced death from Graft-versus Host Disease (GVHD) (Jenq et al., 2015), but its functional association with helminths is not well understood. The results presented here highlight the need for future research into its interactions with both helminths and the intestinal microbiome of the host.

(ii) *Clostridium sensu stricto* 1 (**Supplementary Figure S7**) was negatively associated with infection for at least one OTU in

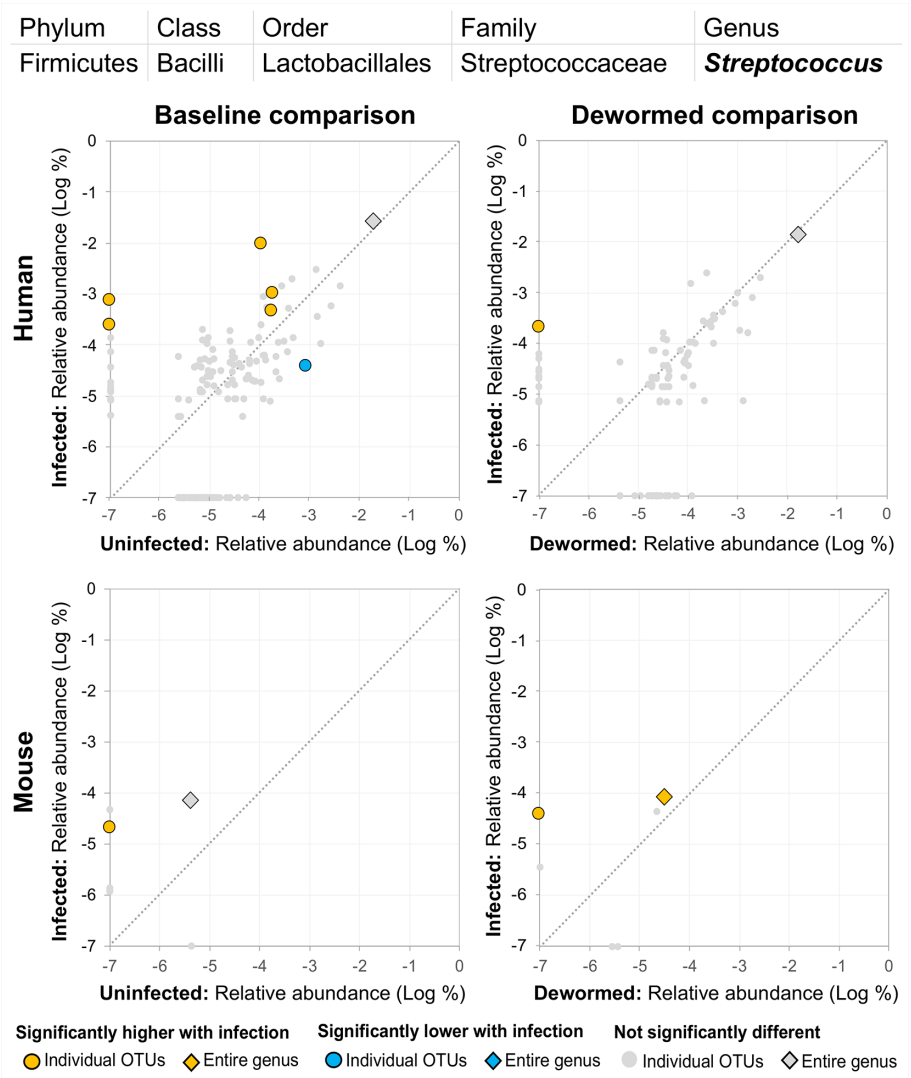



**FIGURE 7** | The relative abundance (%) of all *Streptococcus* (genus) OTUs in each differential comparison among samples from whipworm-infected and uninfected humans and mice. Significant differentially abundant OTUs in each comparison (as identified by LEfSe) are colored in orange (higher with infection) and blue (lower with infection). The sum of all OTUs in the genus is represented with a diamond symbol. This is one of four genera with at least one OTU significantly higher in infection in all four comparisons.

each comparison, however, among mice (but not humans), several OTUs and the genus as a whole showed a significant positive association with infection, both at baseline and following deworming, suggesting multiple roles for different species from this genus and an unclear relationship for the genus as a whole. Additionally, two OTUs from this genus showed lower abundance in the infected untreated mouse control cohort, further complicating its potential association with whipworm infection. In children from Ecuador, *Clostridium sensu stricto* was significantly less abundant in infected compared to uninfected individuals (Cooper et al., 2013).

Three bacterial genera were represented by OTUs that were significantly less abundant in the intestines of infected humans and mice compared to uninfected individuals, and significantly more abundant following deworming (negatively associated with infection) in mice but not significantly in humans:

(i) *Lachnospiraceae* NK4A136 group (**Figure 12**) is not described in association with intestinal parasitic infection in the literature, but is another member of the *Lachnospiraceae* family along with *Blautia* (described above). However, unlike for the other genera, there were many OTUs for *Lachnospiraceae* NK4A136 group which followed the same

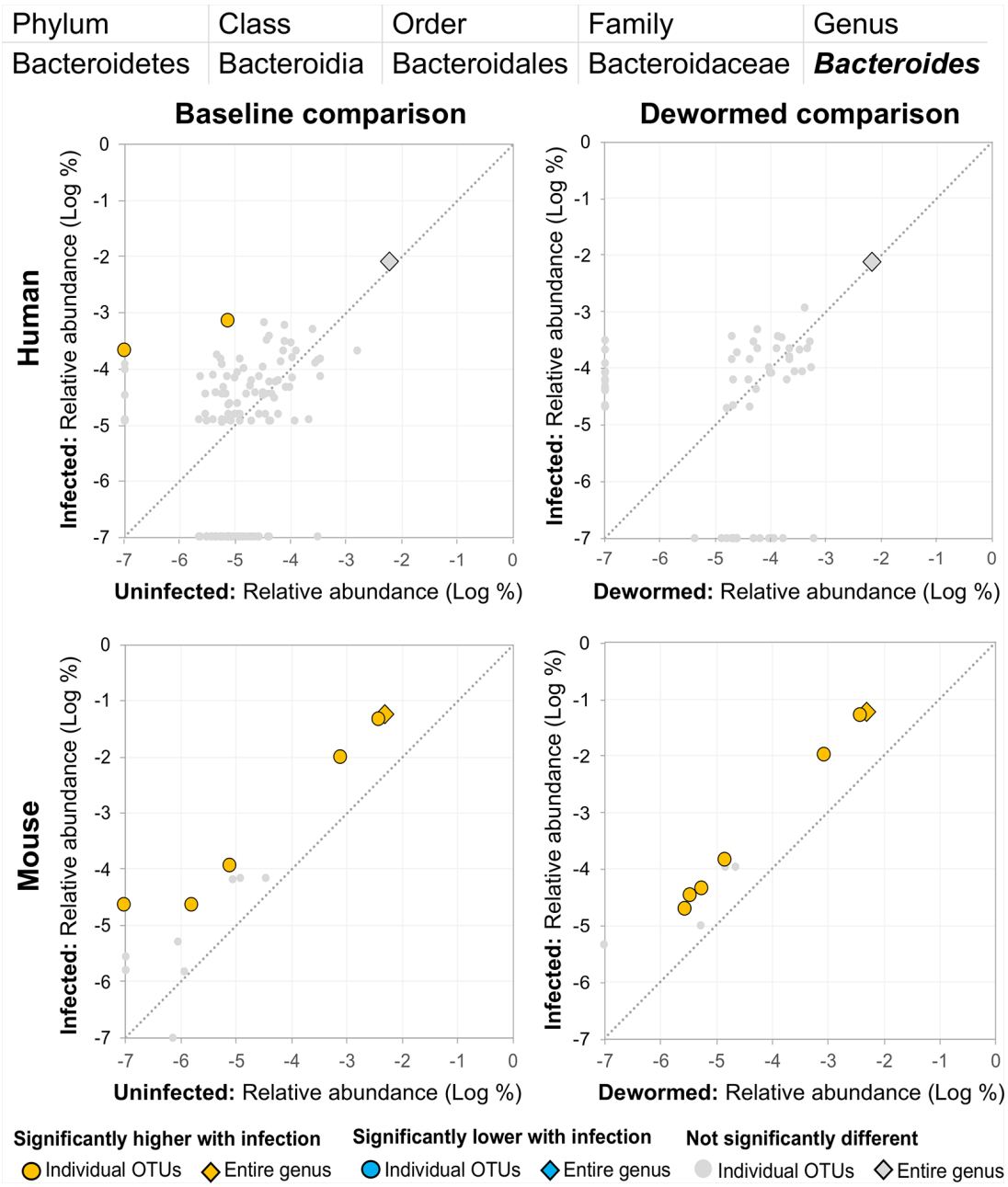



**FIGURE 8** | The relative abundance (%) of all *Bacteroides* (genus) OTUs in each differential comparison among samples from whipworm-infected and uninfected humans and mice. Significant differentially abundant OTUs in each comparison (as identified by LEfSe) are colored in orange (higher with infection) and blue (lower with infection). The sum of all OTUs in the genus is represented with a diamond symbol. This is one of three genera with at least one OTU significantly higher in infection in three of four comparisons.

trend over the time period in the control mouse samples (the entire genus and five OTUs in uninfected untreated mice, and six OTUs in uninfected treated mice, although no differences in the human control samples or in the infected untreated mice). The identification of two different *Lachnospiraceae* members (*Blautia* and *Lachnospiraceae* NK4A136) among just five genera overall that consistently showed a negative association with infection across species highlights the importance of this bacterial family, which has also been observed to have a significant negative association with whipworm infection among humans from Malaysia (Lee et al., 2019) and mice with chronic whipworm infections (represented by the species *Roseburia* (20-egg infection, C57BL/6 mice, 35 days post-infection) (Holm et al., 2015)).

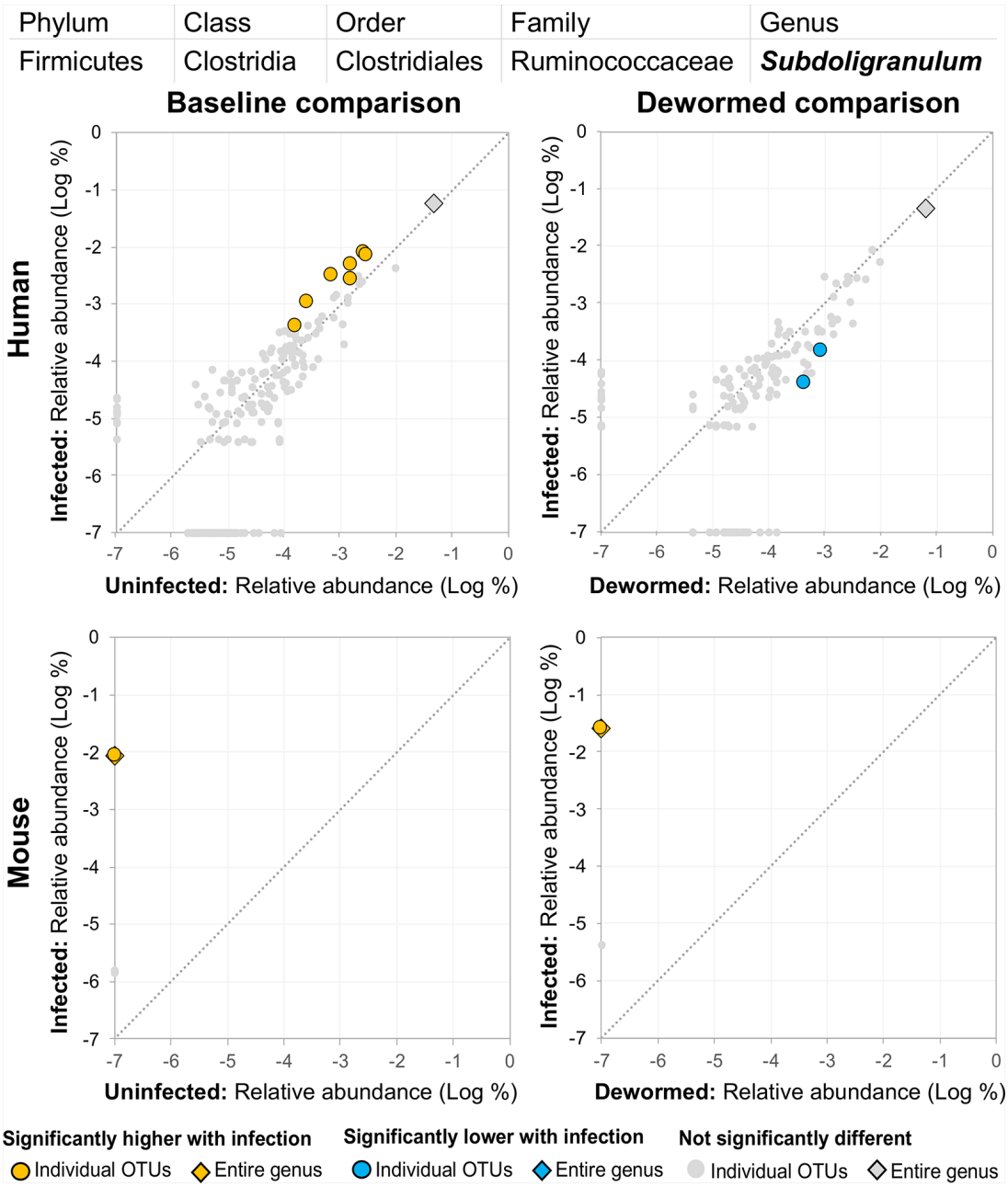



**FIGURE 9** | The relative abundance (%) of all *Subdoligranulum* (genus) OTUs in each differential comparison among samples from whipworm-infected and uninfected humans and mice. Significant differentially abundant OTUs in each comparison (as identified by LEfSe) are colored in orange (higher with infection) and blue (lower with infection). The sum of all OTUs in the genus is represented with a diamond symbol. This is one of three genera with at least one OTU significantly higher in infection in three of four comparisons.

(ii) *Ruminococcaceae* UCG 014 (**Supplementary Figure S8**), like *Clostridium sensu stricto* 1 (above), was significantly represented by individual OTUs that were negatively associated with whipworm infection, but the entire genus is positively associated with infection in mice, highlighting a complicated relationship with infection. One OTU also increased over the deworming time period in uninfected treated mice. *Ruminococcaceae* showed a significant negative association (after 27 days) with chronic whipworm infection in a previous mouse study (Holm et al., 2015).

(iii) *Prevotella* 9 was previously described here as being positively associated with infection (**Figure 6**). However, although

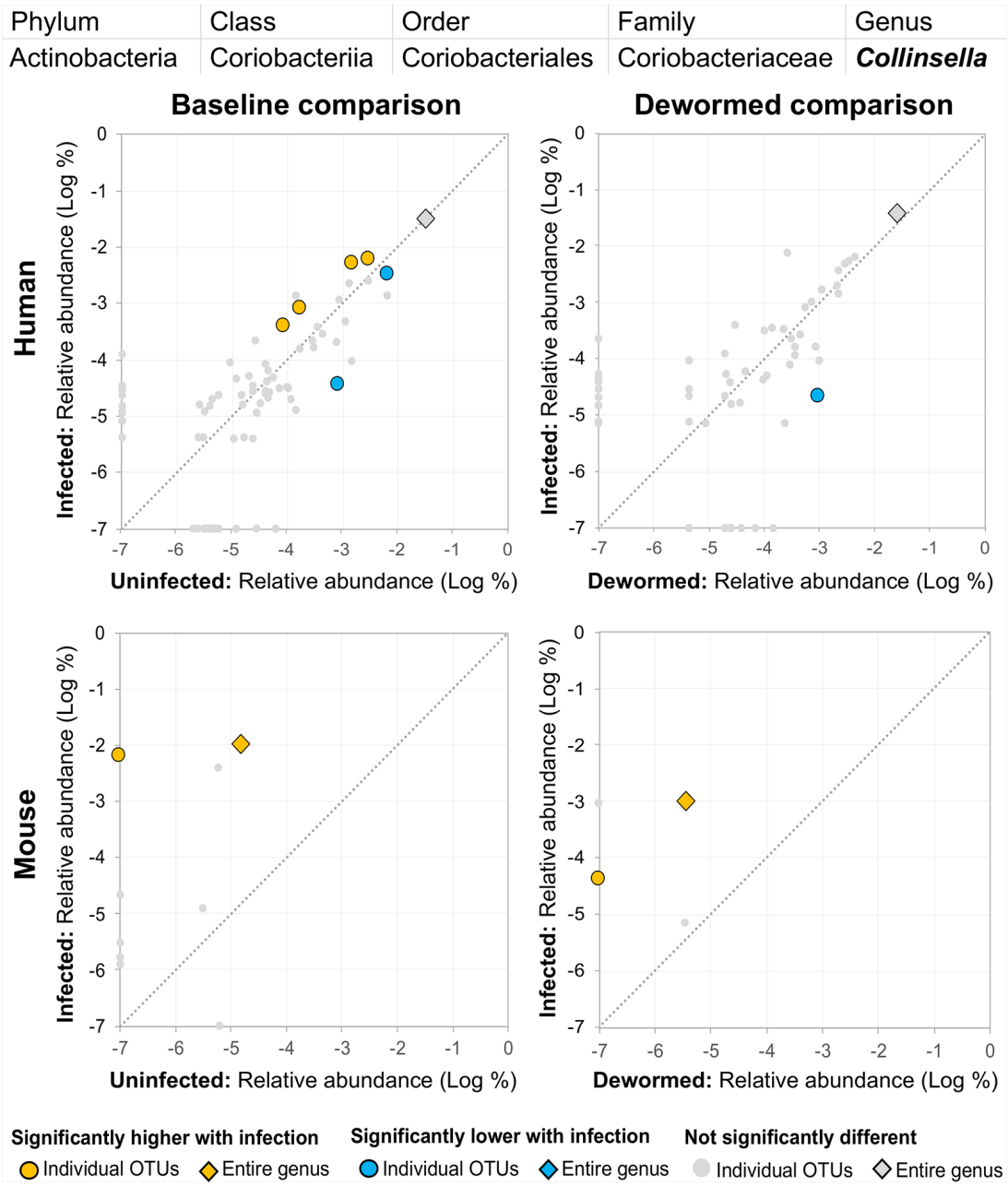



**FIGURE 10** | The relative abundance (%) of all *Collinsella* (genus) OTUs in each differential comparison among samples from whipworm-infected and uninfected humans and mice. Significant differentially abundant OTUs in each comparison (as identified by LEfSe) are colored in orange (higher with infection) and blue (lower with infection). The sum of all OTUs in the genus is represented with a diamond symbol. This is one of three genera with at least one OTU significantly higher in infection in three of four comparisons.

some OTUs from this genus were shown to have an overwhelmingly positive association with infection, at least one OTU in each of the three comparisons described for this genus were negatively associated with infection. This suggests the existence of different roles and functions for different species or strains within this genus.

A negative association with infection was consistently shown in both humans and mice for five taxa, four of which belong to the order *Clostridiales* (*Blautia*, *Clostridium sensu stricto* 1, *Lachnospiraceae* NK4A136 group, and *Ruminococcaceae* UCG 014), which had been described in association with infection in previous studies (as described above for individual genera). The

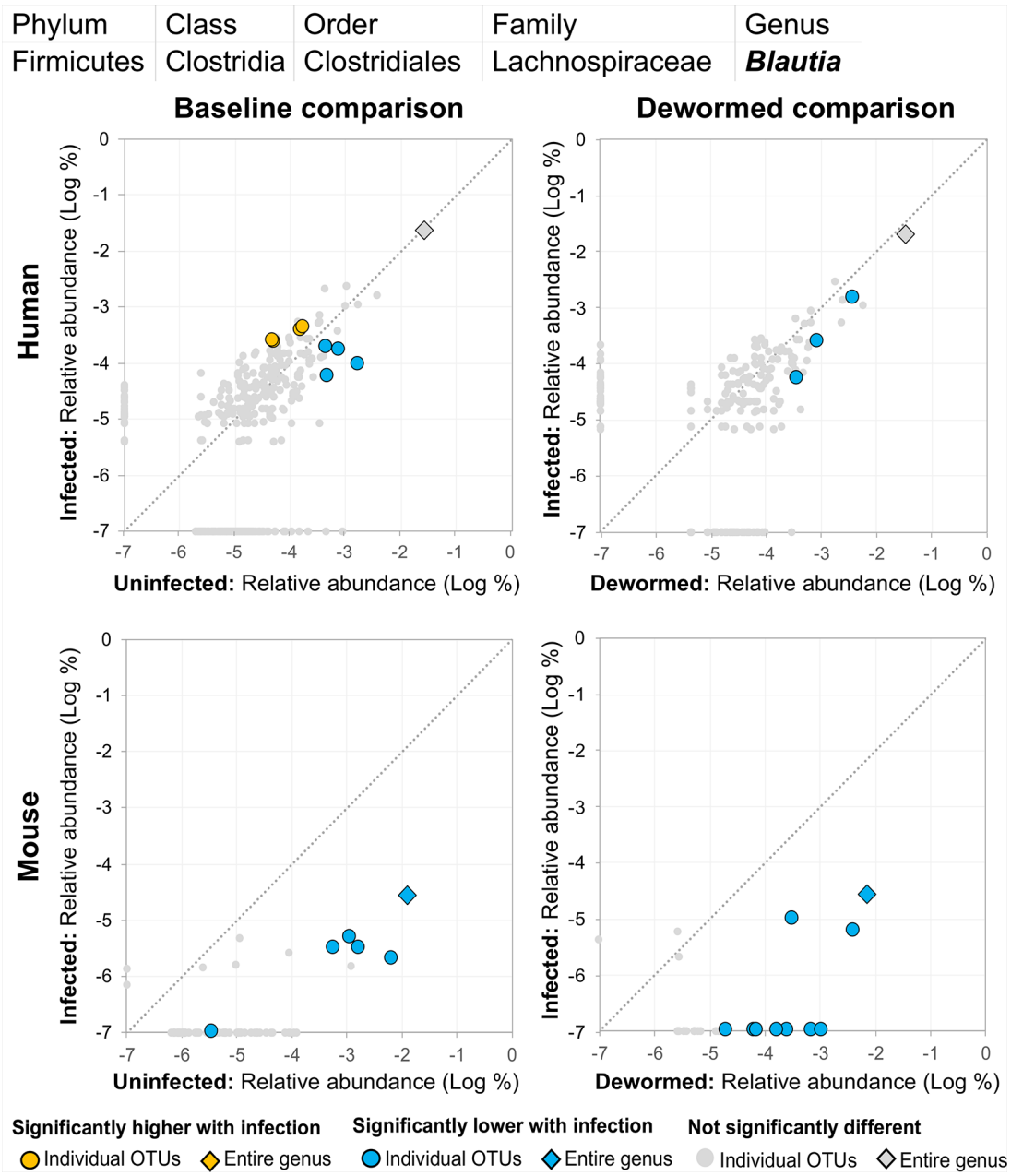



**FIGURE 11** | The relative abundance (%) of all *Blautia* (genus) OTUs in each differential comparison among samples from whipworm-infected and uninfected humans and mice. Significant differentially abundant OTUs in each comparison (as identified by LEfSe) are colored in orange (higher with infection) and blue (lower with infection). The sum of all OTUs in the genus is represented with a diamond symbol. This is one of two genera with at least one OTU significantly lower in infection in all four comparisons.

*Clostridia* class that *Clostridiales* belongs to was the only taxa found to be negatively associated with whipworm/*Ascaris* infections in school children from Ecuador (Cooper et al., 2013). Following albendazole treatment of STH (hookworm and *Ascaris*)-infected patients in Kenya, there was a significant increase observed in *Clostridiales* (Easton et al., 2019), supporting the post-deworming increase of the order that we observed. Here, we highlighted specific genera, particularly those from the *Lachnospiraceae*, that were negatively associated with whipworm infection, both before infection and after infection, many of which are supported by previous additional human and mouse studies (as described above for individual genera).

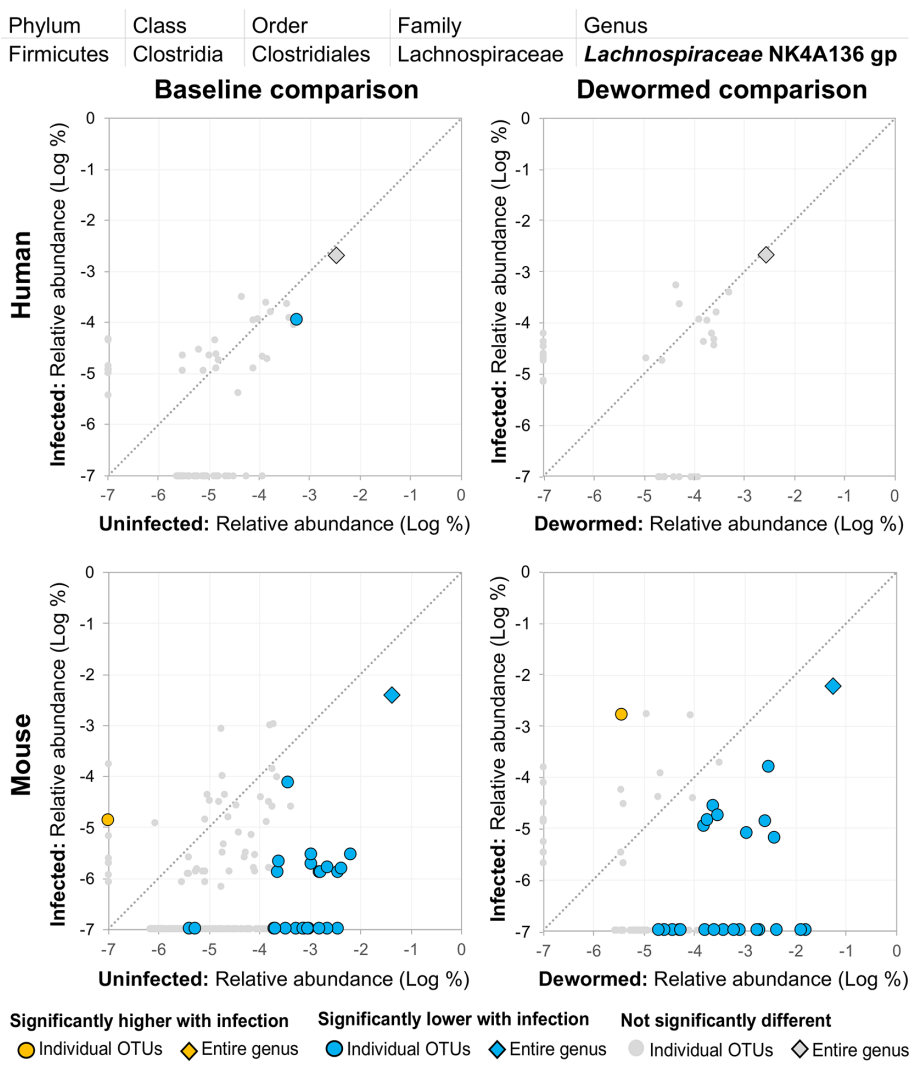



**FIGURE 12** | The relative abundance (%) of all *Lachnospiraceae* NK4A136 group (genus) OTUs in each differential comparison among samples from whipworm-infected and uninfected humans and mice. Significant differentially abundant OTUs in each comparison (as identified by LEfSe) are colored in orange (higher with infection) and blue (lower with infection). The sum of all OTUs in the genus is represented with a diamond symbol. This is one of three genera with at least one OTU significantly lower in infection in three of four comparisons.

## CONCLUSIONS

Interactions between the whipworm and the host intestinal microbiome where it lives are complicated and often identified inconsistently across the literature. Here, for the first time, we undertook a combined 16S rRNA gene OTU analysis to consistently identify bacterial taxa that were associated with infection of two closely related whipworm species in both human and mouse hosts. While many of the taxa identified by this analysis were consistent with the results of various human and/or mice studies in the literature, we validated these previous identifications by analyzing two host species in a combined bioinformatic analysis comparing different uninfected vs infected individuals at baseline as well as before and after deworming. Despite substantial differences in intestinal microbial ecology between these species, using this approach, we validated the use of murine whipworm infection as a model to evaluate whipworm-induced disruption in the human intestinal microbiome and subsequent restoration after deworming. While the specific mechanistic roles and host-parasite interaction consequences of identified microbiome genera are not necessarily conserved across the hosts, we identified 11 specific bacterial genera of interest for further association and mechanistic studies (particularly *Escherichia* and *Blautia*) which we suggest can be explored using a murine model of human whipworm infection. Overall, this novel approach to cross-species analysis provides a valuable tool to study the interaction between whipworm infection and the host intestinal microbiome.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the National Center for Biotechnology (NCBI) BioProject repository (https://www.ncbi.nlm.nih.gov/bioproject/), accession number PRJNA679627. **Supplementary Table S1** and **Supplementary Table S2** contain all relative abundance data per OTU per species, and all statistical comparison results.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Boards at Washington University School of Medicine in St. Louis, University of Liberia, Monrovia, and Ethical Committee for Research of the University of Indonesia, Jakarta. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by USDA Beltsville Area Institutional Animal Care Committee.

## AUTHOR CONTRIBUTIONS

MM, JU, and PF designed the study. BR and MM wrote the manuscript. JM processed and mapped 16S rRNA reads. BR and CS performed bioinformatic analysis. BR prepared figures and tables. JU performed mouse experiments and EB performed DNA extractions. TS and LG produced the human datasets. KF and JK performed RT-qPCR experimentation. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.637570/full#supplementary-material

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# Control Strategies for Carcinogenic-Associated Helminthiases: An Integrated Overview

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Helminthiases are extremely prevalent in the developing world. In addition, the chronic infection with some parasitic worms are classified as carcinogenic. Therefore, it is utmost importance to understand the parasite-host interactions, the mechanisms underlay carcinogenesis and how they could be counteracted. This knowledge may ultimately guide novel control strategies that include chemotherapy-based approaches targeting these pathogens and associated pathologies caused by their infections. Little is known on how some helminthiases are associated with cancer; however, it has been hypothesized that chemical carcinogenesis may be involved in the process. Here, we summarize the current knowledge on chemical carcinogenesis associated with helminthiases, along with available therapeutic options and potential therapeutic alternatives including chemotherapy and/or immunotherapy. Ideally, the treatment of the carcinogenic helminthiases should target both the parasite and associated pathologies. The success of any chemotherapeutic regimen often depends on the host immune response during the infection and nutritional status among other factors. The close association between chemotherapy and cell-mediated immunity suggests that a dual therapeutic approach would be advantageous. In addition, there is a pressing need for complementary drugs that antagonize the carcinogenesis process associated with the helminth infections.

**Keywords: helminths, carcinogenesis, chemotherapy, immunotherapy, antioxidants**

## HELMINTH INFECTIONS AND CANCER

More than 10% of all cancers in the developing world are believed to be associated with infections (IARC, 2012). Whereas the infection with viruses as human papilloma virus (HPV), hepatitis C and D virus (HCV, HDV) (Ji et al., 2012; Yi and Yuan, 2017) and some bacteria are well-established biological carcinogens, helminthiases associated with malignancy remain largely unexplored (Blattner, 1999; Parkin, 2006; Brindley et al., 2015; Mentis et al., 2019). Infections with the blood fluke, *Schistosoma haematobium* (*S. haematobium*) and the Asian liver flukes, *Opisthorchis viverrini*

(*O. viverrini*) and *Clonorchis sinensis* (*C. sinensis*) have been classified as Group 1 biological carcinogens: definitive causes of cancer according to International Agency for Research in Cancer (IARC), (IARC, 2012). Some close relatives of these parasites, e.g. *Schistosoma japonicum* (Group 2b) and *Opisthorchis felineus* (Group 3) are not classified as definitive biological carcinogens (Vennervald and Polman, 2009; IARC, 2012). Yet, recent findings have indicated that infection with the European liver fluke *O. felineus* may eventually lead to cholangiocarcinoma (CCA) (Gouveia et al., 2017; Pakharukova et al., 2019; Fedorova et al., 2020) and that infection with *S. japonicum* may be a risk factor for colorectal cancer (Wu et al., 2020). Curiously, chronic infections with related trematodes, the blood flukes *Schistosoma mansoni* and the liver fluke *Fasciola hepatica* have not been classified as biological carcinogens. These observations prompt questions related to the mechanisms underlying carcinogenesis during the helminth infection: how might these infections trigger cancer? (Brindley et al., 2015). Infections with parasites are recognized as both biological and chemical insults to host tissues (chemical carcinogenesis promoters) leading to inflammation, fibrosis, and changes in tissue microenvironment (Brindley and Loukas, 2017; Gouveia et al., 2017).

## CHEMICAL CARCINOGENESIS MAY BE RESPONSIBLE FOR HELMINTH INDUCED MALIGNANCY

Chemical carcinogenesis (ChC), as Experimental Science, started in 1915 by Yamagiwa and Ichikawa who reproduced the carcinogenicity of coal tar in rabbit skin (Yamagiwa and Ishikawa, 1917). Later on, Elizabeth C. Miller and James A. Miller showed that ChC may occur through direct interaction of electrophilic compounds with the DNA (Miller and Miller, 1981). Several environmental factors, including physical (e.g., ionizing radiation), biological (e.g., viral pathogens), and chemicals underly the development of several human cancers (Minamoto et al., 1999). Recently, Ercole L. Cavalieri, Eleanor G. Rogan and collaborators hypothesized that catechol estrogen-3,4-quinones (CEQ) can initiate cell transformation by reacting with DNA. The production of DNA adducts creates apurinic sites in the DNA (Cavalieri et al., 1994; Cavalieri et al., 2006; Cavalieri and Rogan, 2011) and/or oxidizes the DNA. If this metabolism becomes unbalanced and generates excessive CEQ, the formation of CEQ-DNA adducts would consequently increase (Cavalieri et al., 1994; Cavalieri and Rogan, 2011). We have previously identified estrogen-like metabolites in both sera and urine from *S. haematobium*-infected patients, and parasite extracts (Botelho et al., 2013; Gouveia et al., 2015). Similarly, oxysterols were identified in *O. viverrini* and *O. felineus* (Vale et al., 2013; Gouveia et al., 2017). In both cases, tentative products derived from the interaction between parasitic-derived metabolites (e.g. estrogen- and oxysterol-like metabolites) and host DNA were identified, i.e. DNA-adducts in patients with urogenital schistosomiasis and rodent model of opisthorchiasis. These findings suggest that chemical-mediated carcinogenesis processes may underlie, at least partially, the helminthiases-associated malignancies. They also support the hypothesis that reactive oxysterol-like metabolites and oestrogen-like precursors of presumably parasite origin may be genotoxic to the host genome (Correia da Costa et al., 2014; Gouveia et al., 2015; Santos et al., 2015; Gouveia et al., 2017).

In addition to oestrogen-derived metabolites acting as chemical toxins, recent findings suggest estrogen receptors may also be involved in urogenital schistosomiasis (UGS)-associated carcinogenesis. Differential gene expression of the estrogen receptor α (ERα) is evident between UGS-associated bladder cancer and non-UGS-associated bladder cancer (Bernardo et al., 2020). There is a direct correlation between the ERα expression levels, tumor proliferation and expression of p53. The expression of ERα has been also related with the presence of parasitic eggs in bladder. On the other hand, ERβ is widely expressed in both non-UGS- and UGS-associated bladder cancer. Remarkably, these findings were supported by proteomic studies on *S. haematobium* parasites and, moreover, the *in vitro* activation of ERα promotes cell proliferation in ERα-expressing bladder cancer cells (Bernardo et al., 2020).

We hypothesized that the interaction of estrogen-like metabolites with host DNA may have a potential role in inducing dysregulation of p53 during UGS (Vale et al., 2017). Bladder tissue from schistosomiasis patients expressed p53 (**Figure 1**) and most likely it is mutated without capacity to repair DNA (Santos et al., 2015). *S. haematobium* eggs express sialyl-Lewis, sLea and sLex antigens, in mimicry of human leukocytes glycosylation, and may play a role in cancer metastasis (Santos et al., 2015). Recently, we reported the effects of culturing human epithelial cells established from normal urothelium (HCV29) and normal cholangiocytes (H69), in the presence of *S. haematobium* or *S. mansoni* eggs. Intriguingly, the estrogen receptor and β-estradiol were predicted to be altered in urothelial cells exposed only to *S. haematobium* but not *S. mansoni* eggs. In addition, genes involved in the p53 pathway were downregulated when exposed to eggs from species of both schistosomes (Nacif-Pimenta et al., 2019). Concerning liver flukes, a comparison between the mutation profiles between opisthorchiasis-associated CCA and non-opisthorchiasis-associated CCA reveals a significant higher number of mutations in p53, along with other somatic and epigenetic lesions, in the former compared to the latter (Jusakul et al., 2015; Jusakul et al., 2017). Whereas the full complement of metabolites released by this parasite remains generally to be investigated (Brindley and Loukas, 2017), a granulin secreted by the liver fluke, termed Ov-GRN-1, has been studied in depth for several years now (Smout et al., 2015; Dastpeyman et al., 2018). This protein induces proliferation of cholangiocytes, the cell of origin of CCA. Remarkably, it was recently reported that infection of hamsters with gene-edited liver flukes, mutated by CRSPR/Cas programmed editing of the granulin gen locus, resulted in

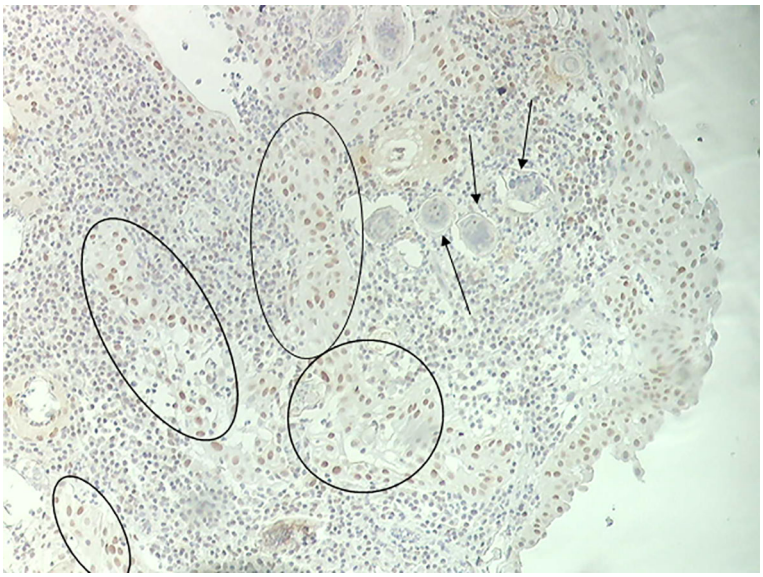

**FIGURE 1** | Immunohistochemistry of bladder mucosa evidencing p53 tissue deposition (black circles) and *S. haematobium* eggs (black arrows).

markedly reduced pathology (Arunsan et al., 2019). Besides the risk factors mentioned above, infections with schistosomes disturb the host microbiota leading to intestinal dysbiosis before oviposition (Jenkins et al., 2018). In addition, the original composition of host microbiota might affect the susceptibility to schistosome infection (Córtes et al., 2020). In the context of UGS-bladder cancer, the urinary microbiome might be relevant for biomarker discovery to advance novel diagnostics and treatment (Adebayo et al., 2017). In rural regions of Thailand and Laos where opisthorchiasis and opisthorchiasis-associated CCA are highly endemic, traditional dietary practices involved eating of raw fish, and hence repeated exposure to liver flukes, and consumption of nitrosamine-contaminated food, particularly fermented fish products, represent the major risk factors for CCA (Sripa et al., 2007; Siriraj et al., 2016). Additional risk factors that are now coming under closer scrutiny include the carriage of *Helicobacter* species and other microbiome changes within the biliary tract that influence the inflammatory milieu (Sripa et al., 2018). A new paradigm has been proposed that indicates that most cancers originate from biological or chemical stimuli followed by chronic inflammation, fibrosis, and changes in the tissue microenvironment that eventually lead to a pre-cancerous lesion (Brücher and Jamall, 2014). It is reasonably believed that that paradigm could reflect the progression of UGS and associated bladder cancer. Or more likely, knowing that cancer is a multifactorial diseases (Clavel, 2007), the risk factors noted above may act in concert during the development of SCC-associated to UGS (**Figure 2**). Accordingly, therapeutic programs against carcinogenic helminthiases should consider a dual approach: treatment against both the parasite and associated pathology, including pre-carcinogenic lesions (**Figure 2**).

## CAN CHEMOTHERAPY AND IMMUNOTHERAPY COMBINED BE AN EFFICIENT OPTION TO TREAT HELMINTHIASES AND ASSOCIATED PATHOLOGIES?

Currently, praziquantel (PZQ) is used to treat schistosomiasis and opisthorchiasis, and currently employed in mass drug administration (MDA) programs across endemic regions (Cioli and Pica-Mattocia, 2003; Fenwick et al., 2003; Utzinger and Keiser, 2004; Caffrey, 2007). Of concern, parasite isolates collected in the field with decreased sensitivity to PZQ have been reported (Ismail et al., 1996; Fallon, 1998; Pica-Mattocia and Cioli, 2004; Melman et al., 2009; Crellen et al., 2016). Although PZQ can eliminate the parasites, it does not reverse the pathological sequelae after the chronic infection. Altogether, the evidence points to the need for novel therapeutic approaches against parasites. In many cases, the new drugs or derivatives exhibit lower therapeutic potency (Utzinger et al., 2011). Alternative approaches, e.g., drug repurposing, combination of drugs or other active agents and immunotherapy would be promising for the treatment of these helminthiases. Ideally, their treatment would not only treat the infection, by eliminating the parasite and ameliorating associated disease, and in addition, thwart the development of malignancy.

Combinations of drugs have been used in other infectious diseases such as malaria, and tuberculosis (Kerantzas and Jacobs Jr, 2017; Mokhtari et al., 2017; Alven and Aderibigbe, 2019). Using drugs with diverse modes of actions may be advantageous and render the treatment more effective than single drug. In the context of helminth infections, using a combination of two drugs, one active

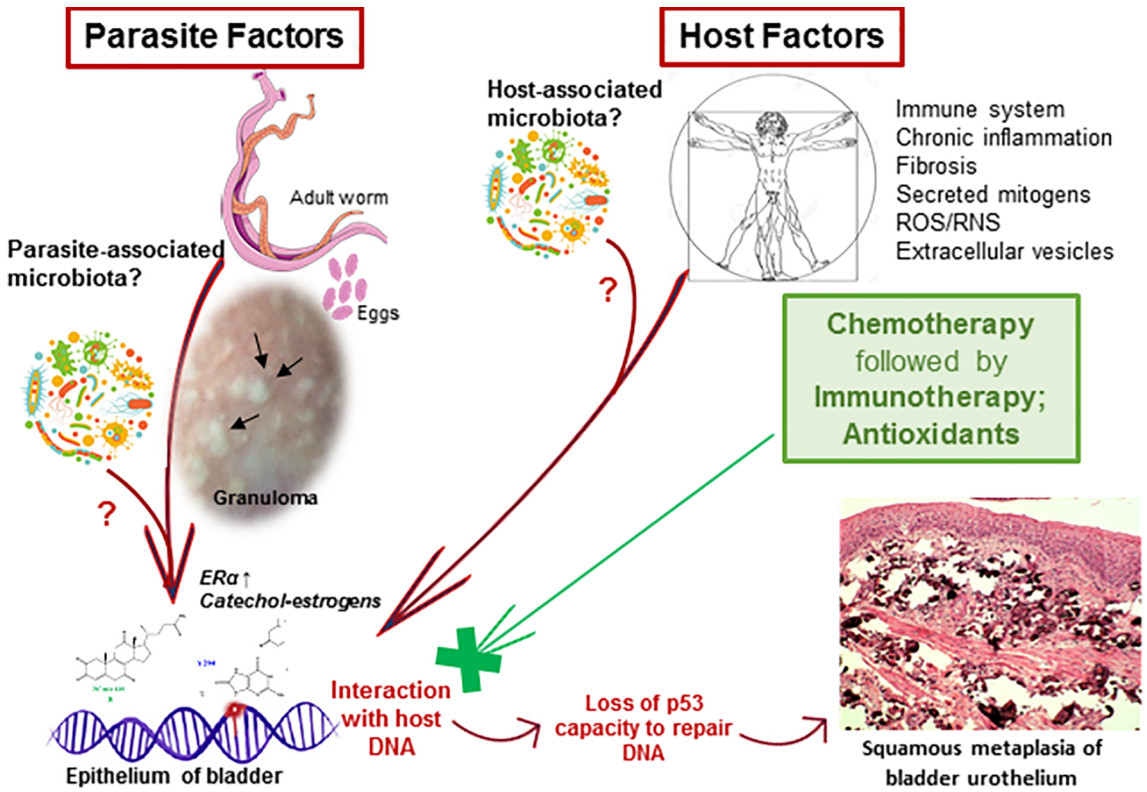


**FIGURE 2** | Schematic depiction of hypothesized carcinogenesis induced by urogenital schistosomiasis (UGS). Several risk factors may play a role in the onset of UGS-associated bladder cancer. Both parasite- and host-associated factors may be involved during carcinogenesis. Chronic inflammation, fibrosis and granulomas (black arrows) induced by the eggs of the parasite may have a role in carcinogenesis. Also, mitogens and reactive metabolites of schistosome origin (e.g., catechol estrogens) could interact with host DNA to form adducts and to induce apurinic sites. These could eventually lead to p53 mutations that consequently lost the ability to repair DNA. Therefore, the mutations may accumulate and ultimately underlie the development of UGS-associated squamous cell carcinoma. Co-factors, such as a tentative microbiota associated with the parasite (Formenti et al., 2020), host microbiota dysbiosis during the infection, and other host-associated factors such as the formation of ROS/RNS during chronic inflammation, and fibrosis, tobacco smoke, gender, age and impaired immune system, are known to influence the development of bladder cancer. Laboratory studies have suggested that the administration of antioxidants may counteract the interaction of reactive metabolites with host DNA, tentatively, preventing the development of pre- and carcinogenic lesions and may contribute to the amelioration of the pathogenesis associated to the UGS.

against adult worm and the other against larval stages should be more effective to eliminate the parasite. Several clinical trials have been evaluated the combination of antiparasitic drugs for UGS and compared with single dose (Pugh and Teesdale, 1983; Wang et al., 2004; Keiser et al., 2010; El-Beshishi et al., 2013; Keiser et al., 2014; Gouveia et al., 2018). Most trials confirmed that a combined regimen is more effective than a single treatment leading to an elevated cure rate (Gouveia et al., 2018). Our research group reported an increase of the PZQ and artesunate (AS) efficiency against larval stages of the parasite when combined with antioxidants *N*-acetylcysteine (NAC) and resveratrol (RESV) (Gouveia et al., 2019a). The use of antioxidants either alone or combined with drugs might be valuable for therapy of helminthiasis-induced malignancy. During schistosomiasis and opisthorchiasis, alterations of cellular antioxidant systems originated during the host immunological response have been described, with the production of reactive oxygen species (ROS) (Maizels et al., 1993; Gharib et al., 1999). The protective effects of NAC and resveratrol (RESV) against host tissue fibrosis maybe due to the inhibition of genotoxic metabolites produced by the parasite, that could eventually initiate cell transformation that leads to SCC and CCA (Seif el-Din et al., 2011; Douiev et al., 2017). Indeed, *in vitro* studies demonstrated that RESV and NAC inhibited the formation of these potentially parasitic genotoxic metabolites (Gouveia et al., 2019b). Thus, antioxidants would be an attractive therapeutic option to counteract reactive xenobiotics arising from oxidation (Soliman et al., 2008; Charoensuk et al., 2011), and inflammatory responses directed at schistosome eggs (Seif el-Din et al., 2011). The combination of one drug that exerts anthelmintic activity with antioxidants might improve biochemical, pathological, and immunological parameters associated to the infection (see Gouveia et al., 2018).

Other advantages of using combinatorial therapy include the delay of drug resistance development, synergy of action and hence a tentative reduction of the therapeutic doses required (Chen and Lahav, 2016). However, there are some limitations of combinatorial therapy such as potential increase of cytotoxicity (Humpfrey et al., 2011). In the specific case of the use of antiparasitic drugs with

antioxidants, this is not expected since antioxidants are considered very safe agents (Saso and Firuzi, 2014). Nonetheless, this should be evaluated in future studies.

Novel control strategies based on immunotherapy against UGS and opisthorchiasis are being tested. These involved the prevention and/or treatment of disease with drugs that stimulate the immune response (Naran et al., 2018). This could be achieved by either the exploitation of parasite-derived antigens or the administration of drugs or other active compounds. Hundreds of schistosome antigens have been studied as promising vaccine candidates. Remarkably, four vaccines (Sh28GST, Sm-14, Sm-TSP-2, and Sm-P80) are currently at differing clinical phases (reviewed in McManus, 2020). The most promising parasite-derived antigen is schistosome 28-kDa glutathione S-transferase of *S. haematobium* (Sh28GST, Bilhavax) that currently is in phase 3 of human clinical trials (Riveau et al., 2018). This candidate induces a strong mucosal immune response associated with Th2-type and regulatory IL-10 cytokines either in animal model, or in human (Riveau et al., 2012; Riveau et al., 2018). Hypothetically, one of the most effective way to control the disease may be a combined action between the use of drugs such as PZQ and vaccines. In a recent study, a simulation was performed to compared the effect of mass drug administration (MDA) alone with vaccination plus MDA against schistosomiasis. The findings indicate that vaccination accompanied with MDA would accelerate and prolong the impact by reducing the reinfection rate and the number of eggs released by residual worms (Alsallaq et al., 2017; Kura et al., 2019). In case of opisthorchiasis, a recombinant protein termed rOv-LEL-TSP-2 of the large extracellular loop of tetraspanin-2 of *O. viverrini* was evaluated in a rodent model increasing levels of several Th1 type cytokines (Phung et al., 2019) and extracellular vesicles (EVs) resulted in partial of protective efficacy against *O. viverrini* infection (Chaiyadet et al., 2019). To the best of our knowledge, vaccines for opisthorchiasis or clonorchiasis have not been evaluated in human trials yet.

The use of antioxidants have been evaluated in animal model of schistosomiasis and opisthorchiasis, either alone or in combination with antiparasitic drugs. This scheme of treatment may enhance the host immune response against the infection and, in parallel ameliorate associated pathologies (Allam, 2009; Aires et al., 2012; Wonchalee et al., 2013; Kamel and El-Shinnawy, 2015; Sheir et al., 2015).

Despite these encouraging findings further studies are needed. It is important to note that the immune response observed in animal models depends on the rodent species and strain and there is a possibility that any given response in the rodent model may not mirror the pathophysiological and immunological events in humans (Herati and Wherry, 2018). Therefore, ideally more reliable models of these diseases to perform immunologic studies are warrant. Also, the identification of key immunological targets should be pursued as well as the role of Th1 and Th2 responses and the balance between these two require further elucidation (McManus, 2020). We believe that the combination of chemotherapy and immunotherapy should be further investigated. Additionally, immunotherapy could play a valuable role in prevention or treatment of cancers induced by infections. This approach has been considered in the treatment of several other cancers (Farkona et al., 2016).

Thus far, it is unclear which components of *S. haematobium* eggs are pro-oncogenic, but it is known that eggs alone without the presence of adult worms could trigger the inflammatory response and cell proliferation (Fu et al., 2012; Nacif-Pimenta et al., 2019). Notably, Santos et al. (2015) outlined findings that provide insights on the glycosylation patterns of *S. haematobium* eggs and speculate about a possible model for the recruitment of eggs to the bladder wall. These observations led to important questions: Has the parasite evolved glycosylation patterns that mimic those of the human host? Would these insights represent informative guides to develop novel therapeutic strategies, namely glycoconjugate vaccines?

## Concluding Remarks—The Critical Need for an Integrated Approach

Combined chemotherapy alone or in association with vaccines, will develop into novel therapeutic approaches. However, it is unlikely that they will achieve sustainable medium- to long-term control for schistosomiasis and opisthorchiasis *per se*. A better understanding of socioecological context of parasite transmission and links of schistosomiasis with poverty are crucial to achieve effective control programs (Utzinger et al., 2003; Singer and Castro, 2007; Bruun and Aagaard-Hansen, 2008; Parker et al., 2008; Utzinger et al., 2009; Gray et al., 2010; Tangkawattana and Sripa, 2018). Consensually, such programs need to include education, snail control, better access to clean water and sanitation. Incorporate local resources and leadership to improve and enhance the existing health system may be critical (Utzinger et al., 2011). In this regard, China provides a good example of what can be achieved by implementing an integrated and sustainable control strategy against schistosomiasis. Combining experience among all the actors involved, using local resources for control and implementing an integrated control approach adapted to the local ecological settings will be crucial. This will ultimately lead to a reduction or elimination of the transmission of schistosomiasis and other helminthiasis, and the severe associated pathologies including cancer (Utzinger et al., 2005; Wang et al., 2008; Wang et al., 2009; Gray et al., 2010; Spiegel et al., 2010).

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

JC conceptualized and wrote the first draft of the manuscript. MG, PB, GR, JS, and LS reviewed and suggested alterations that were included in the manuscript. All authors contributed to the article and approved the submitted version.

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# Modulation of Rumen Microbes Through Extracellular Vesicle Released by the Rumen Fluke *Calicophoron daubneyi*

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Parasite derived extracellular vesicles (EVs) have been proposed to play key roles in the establishment and maintenance of infection. *Calicophoron daubneyi* is a newly emerging parasite of livestock with many aspects of its underpinning biology yet to be resolved. This research is the first in-depth investigation of EVs released by adult *C. daubneyi.* EVs were successfully isolated using both differential centrifugation and size exclusion chromatography (SEC), and morphologically characterized though transmission electron microscopy (TEM). EV protein components were characterized using a GeLC approach allowing the elucidation of comprehensive proteomic profiles for both their soluble protein cargo and surface membrane bound proteins yielding a total of 378 soluble proteins identified. Notably, EVs contained Sigma-class GST and cathepsin L and B proteases, which have previously been described in immune modulation and successful establishment of parasitic flatworm infections. SEC purified *C. daubneyi* EVs were observed to modulate rumen bacterial populations by likely increasing microbial species diversity *via* antimicrobial activity. This data indicates EVs released from adult *C. daubneyi* have a role in establishment within the rumen through the regulation of microbial populations offering new routes to control rumen fluke infection and to develop molecular strategies to improve rumen efficiency.

**Keywords:** ***Calicophoron daubneyi*, extracellular vesicle, proteomics, rumen microbiome, mass spectrometry**

## INTRODUCTION

Paramphistomes, commonly known as rumen fluke, have been found to infect ruminant animals worldwide (Huson et al., 2017; Huson et al., 2018). Within tropical and sub-tropical regions, rumen fluke infections cause significant production losses; yet only in recent years have rumen fluke infections been observed throughout Europe with the major species responsible confirmed as *Calicophoron daubneyi* (Sanabria and Romero, 2008; Jones et al., 2017). Clinical disease *via* adult rumen fluke is rarely reported in temperate areas, but mortality to large burdens of immature parasites has been observed in adolescent sheep and cattle (Mason et al., 2012; Millar et al., 2012).

Parasitic helminths establish long-term infections by manipulating the immune system in order to create an anti-inflammatory environment within the host (Coakley et al., 2016; Maizels and McSorley, 2016). In recent years parasite extracellular vesicles (EVs) have been recognized as key components of this strategy by transporting immunomodulatory cargo molecules (Marcilla et al., 2012). To date, there is fragmented understanding of the mechanisms underpinning EV activity with respect to immune-modulation and successful establishment of infection (Coakley et al., 2016). EVs appear the major route for macromolecule exportation from parasitic helminths, with some EVs even containing host mimicking components (Marcilla et al., 2012). EVs released by several helminth parasites have been found to deliver bioactive molecules and miRNA to host cells where they modulate host gene expression and suppress cytokine formation (Buck et al., 2014). The packaged cargo is developmentally regulated likely allowing parasite migration and establishment within the definitive host (Marcilla et al., 2012; Montaner et al., 2014; Cwiklinski et al., 2015). EVs from parasitic flatworms contain a number of established immune modulating proteins, such as FhGST-S1, the Sigma class GST (Prostaglandin synthase) from *F. hepatica* (LaCourse et al., 2012; Davis et al., 2019).

EVs have been confirmed to be released from the rumen fluke *C. daubneyi* (Huson et al., 2018). However, these EVs are yet to be studied at a molecular level or in relation to their effects on rumenal microbes. Previous studies of helminth parasites have shown they interact with their hosts gut microbiota in order to successfully establish infection whilst interrupting the 'healthy' microbiome that ultimately promotes the hosts health (Jenkins et al., 2018). With this in mind it is notable that relationships between gut microbiota and parasites are not fully resolved. However, evidence suggests the microbiotas involvement in regulation of the immune system ensuring appropriate responses to pathogenic organisms. However, evidence suggests the microbiotas involvement in regulation of the immune system ensuring appropriate responses to pathogenic organisms (Gensollen et al., 2016). Currently, studies into domestic livestock's microbiota in response to helminth infections remain limited and inconsistent (Peachey et al., 2019). Specifically, the rumen microbiota has been extensively studied due to the importance of rumen microbes in the nutrition and health of the animal (Petri et al., 2013; Chaucheyras-Durand and Ossa, 2014). Growing evidence suggest the rumen microbiome is involved in a complex and intimate dialogue with the immune and metabolic functions of the host (Zaiss and Harris, 2016). Owing to the links between rumen microbiota and animal health, disturbances in the rumen ecosystem may hinder rumen functionality and lead to disease in the host (Zaiss and Harris, 2016), with studies showing a causal link between natural and experimental infections of parasitic helminths with qualitative and quantitative alterations to the intestinal microbiota in a variety of animal species (Walk et al., 2010; Broadhurst et al., 2012; Li et al., 2012; Cantacessi et al., 2014; Lee et al., 2014). Here we unravel the proteomic profile of adult *C. daubneyi* EVs and explore the EV impact on the complex microbial environment contributing to their successful establishment within the host.

# METHODS

## *Calicophoron daubneyi* Collection and *In Vitro* Maintenance

Adult *C. daubneyi* were retrieved from naturally infected bovine rumens post-slaughter in a local abattoir (mid-Wales, UK). Following collection, *C. daubneyi* were washed in phosphate buffered saline (PBS), pH 7.4, at 39°C to remove contaminating materials. Flukes were divided into batches of 30 adults and placed in 1 ml/fluke DME culture media (supplemented with 2.2 mM Ca, 2.7 mM $MgSO_4$, 61.1 mM glucose, 1 μM serotonin and gentamycin (5 μg/ml), 15 mM HEPES), 39°C for 6 hours. Subsequently, both flukes and DME media were snap frozen in liquid nitrogen and stored at -80°C.

## EV Purification

Prior to differential centrifugation and size exclusion chromatography EV purification, media was submitted to centrifugation at 300 × *g* for 10 minutes at 4°C, followed by centrifugation at 700 × *g* for 30 minutes at 4°C to remove residual debris.

## Differential Centrifugation (DC)

*C. daubneyi* maintenance media was utilized in order to purify EV populations through differential centrifugation (DC) as previously described (Davis et al., 2019). Media was centrifuged at 120,000 × *g* for 80 min at 4°C in an Optima L-100 XP ultracentrifuge (Beckmann Coulter, High Wycombe, UK). The resulting pellet was washed in 5 ml PBS, pH 7.4 and submitted to 0.2 μm syringe filtering before the centrifugation step being repeated. The resulting pellet was suspended in 500 μl PBS and stored at -20°C.

## Size Exclusion Chromatography (SEC)

*C. daubneyi* maintenance media was utilized in order to purify EV populations through size exclusion chromatography (SEC) as previously described (Davis et al., 2019). Media was concentrated using Amicon ultra-15 centrifugal filters (Merk, Millipore), with a 10 kDa MW cut off. Samples were added to the centrifugal unit and centrifuged at 4000 × *g* for 20 min at 4°C until an approximately 500 μl EV enriched sample remained. EV enriched samples were 0.2 μm filtered and a maximum of 500 μl passed through qEVoriginal SEC columns (IZON science, U.K) following the manufacturers protocol. Briefly, columns were equilibrated with a minimum of 10 ml of PBS prior to addition of the sample. The initial 2.5 ml flow through was discarded with the following 2.5 ml EV enriched fraction retained and stored at -20°C.

## Quantification Using Tunable Resistive Pulse Sensing

A Nanopore NP200 (IZON Science) was utilized in the quantification of SEC purified EV samples. The Nanopore was calibrated using calibration particles (CPC200, 1:1000 filtered PBS). EV samples were measured at 47 mm nanopore stretch at a 100 nA voltage under 7 mbar pressure. Particles were detected

through short pulses of current and the resulting data analyzed using qNano particle analysis software (IZON, version 3.2).

## Transmission Electron Microscopy (TEM)

DC and SEC purified EVs were fixed onto formvar/carbon coated copper grids (Agar Scientific) for TEM analysis following the manufacturer's instructions. Briefly, 10 µl of EV-enriched sample was added to each grid and incubated for 45 min on ice. Grids were placed on the meniscus of 4% w/v uranyl acetate for 5 min on ice. Grids were then stored for a minimum of 24 hours at room temperature prior to visualization on the TEM (Jeol JEM1010 microscope at 80 kV), with EV presence confirmed through size selective criteria (30 – 200nm).

## EV Proteomic Analysis

EV proteins were determined through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970). Protein concentration was first determined using a Qubit protein assay following the manufacturer's instructions (Thermo Scientific, UK). Loading concentrations of 10 µg were aliquoted and centrifuged at 100,000 × *g* at 4°C for 30 minutes (S55-S rotor, Sorval MX120 centrifuge, Thermo scientific) with the resulting supernatant discarded. The EV pellet was suspended in 10 µl loading buffer and heated for 10 min at 95°C before loading into hand-cast 7 cm x 7 cm 12.5% polyacrylamide Tris/glyceine gels and subject to electrophoresis on a Protean III system (Bio-Rad, UK). Tris/Glycine/SDS buffer (25 mM Tris, 192 mM Glycine, 0.1% w/v SDS pH 8.3) (BioRad, U.K) was utilized for electrophoresis, with gels run at 70 V through the stacking gel and 150 V until completion. Gels were fixed (40% v/v ethanol and 10% v/v acetic acid) for one hour prior to overnight staining with colloidal Coomassie Brilliant Blue (Sigma, UK) at room temperature with gentle agitation. De-staining was achieved using 30% (v/v) methanol, 10% v/v acetic acid and gels were subsequently visualized on a GS-800 calibrated densitometer (Bio-Rad, UK) and stored in 1% acetic acid prior to trypsin digestion for mass spectrometry analysis following the protocol of Davis et al. (2019).

## EV Surface Trypsin Hydrolysis

SEC purified EVs were concentrated to a final concentration of 200 µg in 250 µl. Sequencing grade trypsin (Roche, U.K) was diluted to 100 µg/ml and added to the EVs resulting in a final concentration of 50 µg/ml. Samples were incubated for 5 minutes at 37°C followed by centrifugation for 1 hour at 100,000 × *g* at 4°C (S55-S rotor, Sorval MX120 centrifuge, Thermo Scientific). The resulting supernatant was divided into 20 µl fractions and subject to LC MSMS with an injection volume of 1 µl.

## Mass Spectrometry

Trypsin digested protein samples were suspended in 20 µl 0.1% formic acid and loaded into an Agilent 6550 iFunnel Q-TOF mass spectrometer combined with a Dual AJS ESI source 1290 series HPLC system (Agilent, Cheshire, U.K). A Zorbax Eclipse Plus C18 column (2.1 x 50 mm 1.8 micron) was utilized with each sample injected into an enrichment column within the system at a flow rate of 2.5 µl/min using an automated micro sampler with an injection volume of 2 µl in the resuspension buffer 0.1% v/v formic acid and allowed to separate at 300 nl/min. Enrichment and separation were carried out on a polaris chip (G4240-62030, Agilent Technologies, U.K). A system of solvents was utilized over the process, solvent A (milliQ water containing 0.1% formic acid) and solvent B (90% v/v acetonitrile containing 0.1% v/v formic acid). Chromatography was achieved using a linear-gradient of 3-8% solvent B over 6 seconds, 8-35% solvent B over 15 minutes, 35-90% solvent B over five minutes and finally 90% solvent B for two minutes. Resulting peak spectra data was loaded onto Agilent Qualitative analysis software (Agilent technologies LDA UK Limited, UK). Each file had compounds found by molecular feature and were saved to MGF. MASCOT (www.matrixofscience.com) was used for analysis by carrying out an MS/MS ion search, settings were set for the enzyme trypsin – allowing 2 missed cleavages, with a fixed modification of carbamidomethyl (C) and a variable modification of oxidation (M) with a peptide charge of 2+, 3+ and 4+. Each sample was then searched against an in-house database composed of a transcript for *C. daubneyi* (Huson et al., 2018) available to search at https://sequenceserver.ibers.aber.ac.uk/. Each of the contigs returned were then searched within an in-house copy of the transcript and the nucleotide sequence recorded. All of the contigs were then translated using ExPasy (www.expasy.com) and the sequences submitted to BLASTp analysis and subsequently searched in the Interpro database.

## EV Rumen Microbe Interactions

Rumen contents were collected from rumen-fistulated steers at Trawsgoed experimental farm (Aberystwyth, Wales) complying with the authorities of the UK Animal (Scientific Procedures) Act (1986). Rumen contents was squeezed through a sieve allowing retention of strained ruminal fluid (SRF) that was immediately incubated at 39°C. Rumen fluid was added to anaerobic incubation medium following the protocol of Goering and Van Soest (1970), to create a 10% v/v solution. PBS was removed from SEC purified EV samples (5.52E+10 particles/ml) through centrifugation using Amicon ultra-15 centrifugal filters (Merk, Millipore) 10 kDa MWCO, with EVs resuspended in an equal volume of modified Van Soest digestion buffer. 1 ml EV solution was added to 9 ml rumen fluid/anaerobic incubation medium (n =3) and allowed to incubate for 24 hrs. For controls, EV solution was replaced with modified Van Soest digestion buffer. Rumen fluid sampling was carried out at 5 time points during the incubation period (0 h, 2 h, 4 h, 6 h, and 24 h) and stored for downstream qPCR analysis.

## Rumen Fluid DNA Extraction

DNA extractions were carried out on 1 ml rumen fluid from each of the aforementioned time points (0 hrs, 2 hrs, 4 hrs, 6 hrs and 24 hrs). Extractions were carried out using a FastDNA spin kit for soil (MP Biomedicals, USA) according to the manufacturers protocol, as described by Huws et al. (2010). Extracted DNA was quantified using the Biotech Epoch Microplate Spectrophotometer (Biotek Instruments Inc, USA). The Epoch Microplate Spectrophotometer was calibrated prior to quantification using 1.25 µl DNase/pyrogen free water.

Following quantification samples were stored at -20°C for qPCR analysis.

## Bacterial qPCR Analysis

qPCR of 16S rDNA was undertaken to determine the effects of incubation of EVs with rumen fluid on total bacteria population as well as specifically *Ruminococcus albus, Fibrobacter succinogenes*, and *Prevotella* spp. Extracted DNA was diluted 10-fold with $ddH_2O$. The reaction mixture (1215 µl) for each qPCR run was prepared with 1 x SYBR Geen I master mix (Applied Biosystems), 5.4 µl of each primer (**Table 1**) and $ddH_20$. 10 µl of reaction mixture was added to 1 µl of each DNA sample analyzed using a Roche lightcycler 480 II (Roche diagnostics Ltd.) on a 384 well qPCR plate. A bacterial standard was prepared with equal amounts of genomic DNA as outlined by Huws et al. (2010). For each qPCR, with the exception of *Prevotella* spp., amplification was performed at 95°C for 10 minutes, followed by 35 cycles of 95°C for 15 seconds, 58°C for 15 seconds, and 72°C for 15 seconds, and then an extension step of 72°C for 5 minutes. For the *Prevotella* spp. qPCR amplification was performed at 95°C for 10 minutes followed by 35 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 15 seconds, and then an extension step of 72°C for five minutes. All qPCR reactions were performed in triplicate and assay qPCR efficiency was calculated as: efficiency=10(- 1/slope) x100. The bacterial standards were used to create a standard curve to allow for quantification of the samples. Statistical analysis of the qPCR values was undertaken in Microsoft Excel, and using repeated measures ANOVA to test for significant differences in IBM SPSS Statistics 23.0.

# RESULTS

## Confirmation of EVs in Adult *C. daubneyi* Maintenance Media

The presence of extracellular vesicles in both DC and SEC purified adult *C. daubneyi* samples was confirmed by the identification of membrane bound vesicles ~30-100 nm in size through transmission electron microscopy (TEM). TEM imaging demonstrated EVs present to have diverse morphologies with ruptured vesicles only identified in DC purified samples. A large number of aggregated vesicles were also observed in DC samples (**Figure 1A**) whilst reduced aggregation was observed in the samples isolated through SEC (**Figure 1B**) and, despite the inclusion of 0.2 µm filtering, background contamination was visibly present following both purification methods.

## Adult *C. daubneyi* Whole EV Proteome

DC purified EVs were utilized to resolve the *C. daubneyi* whole lysed EV proteome. A GelC strategy was exploited to identify lysed EV proteins with proteins resolved on a 12.5% one-dimensional sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) followed by LC-MSMS analysis with the mass spectrometry proteomics data deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD024182. Replication of lysed EV proteomic profiles confirmed reproducibility (**Figure 2A**). A total of 378 proteins were found to be consistent across three biological replicates (n = 3) following LC-MSMS analysis (**Supplementary Material 1**). EV protein abundance was quantified by the number of unique peptides present, with only protein hits above the significance threshold (>47) included as a positive identification. Quantification by the number of unique peptides elucidated the top 50 protein hits (**Table 2**). Further analysis of the returned proteome identified a number of common EV markers through comparison with the Exocarta database (http://exocarta.org). All 378 sequences resolved in the EV proteome were further characterized by their functionality through use of the Interpro database and sorted into 9 distinct categories: Cytoskeleton, Proteases, Enzymes, Chaperones, Metabolism, Transporters, Carrier, Exosome Biogenesis and Others as previously described by (Cwiklinski et al., 2015) (**Figure 2B**). Interestingly, the category with the greatest number of sequences assigned was 'other' encompassing all sequences with no BLAST result or a BLAST result to a hypothetical or unassigned protein accounting for 36% of the sequences. This was followed by cytoskeletal proteins accounting for 24% of proteins. The category representing the fewest number of proteins were carriers accounting for 1% of the total proteome.

## Trypsin Hydrolysis of External Surface Proteins on Adult *C. daubneyi* EVs

Following resolution of the whole EV proteomic profile, the proteins present on the external surface of EVs were investigated through trypsin cleavage from the membrane. Transcript IDs identified through LC-MSMS were translated before submission to BLASTp investigation allowing identification of protein IDs. In total, 89 proteins were identified as present upon the external surface of EVs release by adult *C. daubneyi* (**Table 3**), including a variety of well-known exosomal markers such as heat shock protein 70 and members of the tetraspanin family as defined by the Exocarta database (http://www.exocarta.com). Several membrane channel and transporter proteins were identified including ATPase, V-type H+- transporting ATPase, phospholipase and glucose transporters.

**TABLE 1** | Forward and reverse primer sequences used to target 16S rDNA in qPCR analysis of total bacteria, *Ruminococcus albus, Fibrobacter succinogenes* and *Prevotella* spp. DNA concentrations.

| Target | Forward primers (5'-3') | Reverse primers (3'-5') |
|---|---|---|
| **Total Bacteria** | GTGSTGCAYGGYTGTCGTCA | GAGGAAGGTGKGGAYGACGT |
| ***Ruminococcus albus*** | CCCTAAAAGCAGTCTTAGTTCG | CCTCCTTGCGGTTAGAACA |
| ***Fibrobacter succinogenes*** | GGTATGGGATGAGCTTGC | GCCTGCCCCTGAACTATC |
| ***Prevotella* spp.** | CACRGTAAACGATGGATGCC | GGT CGG GTT GCA GAC C |

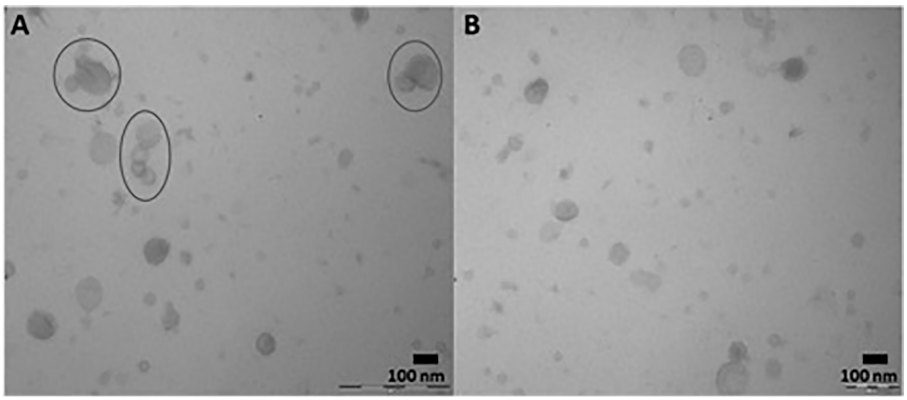


**FIGURE 1** | Representative developed TEM micrographs identifying extracellular vesicles secreted by *C. daubneyi in vitro* through DC and SEC isolation. **(A)** DC purified samples with visible aggregation of vesicles (circled) **(B)** SEC purified samples demonstrating a reduction in EV aggregation.

## EV Interaction With the Rumen Microbiome

The impact of adult *C. daubneyi* EVs on rumen microbial populations was completed on three key ruminant bacterial species, *Fibrobacter succinogenes, Ruminococcus albus* and *Prevotella spp*, as well as on the total bacterial microbiome using 16S rDNA analysis. Quantitative PCR was undertaken on samples at five timepoints (0 hrs, 2 hrs, 4 hrs, 6 hrs and 24 hrs) to assess the impact of EVs on the rumen microbiome (**Figure 3**). Total Bacteria DNA concentrations ranged from 500.93 to 1236.95 ng/ml in cultures incubated in the presence of rumen fluke EVs, and from 229.93 to 656.22 ng/ml in cultures with absence of rumen fluke EVs. *Fibrobacter succinogenes* DNA concentration ranged from 0.471 to 7.871 ng/ml with EVs and from 0.207 to 5.384 ng/ml in the absence of EVs. *Ruminococcus albus* DNA concentrations ranged from 0.175 to 0.509 ng/ml with EVs, and from 0.049 to 0.526 ng/ml in the absence of rumen fluke EVs. Finally, the DNA concentrations for *Prevotella* spp. ranged from 70.60 to 298.03 ng/ml with EVs, and from 30.45 to 326.97 ng/ml in the absence of rumen fluke EVs.

In terms of overall treatment, EV presence or absence, a significant effect of treatment with *C. daubneyi* EVs was only observed for total bacteria ($P = 0.002$). Whereby, the incubation of rumen fluid with EVs led to an increase in total bacterial concentration, with no significant overall effect of treatment on bacterial concentrations for *F. succinogenes*, *R. albus*, or *Prevotella* spp. Analysis of overall treatment between time points (0-24 hrs) showed significant interaction for total bacteria ($P=0.008$), *R. albus* ($P<0.05$), and *Prevotella* spp. ($P<0.05$). However, there was no significant interaction between timepoint and treatment for *F. succinogenes* ($P>0.05$). For total bacteria concentrations, there was a significant difference between treatment means at the zero hours and six-hour timepoints. For *F. succinogenes*, the only timepoint at which there was a significant difference between treatment means was at zero hours ($P=0.001$). There was a significant difference in the mean concentration of *R. albus* DNA between treatments at all except the four-hour timepoint. *Prevotella* spp. had a significant difference between treatment means at the two and six hour timepoints ($P >0.01$).

## DISCUSSION

Utilization of the recently reported adult *C. daubneyi* transcriptome (Huson et al., 2018) has allowed a comprehensive proteomic characterization of the adult helminth's membrane bound vesicle secretions, leading to identification of 378 proteins consistent across biological replicates. Comparison with resolved eukaryote EV proteomes highlighted a number of common proteins including, tetraspanins (TR20913|c0_g1_i1, TR22166|c0_g1_i1, TR22094|c0_g1_i1 and TR22869|c0_g1_i12), Heat shock proteins (TR17741|c0_g1_i1 and TR20530|c0_g1_i1) and Annexins (TR22803|c1_g1_i2, TR20643|c0_g1_i1 and TR17648|c0_g1_i1). This is in addition to EV associated cytoskeletal proteins such as Actin (TR9358|c0_g1_i1, TR17779|c0_g1_i1 and TR28482|c0_g1_i1) and Ezrin (TR19715|c0_g1_i1) as well as proteins involved in metabolic processes such as enolase (TR17367|c0_g1_i1, TR24268|c0_g1_i1, TR24268|c0_g2_i1 and TR19628|c0_g2_i1), Peroxidases (TR17193|c0_g1_i1 and TR12513|c0_g1_i1) and pyruvate kinases (TR21788|c0_g1_i1) (Choi et al., 2013; Nowacki et al., 2015). The consistency in proteins with established EV proteomes further supports the identification of the membrane bound vesicles by TEM imaging as EVs, suggesting the *C. daubneyi* secretome is more complex than previously demonstrated (Huson et al., 2018).

Protein cargo packaged into EVs prior to their release is dependent upon cellular source and release cell associated activity (Simons and Raposo, 2009). Similar to the closely related trematode *F. hepatica* and in contrast to several trematode species such as *E. caproni*, *S. mansoni* and *D. dendriticum*, rumen fluke EVs returned a large quantity of proteases and peptidases including Xaa-pro peptidase, cathepsins and metalloproteases (Marcilla et al., 2012; Bernal et al., 2014; Sotillo et al., 2016). Differences observed in protein cargo packaged between species could be due to their

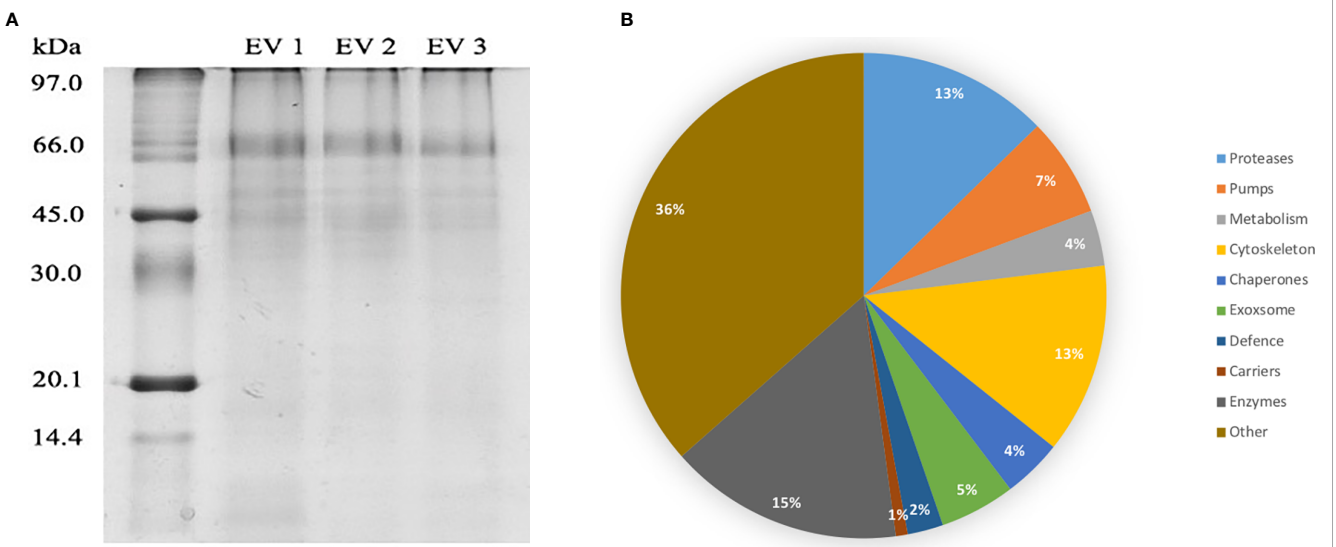


**FIGURE 2** | **(A)** EV proteome arrays of lysed *C. daubneyi* EVs (n=3). EVs released *in vitro* were lysed and subjected to 12.5% 1D polyacrylamide gel analysis and colloidal Coomassie blue stained. All biological replicates produced a highly reproducible profile. **(B)** Categorization of all sequences returned from the *C. daubneyi* EV proteome. Proteins consistent across three replicates were submitted to Interpro and GeneOntology searches and assigned to 9 functional categories as defined by Cwiklinski et al. (2015). Proteins that did not fit any of the nine categories were placed into a final category classified as 'other'. Cytoskeleton associated proteins accounted for 13% of the sequences resolved, Proteases 14%, Enzymes 8%, Chaperones 7%, Transporters 4%, Exosome biogenesis 3%, Metabolism 5%, Carriers 1% with Others filling the remaining 36%.

residency within the definitive host but could also be attributed to conditions during time of release (Nowacki et al., 2015). Hits to hypothetical proteins and proteins with 'no confirmed identity' highlighted the variability in proteins packaged and their likely roles in parasite establishment that are likely unique to the rumen fluke. In total, 14.2% of the proteins identified represented these undefined proteins and their further investigation could allow insight into infection, migration and successful establishment of infection (Dalton et al., 2003). Consistent to studies in *F. hepatica* a plethora of molecules including fatty-acid binding proteins, sigma-class glutathione transferases and cathepsin B were identified which are known to be internalized by host cells with their immunomodulation activity leading to a TH2-mediated environment that is favorable for parasite establishment (Dalton et al., 2003; Donnelly et al., 2010; Dowling et al., 2010). As with previous trematode studies, the presence of uncharacterized proteins allows the hypothesis that they could contain a plethora of novel sequences with potential roles in parasite pathogenesis. Investigation of uncharacterized proteins with no homology to resolved sequences provides an assortment of possible research avenues into future control and intervention of infection (Mulvenna et al., 2010).

Proteins present upon the surface of parasite derived EVs have been found critical to EV function as they interact directly with cells mediating cellular uptake and affecting immune recognition whilst also allowing identification, isolation and classification of EV subpopulations (Buzás et al., 2018). Trypsin hydrolysis of the surface of *C. daubneyi* EVs identified a total of 86 proteins including a variety of well-known exosomal markers such as heat shock protein 70 and members of the tetraspanin family as defined by the Exocarta database (http://www.exocarta.com). As expected, a trematode specific tetraspanin (CD63) was identified within the proteome, as common EV markers, members of the tetraspanin family have been widely investigated with studies on *O. viverrini* highlighting the potential use of EV derived tetraspanins as vaccine candidates due to their ability to prevent EV uptake and internalization into host cells (Chaiyadet et al., 2015). As with studies into closely related trematode *F. hepatica* EVs, many of the surface proteins resolved represented metabolic enzymes such as enolase, Glyceraldehyde-3-dehydrogenase and annexins with primary roles as adhesion molecules interacting directly with the surface of host cells and so represent possible targets in preventing *C. daubneyi* successful establishment through interruption of EVs internalization by target cells (Bernal et al., 2004; Lama et al., 2009; de la Torre-Escudero et al., 2012).

Following resolution of both the cargo and membrane bound proteome, the potential of *C. daubneyi* EVs to modulate the microbiome were investigated on a range of ruminant bacterial species. Both helminths and bacterial species residing within the gut have been found to have strong immunomodulatory effects on the mammalian host, with a variety of studies showing helminths effect on the microbiota correlating to the helminths successful establishment (Reynolds et al., 2015). Helminths ability to regulate gut microbiota is important due to the ability of certain species to elicit the host immune response favorable for survival (Reynolds et al., 2015), with several previous helminth studies highlighting their ability to regulate bacterial populations within the gut (Su et al., 2018). Here, the effect of EVs on bacterial species encompassed three bacterial species found within the rumen as well as the total bacterial counts within the rumen microbiome.

**TABLE 2 |** Top 50 proteins resolved in *C. daubneyi* extracellular vesicles following BLAST analysis of transcript identifiers.

| Transcript ID | Isoform | Unique peptides | Blast description | Organism | NCBI accession |
|---|---|---|---|---|---|
| TR26097\|c0_g1 | i1 | 76 | ATPase family protein | *Opisthorchis viverrini* | OON14744.1 |
| TR18968\|c0_g1 | i1 | 58 | Tubulin beta-3 | *Fasciola hepatica* | CAP72051.1 |
| TR17099\|c2_g1 | i1 | 57 | Tubulin beta | *Clonorchis sinensis* | GAA51682.1 |
| TR21569\|c0_g5 | i1 | 44 | No hit | No hit | No hit |
| TR9358\|c0_g1 | i1 | 39 | Actin | *Gossypium arboreum* | XP_017626052.1 |
| TR19715\|c0_g1 | i1 | 36 | Radixin | *Carlito syrichta* | XP_008054748.1 |
| TR21569\|c0_g5 | i2 | 36 | No hit | No hit | No hit |
| TR17877\|c2_g2 | i1 | 31 | Alpha-tubulin | *Fasciola hepatica* | CAO79602.1 |
| TR18958\|c0_g1 | i1 | 28 | Alpha tubulin | *Schistosoma japonicum* | AAW27478.1 |
| TR19159\|c0_g1 | i1 | 26 | Alpha tubulin | *Clonorchis sinensis* | GAA56421.1 |
| TR23254\|c0_g1 | i1 | 24 | Leucyl aminopeptidase | *Clonorchis sinensis* | ABL11479.1 |
| TR24554\|c0_g1 | i1 | 23 | alpha-glucosidase | *Schistosoma mansoni* | XP_018647945.1 |
| TR18070\|c0_g1 | i1 | 22 | Acid sphingomyelinase phosphodiesterase | *Clonorchis sinensis* | GAA33847.2 |
| TR23757\|c0_g1 | i1 | 22 | Alpha tubulin | *Clonorchis sinensis* | GAA38337.2 |
| TR24153\|c0_g1 | i1 | 22 | Hypothetical protein | *Opisthorchis viverrini* | OON14506.1 |
| TR23969\|c0_g1 | i1 | 21 | Tektin | *Clonorchis sinensis* | GAA33438.1 |
| TR23279\|c0_g1 | i1 | 21 | Alpha tubulin | *Fasciola hepatica* | CAO79606.1 |
| TR18525\|c0_g1 | i1 | 20 | 14-3-3 epsilon | *Opisthorchis viverrini* | OON22058.1 |
| TR21014\|c0_g1 | i1 | 20 | SNaK1 | *Schistosoma mansoni* | AAL09322.1 |
| TR20466\|c0_g1 | i1 | 19 | Calpain | *Schistosoma mansoni* | CCD74981.1 |
| TR20643\|c0_g1 | i1 | 18 | annexin a7 | *Schistosoma haematobium* | KGB33756.1 |
| TR18939\|c0_g1 | i1 | 18 | No hit | No hit | No hit |
| TR22034\|c1_g4 | i3 | 18 | Aldolase | *Opisthorchis viverrini* | OON20700.1 |
| TR25036\|c3_g1 | i13 | 17 | Cathepsin D | *Fasciola gigantica* | AEE69372.1 |
| TR25036\|c3_g1 | i2 | 17 | Cathepsin D | *Fasciola gigantica* | AEE69372.1 |
| TR23288\|c0_g1 | i1 | 16 | EF-hand domain | *Schistosoma mansoni* | CCD76447.1 |
| TR19073\|c1_g2 | i1 | 16 | Hypothetical protein | *Opisthorchis viverrini* | OON16570.1 |
| TR18454\|c0_g1 | i1 | 16 | Hypothetical protein | *Opisthorchis viverrini* | OON20759.1 |
| TR25036\|c3_g1 | i1 | 16 | eukaryotic aspartyl protease | *Opisthorchis viverrini* | OON23093.1 |
| TR21569\|c0_g5 | i7 | 16 | No hit | No hit | No hit |
| TR25036\|c3_g1 | i14 | 14 | Cathepsin D | *Clonorchis sinensis* | GAA56870.1 |
| TR23782\|c0_g2 | i1 | 14 | Leukotriene-A4 hydrolase | *Clonorchis sinensis* | GAA49617.1 |

*(Continued)*

**TABLE 2** | Continued

| Transcript ID | Isoform | Unique peptides | Blast description | Organism | NCBI accession |
|---|---|---|---|---|---|
| TR17046\|c0_g1 | i1 | 15 | 14-3-3 epsilon | *Clonorchis sinensis* | AEO89649.1 |
| TR9216\|c0_g1 | i1 | 15 | 14-3-3 protein | *Opisthorchis viverrini* | OON14987.1 |
| TR25395\|c0_g2 | i2 | 15 | Hypothetical protein | *Opisthorchis viverrini* | XP_009165006.1 |
| TR17779\|c0_g1 | i1 | 15 | Actin | *Opisthorchis viverrini* | XP_009173847.1 |
| TR25395\|c0_g1 | i1 | 15 | Hypothetical protein | *Opisthorchis viverrini* | XP_009165006.1 |
| TR22003\|c1_g5 | i1 | 14 | Tubulin beta | *Cricetulus griseus* | XP_007606483.1 |
| TR19892\|c0_g1 | i1 | 14 | Hypothetical protein | *Opisthorchis viverrini* | OON16605.1 |
| TR18374\|c0_g1 | i1 | 14 | Triose phosphate isomerase | *Fasciola hepatica* | AGJ83762.1 |
| TR25036\|c3_g1 | i12 | 14 | Cathepsin E-A | *Apaloderma vittatum* | KFP91951.1 |
| TR17173\|c0_g1 | i1 | 14 | leishmanolysin peptidase | *Clonorchis sinensis* | GAA54636.1 |
| TR17164\|c0_g1 | i1 | 13 | glyceraldehyde 3- phosphate dehydrogenase | *Clonorchis sinensis* | GAA28380.1 |
| TR15297\|c0_g1 | i1 | 13 | Chloride intracellular channel | *Clonorchis sinensis* | GAA38512.2 |
| TR19675\|c0_g1 | i1 | 13 | JF-2 | *Schistosoma japonicum* | AAB49033.1 |
| TR25036\|c3_g1 | i8 | 13 | Cathepsin D | *Clonorchis sinensis* | GAA56870.1 |
| TR23072\|c0_g1 | i1 | 13 | EF-hand calcium-binding domain | *Clonorchis sinensis* | GAA51832.1 |
| TR24199\|c0_g4 | i1 | 12 | Plastin-1 | *Clonorchis sinensis* | GAA29911.1 |
| TR21252\|c0_g1 | i1 | 12 | 33kDa inner dynein arm light chain | *Schistosoma japonicum* | CAX73643.1 |
| TR25837\|c0_g3 | i3 | 12 | EF-hand domain-containing family member | *Clonorchis sinensis* | GAA35263.2 |

*Proteins were ranked based on the number of unique peptides sequenced during tandem LC-MSMS and BLAST identifiers chosen based on E-values.*

*F. succinogenes*, *R. albus*, and *Prevotella* spp. were chosen for quantification using qPCR to investigate the effect of EVs on the rumen microbiome and any subsequent effects on metabolism. Of these three species, *F. succinogenes* and *R. albus* are considered to be the main cellulolytic bacteria in the rumen (Forsberg et al., 1997), with these species extensively studied using a combination of pure culture and molecular techniques (Minato and Suto, 1978; Mosoni et al., 1997; Koike et al., 2003; Shinkai and Kobayashi, 2007; Zeng et al., 2015). The third species utilized, *Prevotella* spp. represent non-cellulolytic bacteria that play a vital role in ruminal protein degradation (Wallace, 1996; Alauzet et al., 2010).

Exposure of *C. daubneyi* EVs to ruminant bacterial populations showed no significant effect between EV treatment and controls, suggesting there is no significant effect of *C. daubneyi* EVs on rumen microbial facilitated metabolism, or in particular fiber and protein digestion. As expected, given the absence of a feed source, a drop in bacterial DNA concentrations at the 24-hour time point was common across all samples, as feed associated bacteria comprise 70-80% of the ruminal microbial matter (McAllister et al., 1994). The only timepoint found to have a significant difference between treatment means for *F. succinogenes* was timepoint zero. This could be due to the use of rumen fluid inoculum which is deemed as the largest source of variation in *in vitro* rumen studies, due to variations that can occur due to its microbial activity, the preparation method, the concentration of the rumen fluid used, the donor animal from which it is derived and their diet, and even variances within the day have been reported (Cone et al., 1996; Jessop and Herrero, 1998; Rymer et al., 1999; Mould, 2003; Váradyová et al., 2005). For *R. albus* the only timepoint at which there was no significant difference between treatments was the 4-hour timepoint. Whilst, not large enough to be significant the inclusion of EVs appears to slow the decrease in the concentration of *R. albus* over time. A similar effect of EV inclusion was observed for *Prevotella* spp. although again this was not significant.

However, a significant difference was observed for total bacteria DNA concentrations between EV treatment and controls. An increase observed in total bacteria populations alongside no significant differences between treatment and controls for *F. Succinogenes*, *R. albus*, and *Prevotella* spp. may indicate that addition of EVs leads to an increase in total bacterial diversity. This increase in diversity could be due to rumen fluke EVs promoting the survival of several bacterial species in the rumen with previous whole parasite studies reporting increases in

**TABLE 3 |** Putative proteins identified in SEC purified *C. daubneyi* EVs surface trypsin shave (n = 3).

| Transcript ID | Blast ID |
|---|---|
| TR19715\|c0_g1_i1 | Moesin ezrin radixin homolog 1 isoform X1 |
| TR18070\|c0_g1_i1 | Acid sphingomyelinase-like phosphodiesterase 3a |
| TR20146\|c0_g1_i2 | No hit |
| TR9358\|c0_g1_i1 | Actin-7 |
| TR17099\|c2_g1_i1 | Tubulin beta chain |
| TR18542\|c0_g1_i1 | Tubulin beta chain |
| TR22003\|c1_g6_i1 | Tubulin beta-2C chain |
| TR22003\|c1_g4_i3 | Tubulin beta chain isoform X1 |
| TR23322\|c0_g3_i1 | Tubulin beta-2C chain |
| TR22003\|c1_g2_i1 | Beta tubulin |
| TR22003\|c0_g1_i1 | Tubulin beta chain |
| TR21569\|c0_g5_i1 | No hit |
| TR21569\|c0_g5_i2 | No hit |
| TR25036\|c3_g1_i12 | Cathepsin E- |
| TR25036\|c3_g1_i14 | Lysosomal aspartic protease |
| TR3846\|c0_g1_i1 | Cathepsin D (lysosomal aspartyl protease) |
| TR6048\|c0_g1_i1 | Asparticase oryzasin-1-like |
| TR25036\|c3_g1_i1 | Cathepsin E-A-like |
| TR25036\|c5_g1_i1 | Renin |
| TR25036\|c0_g1_i1 | Lysosomal aspartic protease-like |
| TR25036\|c3_g1_i2 | Cathepsin D (lysosomal aspartyl protease) |
| TR25036\|c3_g1_i8 | Lysosomal aspartic protease-like |
| TR55450\|c0_g1_i1 | No hit |
| TR16856\|c0_g1_i1 | DM9 domain-containing |
| TR18939\|c0_g1_i1 | No hit |
| TR17640\|c0_g1_i1 | No hit |
| TR20530\|c0_g1_i1 | Heat shock 90 |
| TR21065\|c0_g1_i1 | Heat shock 75 mitochondrial |
| TR24356\|c0_g1_i3 | Program cell death 6-interacting |
| TR15792\|c0_g1_i1 | Golgi-associated plant pathogenesis-related 1 |
| TR23254\|c0_g1_i1 | Leucyl aminopeptidase |
| TR17173\|c0_g1_i1 | Leishmanolysin-like peptidase |
| TR19239\|c0_g1_i1 | No hit |
| TR12225\|c0_g1_i1 | Erythrocyte band 7 integral membrane |
| TR16040\|c0_g1_i1 | Lysosomal Pro-X carboxypeptidase precursor |
| TR18162\|c0_g1_i1 | Liver basic fatty acid binding |
| TR26002\|c2_g1_i1 | Cytoplasmin type 5 |
| TR33621\|c0_g1_i1 | No hit |
| TR29071\|c0_g1_i1 | Actin |
| TR4440\|c0_g1_i1 | No hit |
| TR23598\|c0_g2_i1 | Adenylate kinase 9 |
| TR17877\|c2_g2_i1 | Tubulin alpha-1A chain-like |
| TR19159\|c0_g1_i1 | Tubulin alpha-1A chain |
| TR18958\|c0_g1_i1 | Tubulin alpha-1A chain-like |
| TR12612\|c0_g1_i1 | Alpha tubulin |
| TR21082\|c0_g1_i1 | Tubulin GTPase domain |
| TR17328\|c0_g1_i1 | Na(+) H(+) exchange regulatory cofactor NHE-RF1 |
| TR20466\|c0_g1_i1 | Leucine-rich repeat-containing 23 |
| TR9216\|c0_g1_i1 | Tyrosine 3-monooxygenase tryptophan 5-monooxygenase |
| TR16536\|c0_g1_i1 | 14-3-3 beta alpha-1 |
| TR17046\|c0_g1_i1 | 14-3-3 epsilon |
| TR20794\|c0_g1_i1 | Phosphoglycerate kinase 1 |
| TR23598\|c0_g1_i1 | Adenylate kinase 9-like |
| TR20586\|c0_g1_i2 | Regulator of microtubular dynamics 1-like |
| TR13665\|c0_g1_i1 | Calcyphosin isoform X5 |
| TR19675\|c0_g1_i1 | Radixin isoform X1 |
| TR20928\|c0_g1_i1 | Cathepsin B-like cysteine ase precursor |
| TR11284\|c0_g1_i1 | Histone H4 |
| TR16097\|c0_g1_i1 | Chloride intracellular channel 4 |
| TR16168\|c0_g1_i1 | Actin depolymerizing factor |
| TR15827\|c0_g1_i1 | Lysosomal protective |
| TR17138\|c0_g1_i1 | Fatty acid binding brain |
| TR17367\|c0_g1_i1 | Enolase |
| TR19538\|c0_g1_i1 | Charged multivesicular body 1a |
| TR22854\|c0_g1_i4 | Aquaporin-1 |
| TR15761\|c0_g1_i1 | Lysosomal alpha-glucosidase |
| TR20893\|c0_g1_i1 | Methylthioadenosine phosphorylase |
| TR12782\|c0_g1_i1 | 8 kDa calcium-binding |
| TR36972\|c0_g1_i1 | Histone H4-like |
| TR18466\|c1_g2_i1 | Globin-3 |
| TR20091\|c0_g1_i1 | Glucose transport |
| TR17869\|c0_g1_i1 | Phospholipase D3 |
| TR15896\|c0_g1_i1 | Calmodium 6 |
| TR17164\|c0_g1_i1 | Glyceraldehyde 3-phosphate dehydrogenase |
| TR17741\|c0_g1_i1 | Heat shock 70 |
| TR22803\|c1_g1_i2 | Annexin A11 |
| TR3136\|c0_g1_i1 | No hit |
| TR20643\|c0_g1_i1 | Annexin A7 |
| TR17762\|c0_g1_i2 | Lysosomal acid phosphatase |
| TR16407\|c0_g1_i2 | Cathepsin D (lysosomal aspartyl protease) |
| TR22152\|c0_g1_i1 | Hypothetical protein CLF_104825 |
| TR16514\|c0_g1_i1 | No hit |
| TR23279\|c0_g1_i1 | Tubulin alpha testis-specific |
| TR18133\|c0_g1_i3 | CD63 antigen |

*Including transcript identifiers and BLAST description. The top BLAST hit was chosen based on the lowest E-value and transcripts were ordered by number of unique peptides.*

certain bacterial species in response to infection. Walk et al. (2010) and Reynolds et al. (2015) observed an increase in members of the lactobacillaceae family in the ileum of mice infected with *H. polygyrus*, despite the mice having different microbiotas present at the outset of the experiment. Similarly, the administration of a single dose of *Trichuris suis* led to a reduction in the abundance of *Fibrobacter* and *Ruminococcus*, accompanied by an increase of campylobacter in gastrointestinal microbiota of pigs (Wu et al., 2012). Alternatively, rumen fluke EVs could be promoting an increase in overall ruminal bacterial diversity as has been observed in gastrointestinal helminth infections in humans (Sepehri et al., 2007; Monira et al., 2012; Lee et al., 2014). Due to the absence of significant differences between treatments and the observed change in total bacteria concentrations the effects of *C. daubneyi* EVs on further ruminal bacterial species would allow a more in-depth understanding of *C. daubneyi* regulation of the microbiota.

Currently, studies into the effects of parasitic helminths on the gut microbiota of their ruminant hosts remain inconsistent, with investigations showing conflicting results (Li et al., 2011; Li et al., 2016). It is thought these inconsistencies are due to the composition and abundance of gut microbial taxa associated with the parasitic helminths being specific to each species (Kreisinger et al., 2015). A higher species richness may benefit the host, as higher species richness of the gut microbiota has been associated with 'healthier' gut homeostasis (Sepehri et al., 2007; Monira et al., 2012; Lin et al., 2013; Lee et al., 2014; Kreisinger et al., 2015). However, this study was designed to simulate a high, close proximity infection of adult rumen fluke, and so the changes in the microbiome seen here may be indicative of a more local effect on the microbiome. It is likely the effects observed may not be seen across the whole rumen. Additionally, EVs derived from rumen

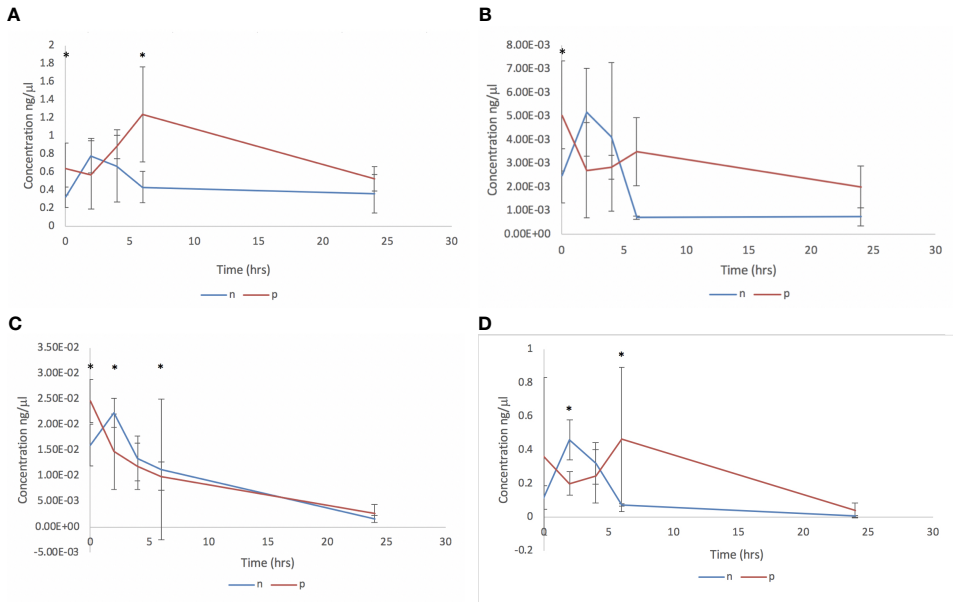


**FIGURE 3** | Bacterial qPCR analysis following *in vitro* culture of rumen fluid incubated with *C. daubneyi* SEC purified EVs (p) and without EVs (n). Data shown for **(A)** Total bacteria, **(B)** *Fibrobacter succinogenes*, **(C)** *Ruminococcus albus* and **(D)** *Prevotella* spp. *indicates a significant difference between treatment means.

fluke may have a greater effect on the bacterial species of the rumen associated with the epithelium and liquid phases, as rumen fluke affix themselves to the rumen epithelium *via* their posterior sucker (McCowan et al., 1978; Michalet-Doreau et al., 2001; Fuertes et al., 2015). EVs ability to regulate the hosts gut microbiota highlights the potential of utilizing EVs in order to promote survival of key bacterial species such as *F. succinogenes* that play a vital role in degradation of plant biomass and so could lead to improved rumen efficiency (Arntzen et al., 2017). A full antimicrobial analysis of all EV proteins characterized would be beneficial as EVs are known to bind to targets in order to become internalized and release their contents and so these may also be influencing the EVs themselves as well as elucidating mechanisms through which EVs released by *C. daubneyi* manipulate the host microbiota leading to conditions favorable for long term establishment.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ProteomeXchange Consortium *via* the PRIDE [1] partner repository with the dataset identifier PXD024182.

## ETHICS STATEMENT

The animal study was reviewed and approved by Aberystwyth University ethics committee.

## AUTHOR CONTRIBUTIONS

NA: data collection, analysis, investigation, methodology, and writing—original draft. AL: experimental design, data collection, and analysis. TW: experimental design and formal analysis. SH: experimental design and technical expertise. HP: technical expertise—LC-MSMS. RM and PB—supervision, writing, review, and editing. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.661830/full#supplementary-material

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REVIEW
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# Rodent Models for the Study of Soil-Transmitted Helminths: A Proteomics Approach

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Soil-transmitted helminths (STH) affect hundreds of millions worldwide and are some of the most important neglected tropical diseases in terms of morbidity. Due to the difficulty in studying STH human infections, rodent models have become increasingly used, mainly because of their similarities in life cycle. *Ascaris suum* and *Trichuris muris* have been proven appropriate and low maintenance models for the study of ascariasis and trichuriasis. In the case of hookworms, despite most of the murine models do not fully reproduce the life cycle of *Necator americanus*, their proteomic similarity makes them highly suitable for the development of novel vaccine candidates and for the study of hookworm biological features. Furthermore, these models have been helpful in elucidating some basic aspects of our immune system, and are currently being used by numerous researchers to develop novel molecules with immunomodulatory proteins. Herein we review the similarities in the proteomic composition between *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus bakeri* and *Trichuris muris* and their respective human counterpart with a focus on the vaccine candidates and immunomodulatory proteins being currently studied.

**Keywords: proteomics, soil-transmitted helminths (STHs), host-parasite interactions, *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus*, *Trichuris muris*, vaccines, immunomodulation**

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## INTRODUCTION

Infection by soil-transmitted helminths (STHs), some of the most common neglected tropical parasites in the world, affects mainly low and middle-income countries (Brooker, 2010). Indeed, it is considered that, globally, nearly 2 billion people are infected with STHs (Brooker, 2010; World Health Organization, 2012), and hookworm infection alone results in >4 million disability-adjusted life years lost annually (DALYs), as well as in significant economic losses (Bartsch et al., 2016). *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworm (mainly *Necator americanus* and *Ancylostoma duodenale*) are the most common species that infect humans (Jourdan et al., 2018). Although competent health care and wide use of available anthelmintic drugs are currently the main approaches for the elimination of most helminth infections, their efficacy varies and chemotherapy does not prevent reinfection (Loukas et al., 2016); thus, it becomes necessary to continue our efforts to improve our understanding of these parasitic diseases. Due to the limited availability and difficulty in obtaining parasite material, researchers have widely used different animal models that

share similarities in the life cycle, immune response elicited or both with their human counterpart (Scott and Tanguay, 1994; Camberis et al., 2003). In this regard, rodent models are, by far, the most popular and frequently used animal models and have been helpful in characterizing many aspects of human helminth infection.

*N. americanus*, one of the most important STHs in terms of morbidity, can survive for decades in the small intestine of their human hosts (Loukas et al., 2016). While *N. americanus* is notably common in most of Africa, southern China, Southeast Asia and the Americas, *A. duodenale* is endemic in northern regions of India and China, in the Mediterranean region and in North Africa. Furthermore, in some parts of Africa, China and India, it is not unusual to observe mixed human infections with *N. americanus* and *A. duodenale* (Pullan et al., 2014). The life cycle of this group of nematodes is very complex, and involves free-living and parasitic stages as well as an intraorganic migration in the definitive host. Hookworm eggs hatch in soil and released rhabditiform larvae moult twice before becoming filariform and infective (iL3). iL3s penetrate the skin of the host and are carried through the bloodstream first to the heart and then to lungs. Following exit from the alveolar capillaries, iL3s ascend the bronchial tree to reach the pharynx and are swallowed. Finally, hookworms complete their migration to the small bowel, typically the distal jejunum, where immature L5 hookworms attach themselves in position to feed and avoid ejection by gut peristalsis (Loukas et al., 2016).

Interestingly, a hamster model susceptible to *N. americanus* is available; however, although adult worms can fully develop without the requirement of corticosteroids, this model was developed after decades of passaging through immunosuppressed hamsters (Jian et al., 2003; Xiao et al., 2008), and the extent of adaptation and genomic and proteomic differences with worms obtained from the human host is yet to be determined. Indeed, worms obtained from hamsters are smaller in size, less fertile and infections do not last longer than a few months (Jian et al., 2003; Xiao et al., 2008), although they do elicit a protective immunity similar to that observed in the related canine hookworm species *Ancylostoma caninum*. This model has also proved useful for the screening of vaccine candidates and the assessment of antihelminthic drugs (Xue et al., 2005; Xiao et al., 2008; Xue et al., 2010; Zhan et al., 2010), however, the impossibility to use hamsters in some countries (e.g. Australia) and the low availability of molecular biology reagents for hamsters can make it challenging to work with.

Because of this, different animal models have been used to study hookworm-host interactions, including the related ancylostomatids *A. caninum* in dogs (Shepherd et al., 2018) and *A. ceylanicum* in hamsters (Alkazmi and Behnke, 2010; Traub, 2013), as well as the murine nematodes *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus bakeri* (both belonging to the Trichostrongyloidea superfamily), all part of the clade V of nematodes. This clade contains members of the suborder Rhabditina with nematodes from the Strongylida and other orders (Blaxter et al., 1998). *A. ceylanicum* infection is a zoonotic disease, and can produce symptomatic infections in humans (Loukas et al., 2016). It can also infect hamsters, where it develops patent infections (Alkazmi and Behnke, 2010), and can elicit acquired immunity, making it a suitable model for the study of hookworm infections (Loukas et al., 2016) with the same limitations listed above.

*N. brasiliensis*, a rodent strongyle nematode widely used by parasitologists, has a similar life cycle to *N. americanus*, including skin penetration, migration through the lungs and establishment in the small intestine of its host, although it is rapidly eliminated and does not recapitulate the long-lasting infections found with *N. americanus* (Camberis et al., 2003). Furthermore, *N. brasiliensis* induces a Th2 type immune response that manifests all the characteristics of a human hookworm infection, including IgE production and eosinophilia, which drive pathology in some allergic diseases (Nair and Herbert, 2016), as well as mastocytosis and mucus production (Camberis et al., 2003). Researchers have taken advantage of the similar life cycle and immunological responses between *N. brasiliensis* and *N. americanus* to conduct immunological studies (both systemic and mucosal) aimed at studying the mechanisms involved in human hookworm infections (Nair and Herbert, 2016).

Despite *H. polygyrus bakeri* does not infect through the skin or migrate through the lungs (as it depends on oral ingestion of infective larvae), it has been extensively employed as a model for human hookworm infections. Indeed, similarly to hookworms, *H. polygyrus bakeri* induces chronic intestinal infections in several mice strains, and the modified Th2 cell responses induced by infection (a Th2-like response linked with the production of anti-inflammatory cytokines and Treg activity) does not completely eliminate the parasites (Wells and Behnke, 1988; Maizels, 2005; Bungiro et al., 2008; Reynolds et al., 2012; Nair and Herbert, 2016). Furthermore, the study of *H. polygyrus bakeri* and *N. brasiliensis* infection in rodents has provided the immunology community with important information about the humoral and cellular mechanisms involved in the induction and development of Th2 immune responses and their capacity in protecting against helminth infections (Ogilvie and Jones, 1971; Ishizaka et al., 1976; Urban et al., 1991; Camberis et al., 2003).

In addition to hookworms, infection with whipworms (mainly *T. trichiura*) largely contributes to the pathological burden caused by STHs. More than 70 species of *Trichuris* (including worms of veterinary, scientific and human interest) have been described so far (Hurst and Else, 2013). All these species were classified within clade I, which groups vertebrate-parasites from the order Trichocephalida together with insect and plant-parasitic nematodes (Blaxter et al., 1998). Due to the difficulty in obtaining live worms from infected people and the impossibility of maintaining *T. trichiura* in the laboratory, *Trichuris muris* has become a widely used laboratory model being physiologically, morphologically, and antigenically similar to the human whipworm species (Grencis, 1993; Dixon et al., 2008). Indeed, the *T. muris* model has allowed researchers to understand relevant features concerning immunity to gut-dwelling nematode parasites as well as to gain a better knowledge of the immune system (Hurst and Else, 2013).

Furthermore, the knowledge attained from this animal model has been applied to better understand human Trichuriasis (Faulkner et al., 2002) and other intestinal helminth infections (Turner et al., 2003).

Ascariasis, mainly caused by *A. lumbricoides*, affects over 800 million people worldwide (Pullan et al., 2014). Similarly to what occurs with other STHs, it is highly challenging to obtain adult worms, and model organisms have been developed (Holland, 2013). The related species *A. suum* is a natural parasite of pigs; however this animal model has not been widely used because of its cost, large size and difficult husbandry (Holland, 2013). This species was found to be able to infect mice and to follow a similar infection behaviour as the one observed in its natural hosts (Slotved et al., 1998), and further research identified mouse strains with different compatibility (e.g. the susceptible C57BL/6 and the resistant CBA/Ca strains), providing a convenient model to investigate the basis of Ascaris biology and for the development of vaccine candidates (Lewis et al., 2007; Deslyper et al., 2019).

Despite the significant advantages of murine models in terms of reproductive capacity, handling, and costs, there are other models used for the study of helminth infections such as the pig whipworm *Trichuris suis*, which pathophysiology is very similar to that occurring in human infections (Dawson et al., 2020) or, as mentioned above, the dog hookworm *A. caninum* (Shepherd et al., 2018). Nevertheless, due to ethical considerations, complex logistics and cost, pig and dog models are less used in parasitological research and will not be the scope of this review.

The need to develop novel and effective treatments against STH is indisputable, and rodent models can provide important information. Understanding, not only the immunological, physiological, anatomical and metabolic similarities that each model has, but also the proteomic and genomic similarities between all species is key for the design of appropriate control approaches. In this review, we compare the available proteomic data between STH of human importance and their murine model counterparts with a focus on the characterization of vaccine candidates and immunomodulatory molecules. This analysis provides the first step towards a rational selection of the most appropriate model for the analysis of a particular protein candidate; however, ideally, a combined approach integrating different transcriptomic, proteomic, lipidomic and metabolomic information will provide a more comprehensive picture of the suitability of a particular model.

## GENOMIC AND PROTEOMIC INFORMATION FROM ANIMAL MODELS

During the last decades, one of the major caveats in the study of host-hookworms interactions has been the lack of comprehensive and thoroughly annotated genomic and proteomic databases. However, in the recent years, the development of novel sequencing platforms and more sensitive mass spectrometers, as well as different initiatives (i.e., 50 helminth genomes project; https://www.sanger.ac.uk/science/collaboration/50hgp) have provided useful information (Sotillo et al., 2017). In the case of *N. americanus*, the first draft genome was published in 2014 (Tang et al., 2014), and a more comprehensive genome version annotated using proteomic and transcriptomic data has recently been published (Logan et al., 2020). Similarly, the genomes and transcriptomes for the whipworms *T. trichiura* and *T. muris* were published in 2014 (Foth et al., 2014). In the case of *A. lumbricoide*s, the Parasite Genomics group at the Wellcome Trust Sanger Institute performed genome predictions as part of the 50 helminth genomes project (International Helminth Genomes Consortium, 2019).

A similarity analysis between the predicted proteome from *N. americanus* and other nematodes from the Ancylostomatidae family as well as hookworm models and other nematodes (all data downloaded from ParasiteWormBase v.14.0) shows that *A. caninum*, *A. duodenale* and *A. ceylanicum* proteins are, in general, more similar to *N. americanus* proteome (**Figure 1**). This analysis also showed that despite *N. brasiliensis* and *H. polygyrus bakeri* do not belong to the Ancylostomatidae family and are, thus, less related to hookworm, they share a high degree of similarity (>65%) in their proteome with *N. americanus* (**Figure 1**), compared to other nematodes. Despite the limitations of analysing the proteins only at the amino acid level, it is well accepted that proteins sharing over 40% (60% in the case of enzymes) sequence identity might share similar functions (Rost, 1999; Tian and Skolnick, 2003).

This is in agreement to what has been found recently, where *N. americanus* and hookworm animal models contained a similar number of predicted proteins encoded by their genomes, and proteins actively secreted by their adult stages presented a similar protein family profile (Logan et al., 2020). Indeed, from the 198 proteins secreted by *N. americanus* adult worms, 173 (>87%) contained homologs in the secretomes from *H. polygyrus bakeri*, *N. brasiliensis* and *A. caninum* (Logan et al., 2020). One of the most represented families in the secretomes of these adult worm species is the sperm-coating protein (SCP)-like extracellular proteins, also called SCP/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS; Pfam accession number no. PF00188). A total of 51 out of the 54 SCP/TAPS proteins found in the secretome of *N. americanus* had homologs in *H. polygyrus bakeri*, *N. brasiliensis* and *A. caninum*, which highlights the usefulness of using these murine models to study this particular family of proteins. Despite a phylogenetic analysis showed *N. americanus* SCP/TAPS proteins cluster more with *A. caninum* proteins than with *N. brasiliensis* or *H. polygyrus bakeri*, there are strong clade-specific similarities (Logan et al., 2020), and the high degree of diversity in the evolution of SCP/TAPS was speculated to be related to host-specific roles for this family of proteins (Logan et al., 2020).

Proteases (aspartyl-, cystein-, metallo- and serine-proteases) are also highly abundant in the secretome of *N. americanus* adult worms (Logan et al., 2020), as well as the murine models (Hewitson et al., 2011; Sotillo et al., 2014). A homology analysis showed that proteases secreted by *N. americanus* had, in general, a higher degree of homology to those from the *H. polygyrus bakeri* and *N. brasiliensis* rather than *A. caninum* (Logan et al., 2020), which would make these models highly

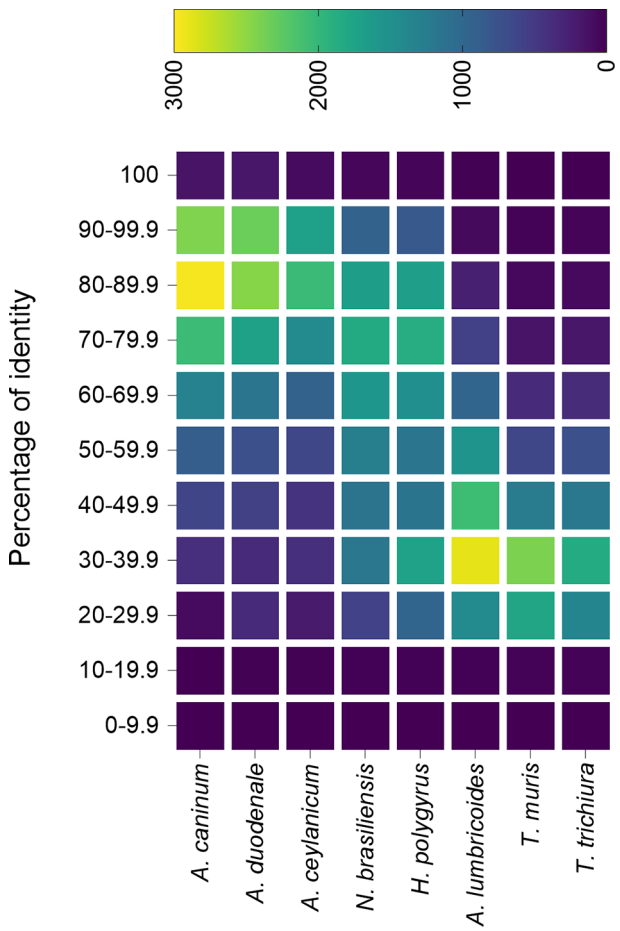


**FIGURE 1** | Percentage identity of *Necator americanus* predicted proteome with the predicted proteins from different nematode species. The predicted proteome from different species of hookworm, hookworm mouse models and unrelated nematodes were compared against the predicted proteome from *N. americanus* and plotted in a heatmap. All predicted proteomes were downloaded from Parasite WormBase (v.14.0) and protein identity was calculated using Blast. Colour represents the number of proteins within a range of identity percentage.

suitable for the development of vaccine studies as discussed in the next section.

Foth et al. sequenced and assembled the genome from both *T. trichiura* and *T. muris*, and found that most Trichuris genes are orthologs shared by both species (Foth et al., 2014). Furthermore, predicted proteomes are highly similar, with over 5,000 proteins having an average homology of 79% and only 2,350 and 3,817 proteins specific from *T. trichiura* and *T. muris* respectively, which highlights the usefulness of the mouse model to study human whipworm infections (Foth et al., 2014). An analysis of the similarity between the *T. trichiura* predicted proteome and other trichurids (i.e. *T. suis* and *T. muris*) as well as unrelated nematodes (all data downloaded from ParasiteWormBase v.14.0) confirms that proteins from both the pig and mouse models are highly similar to the human whipworm, and could be useful for the study of whipworm infections (**Figure 2**).

Only two studies have attempted to characterize the proteins secreted by *T. muris*, identifying 148 (Eichenberger et al., 2018b) and 73 (Tritten et al., 2017) proteins, while in the case of *T. suis* 328 proteins were identified (Leroux et al., 2018). The lower number of identified proteins in the mouse model in comparison with *T. suis* could be a reflection of the more stringent database search settings used. For instance, while Tritten et al. and Eichenberger et al. included databases from the parasite and

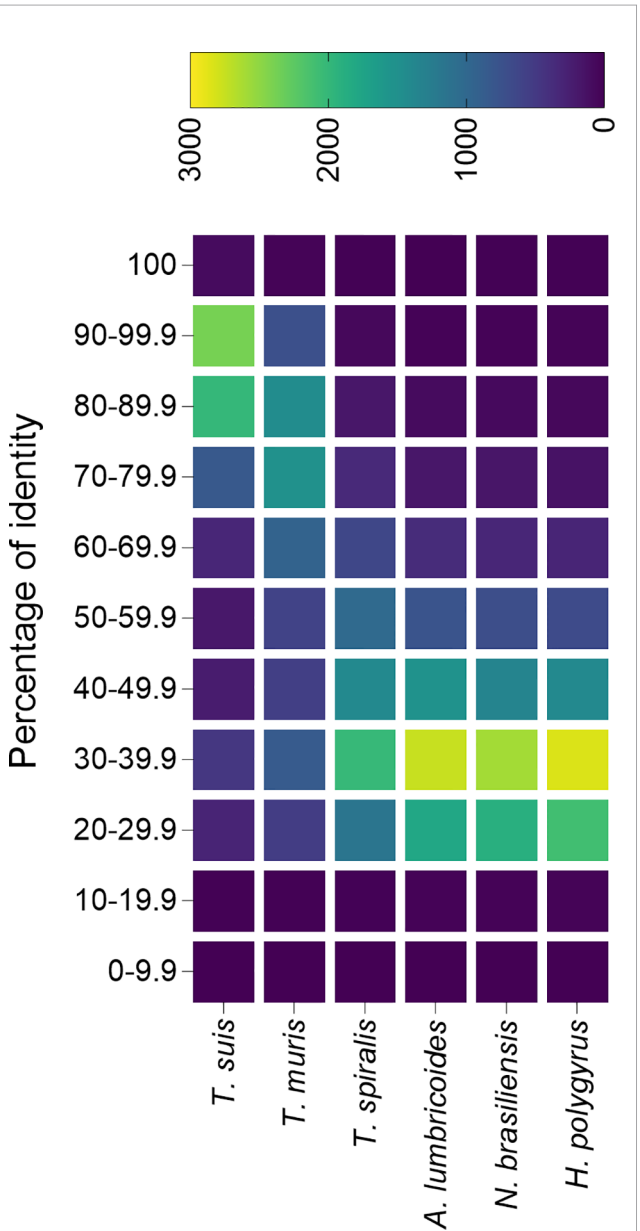


**FIGURE 2** | Percentage identity of *Trichuris trichiura* predicted proteome with the predicted proteins from different nematode species. The predicted proteome from different species of trichurids and unrelated nematodes were compared against the predicted proteome from *T. trichiura* and plotted in a heatmap. All predicted proteomes were downloaded from Parasite WormBase (v.14.0) and protein identity was calculated using Blast. Colour represents the number of proteins within a range of identity percentage.

the host (to eliminate host-associated proteins) and only proteins identified with two or more peptides were used for further analysis, Leroux et al. only used a parasite database (no contaminants were included in the search) and proteins identified with only one peptide were considered as valid identifications (Tritten et al., 2017; Eichenberger et al., 2018b; Leroux et al., 2018). It is noteworthy the low number of SCP/TAPS proteins identified in the *T. muris* secretome compared to parasites from clade V, which agrees with previous observations where this family of proteins is significantly expanded in clades IVa and V but not in clade I (Wilbers et al., 2018; International Helminth Genomes Consortium, 2019). To elucidate the degree of similarity between animal models and human whipworm infections, a comparative analysis of the secretomes from all three parasites would be of high interest, although the difficulty in obtaining viable worms from the human host makes this type of analysis currently very challenging.

## DEVELOPMENT OF VACCINE CANDIDATES IN MURINE MODELS

Since resistance to different antihelminthic drugs is being widely reported in human and animal nematodes, there is an urgent need for vaccines that could complement the current approach to helminth control. In this regard, the different rodent models used to study STHs could be of importance. Indeed, both hookworm hamster models (*N. americanus* and *A. ceylanicum*) have been used for the screening of vaccine candidates (Ghosh et al., 2006; Bungiro et al., 2008; Zhan et al., 2010), and *A. ceylanicum* has been proven a good model for selection of vaccine candidates using bioinformatic and functional approaches (Wei et al., 2016), providing important information for the development of these candidates.

Currently there are no licensed vaccines against human STH, and The Human Hookworm Vaccine initiative is, at present, the only vaccine for hookworm infection in clinical development. This vaccine contains two recombinant antigens, Na-GST-1 and Na-APR-1, both key enzymes involved in the capacity of hookworms to use host blood as source of nutrients (Hotez et al., 2013). Furthermore, challenge studies conducted in laboratory animals have shown the capacity of Na-GST-1 and Na-APR-1 to induce protective efficacy (Hotez et al., 2010; Hotez et al., 2013). Interestingly, both proteins have homologues in other hookworm and hookworm-like parasites, with the highest homology found with *A. ceylanicum*, *A. caninum* and *A. duodenale* (**Figures 3** and **4**) as expected due to closeness of species. Homology found with mice models such as *H. polygyrus bakeri* and *N. brasiliensis* was also high, particularly for Na-APR-1, with >83% aminoacid identity in homologues from both parasites (**Figure 4**). Interestingly, the percentage of identity found with *Trichuris* spp. was ~60% while it was ~80% for *Ascaris* spp., suggesting a key role of this enzyme in ascarids, most likely due to these parasites potentially being blood-feeders (Toh et al., 2010). This could be of interest when ranking and selecting potential candidates against Ascaris infection, and a modified Na-APR-1 could be incorporated into a pan-anthelminthic vaccine as discussed by other authors

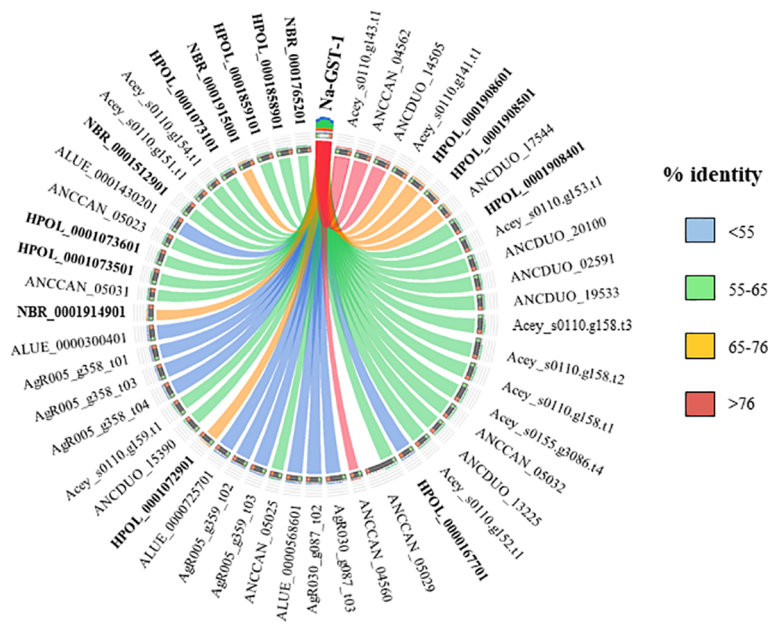


**FIGURE 3** | Similarity plot. Circos plot generated using Circoletto (Darzentas, 2010) showing the percentage of identity between Na-GST-1 and their homologues in different rodent model nematodes. Only homologues with e-values < 1E-50 are shown. Protein names as per Parasite WormBase database (v.15) have been used for comparison. ALUE, *Ascaris lumbricoides*; AgR, *Ascaris suum*; ANCCAN, *Ancylostoma caninum*; Acey, *Ancylostoma ceylanicum*; ANCDUO, *Ancylostoma duodenale*; HPOL, *Heligmosomoides polygyrus*; NBR, *Nippostrongylus brasiliensis*; TMUE, *Trichuris muris*; TTRE, *Trichuris trichiura*.

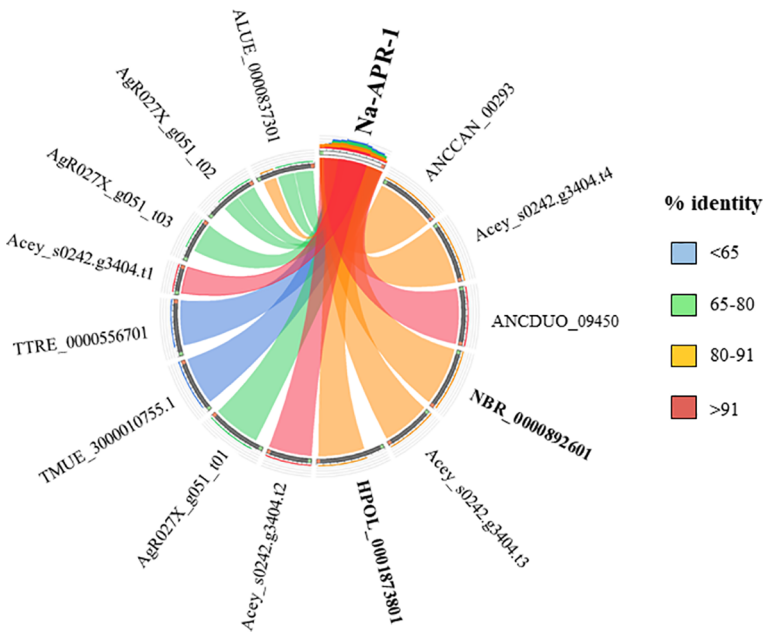


**FIGURE 4** | Similarity plot. Circos plot generated using Circoletto (Darzentas, 2010) showing the percentage of identity between Na-APR-1 and their homologues in different rodent model nematodes. Only homologues with e-values < 1E-50 are shown. Protein names as per Parasite WormBase database (v.15) have been used for comparison. ALUE, *Ascaris lumbricoides*; AgR, *Ascaris suum*; ANCCAN, *Ancylostoma caninum*; Acey, *Ancylostoma ceylanicum*; ANCDUO, *Ancylostoma duodenale*; HPOL, *Heligmosomoides polygyrus*; NBR, *Nippostrongylus brasiliensis*; TMUE, *Trichuris muris*; TTRE, *Trichuris trichiura*.

(Zhan et al., 2014). In the case of Na-GST-1, >65% aminoacid identity was found in different homologues from both mice models (**Figure 3**), while similarity with *Ascaris* spp. was ~50%, which hampers its use as a vaccine candidate in other nematodes as discussed below for Trichuris.

The high similarity found between both vaccine candidates and their *N. brasiliensis* homologues has highlighted the conservation in the blood-feeding pathways with *N. americanus* (Bouchery et al., 2018). Indeed, vaccination with both hookworm vaccine candidates induced protection against *N. brasiliensis* in mice, which made authors suggest that *N. brasiliensis* is a suitable model for vaccine identification and drug screening against hookworms (Bouchery et al., 2018). On the other hand, the fact that *H. polygyrus bakeri* has homologues to these two proteins is intriguingly, since this parasite is believed to feed on epithelial cells and not on blood (Bansemir and Sukhdeo, 1994), and more experiments should be done to ascertain the role of Na-APR-1 and Na-GST-1 homologues in this hookworm-like model. Indeed, vaccination with GST in a mouse model did not confer protection against *H. polygyrus bakeri*, despite eliciting a significant humoral response (Brophy et al., 1994). Thus, it is tempting to speculate that GST might be a potential vaccine candidate only in blood-feeding nematodes, whereas in non-hematophagous nematodes, where this protein is suggested to play a role only as a defence mechanism against toxic substances (Smith, 1992), other candidates must be tested. As mentioned earlier, >60% sequence identity between two proteins usually results in similar functions; however, performing functionality studies and integrating different *omic* technologies is essential to obtain a more holistic picture of the biological problem.

It is also worth highlighting that different studies using experimental infection with helminths show that GST influences the immune responses and cross-reactive allergy (Mitchell, 1989; Smith, 1992; Brophy et al., 1995; Santiago et al., 2012). Furthermore, helminth and cockroach GST cross-react because of their noteworthy molecular and structural similarities, which has led several authors to suggest that vaccine development should take into account the potential impact of cross-reactivity with common allergens (Santiago et al., 2012). In this regard, it is also necessary to consider the ability of vaccines to induce strong Th2 responses, remembering the case described by Diemert et al. (Diemert et al., 2012) where generalized urticarial reactions were developed in several volunteers after vaccination with a single dose of Na-ASP-2. These allergic reactions were linked to pre-existing Na-ASP-2-specific IgE probably induced by previous infection with *N. americanus* (Diemert et al., 2012; Diemert et al., 2018).

Different studies have used the *N. brasiliensis* rodent model to (i) discover new vaccine candidates that could be extrapolated to human hookworm infections and (ii) develop novel administration routes of known vaccine candidates to improve their immunogenicity and reduce undesirable effects. Indeed, since *N. brasiliensis* has a highly conserved orthologue of Na-APR-1 (Bartlett et al., 2020), Bartlet et al. designed a lipopeptide-based vaccine using a B cell epitope derived from Na-APR-1, attached to a T helper epitope and administered it orally. In this study, several lipidated peptides were obtained and tested for

vaccine efficacy using the *N. brasiliensis* hookworm model (Bartlett et al., 2020).

In previous studies, other researchers assessed the use of *N. brasiliensis* as a suitable model for testing vaccine candidates for hookworm infections. Using recombinant acetylcholinesterase B (AChE "B"), the most abundant enzyme isoform secreted by *N. brasiliensis* adult worms (Edwards et al., 1971; Clare Blackburn and Selkirk, 1992), these authors reported a level of protection in AChE-vaccinated animals and concluded that AChE "B" could be considered as a suitable vaccine antigen, with intranasal delivery being the most effective (Ball et al., 2007). Furthermore, the activity of the recombinant enzyme and subtypes of AChE in the somatic extract of *N. brasiliensis* could be inhibited by serum antibody (Ball et al., 2007); however, despite the promising results, no further studies pursued the development of a vaccine using this recombinant protein.

Cystatins, a group of proteins with immunomodulatory properties secreted by helminths, are implicated in several biological and pathological processes such as antigen processing, protein catabolism, and inflammation (Hartmann et al., 1997). Furthermore, cystatins have been identified in numerous parasite species including *N. brasiliensis*, where mice immunized with recombinant nippocystatin became partially resistant to infection, suggesting that *N. brasiliensis* might evade the host defense system using this protease inhibitor (Dainichi et al., 2001), although no other studies have tried to develop this molecule into a vaccine candidate in *N. brasiliensis* or other hookworms.

Coakley et al. showed that extracellular vesicles (EVs) from *H. polygyrus bakeri* are internalized by macrophages and can suppress host macrophage activation and inhibit expression of the IL-33 receptor subunit ST2. Further, vaccination with EVs elicited a protective immunity against *H. polygyrus bakeri* challenge in mice, suggesting EVs might play an important role *in vivo* (Coakley et al., 2017). The similarity of the EV proteomes between *H. polygyrus bakeri* and *N. americanus* is yet to be determined since EVs from the human hookworm have not been characterized yet.

*T. muris* is a well-established model for host immunity. Chronic infections using this model are obtained by a high-dose infection in the susceptible mouse strain AKR or by a low-dose infection in C57BL/6 mice. Furthermore, this model is widely used for assessing the efficacy and immunogenicity of vaccine antigen candidates against whipworm infections (Boes and Helwigh, 2000; Hurst and Else, 2013). Indeed, vaccination with *T. muris* ES products has been shown to elicit protective immunity in murine models (Jenkins and Wakelin, 1977; Jenkins and Wakelin, 1983; Dixon et al., 2008; Dixon et al., 2010; Liu et al., 2017). Furthermore, a recent study showed that immunisation with *T. muris* ES proteins stimulates long-lasting protection against a subsequent low dose infection, which naturally results in chronic infections (Shears et al., 2018). In this study, 11 potential immunogenic proteins were identified, including serpin, TCTP, GSCP and iPMG, all of which have direct homologues in *T. trichiura* (Shears et al., 2018) and could potentially be developed against the human whipworm. Despite these results constitute a great advance in the quest for a vaccine against *T. trichiura*, translation of these molecules into an effective treatment against the human whipworm will be challenging and further studies are needed.

Furthermore, several authors reported the identification of a whey acidic protein in the ES products from *T. muris*, *Tm*-WAP (r*Tm*-WAP49) (Briggs et al., 2018). In this study, the *Tm*-WAP protein was used to evaluate immunogenicity and protective efficacy in a *T. muris* infection mice model and determined that recombinant WAP protein (r*Tm*-WAP49) induces strong type 2 protective immunity (48% worm burden reduction). These authors also confirmed *Tm*-WAP is a potent immunodominant antigen abundantly secreted by *T. muris* adult worms and that recombinant *Tm*-WAP does not elicit antigen-specific IgE response. Furthermore, in this study the immunogenicity of the protein expressed with a Na-GST-1-tag (r*Tm*-WAP-F8+Na-GST-1) was shown to be protective (38% protection) in the susceptible AKR strain, although protection was related to the WAP fragment and not to the GST tag (Briggs et al., 2018), which could reflect the unessential requirement of blood in the parasite's feeding process and impact a potential pan-nematode vaccine using this enzyme as discussed above.

In the case of Ascaris, *A. suum* has been widely used to assess the protection efficacy of different recombinant proteins in a mouse model of infection, and at least 5 candidates have been characterized to date (i.e. As14,As16, As24, As37 and As-Enol-1), all of them having direct homologs in *A. lumbricoides* (reviewed in (Zhan et al., 2014). While As-Enol-1 was developed as a DNA vaccine, having 61% efficacy in terms of larval recovery (Chen et al., 2012), As14,As16, As24 and As37 were tested in recombinant form and elicited a significant protection against subsequent infection ranging from 58-69% (Tsuji et al., 2001; Tsuji et al., 2002; Tsuji et al., 2003; Islam et al., 2005). Despite As-GST-1 has been proposed as a potential candidate mainly due to its homology (>50%) to Na-GST-1 at the aminoacid level (Liebau et al., 1997), its high allergenicity will have an important impact for the design on an anti-Ascaris vaccine (Acevedo et al., 2013). Importantly, since *A. lumbricoides* feeds on the host's luminal content and not on blood, we might also speculate that this protein will not be part of the blood-feeding detoxification pathway, which might hamper its use as a vaccine, similarly to what occurs in *H. polygyrus bakeri* as described above.

## USING MURINE MODELS FOR THE DISCOVERY OF NOVEL IMMUNOMODULATORS

Despite the significant harm caused by parasitic worms, numerous investigations have shown the faculty of helminths, and hookworms in particular, to modulate inflammation and their potential to treat inflammatory diseases (Croese, 2006; Feary et al., 2009; Croese et al., 2015). Indeed, different authors have suggested that allergies and autoimmune disorders are a consequence of our altered and reduced exposure to infectious

antigens, including helminths (Wills-Karp et al., 2001; Yazdanbakhsh, 2002; Rook, 2005; Maizels and Nussey, 2013). Chronic hookworm infections are characterized by a robust and enduring Th2 cell response, and infected individuals do not show any signs of allergy and are, in fact, protected from developing allergies (Scrivener et al., 2001). In the case of hookworm models, rodents develop similar Th2 responses as observed in humans, and different studies have demonstrated the role of secreted proteins and other molecules in the immunomodulatory processes (Hewitson et al., 2009). Since the secretome of *N. americanus* was unknown until very recently (Logan et al., 2020), the scientific community has put the focus on the proteins secreted by the rodent hookworms and other STHs for their potential therapeutic action against allergies and possibly other inflammatory and autoimmune diseases (**Table 1**) (van Riet et al., 2007), including inflammatory bowel disease (IBD), type 1 diabetes, celiac disease and others (Helmby, 2015; Smallwood et al., 2017).

Human trials using live hookworm infections have been and are currently in development, but present strong limitations and challenges such as cost, reproducibility and ethical issues. Consequently, animal models are invaluable research tools that might provide new knowledge about the individual molecules involved in the immunomodulatory processes. For instance, the 41 kDa neutrophil inhibitory factor (NIF) and the tissue inhibitors of metalloprotease Ac-TMP-2 (renamed as Ac-AIP-2) were characterised from *A. caninum* and have been shown to have important anti-inflammatory properties (Xu et al., 2000; Navarro et al., 2016). Similarly, a serine protease inhibitor from *T. suis* (TsCEI), as well as the proteins triosephosphate isomerase and nucleoside diphosphate kinase have been shown to have important immunomodulatory properties (Rhoads et al., 2000; Leroux et al., 2018). Other hookworm proteins and their immunomodulatory roles have been reviewed elsewhere (Abuzeid et al., 2020; Ryan et al., 2020).

The immunomodulatory role of *H. polygyrus bakeri* is indisputable. This role has been attributed, among others, to different secreted proteins, including three proteins that belong to the complement control protein (CCP) superfamily: Hp-TGM (*H. polygyrus bakeri* TGF-β mimic), HpARI (*H. polygyrus bakeri* Alarmin Release Inhibitor) and HpBARI (*H. polygyrus bakeri* Binds Alarmin Receptor and Inhibits). Hp-TGM has been shown to drive Treg production in mice and humans by binding to the mammalian TGF-β complex, despite it has no sequence homology to mammalian TGF-β (Johnston et al., 2017). Furthermore, treatment with rHp-TGM increased the number of Treg cells in draining lymph nodes at the site of graft transplant in mice, resulting in delayed allograft rejection (Grainger et al., 2010; Johnston et al., 2017). HpARI is a cytokine-binding protein that prevents alarmin release within necrotic cells by binding directly to IL-33 and nuclear DNA (Osbourn et al., 2017; Chauché et al., 2020). Indeed, intranasally-administered rHpARI suppressed eosinophil responses and ILC2s in the lungs of mice following the exposure to *Alternaria* allergen, while it increases worm burden and suppresses type 2 responses in *N. brasiliensis*-infected mice (Osbourn et al., 2017). It has also been shown that *H. polygyrus bakeri* can block the IL-33 pathway by blocking the cytokine and its receptor *via* both HpARI and HpBARI, respectively (Vacca et al., 2020). The same authors also identified a close homologue of HpBARI (HpBARI_Hom2), which binds and inhibits the human form of the IL-33 receptor (Vacca et al., 2020). These discoveries highlight a potential use for the referred proteins in a wide variety of inflammatory settings, particularly in asthma (Chauché et al., 2020).

It is also remarkable that calreticulin from *H. polygyrus bakeri* has been shown to promote Th2 cell-responses but no further studies have explored into the immunomodulatory effects of this molecule (Rzepecka et al., 2009).

Although cystatins have been used as vaccination targets, rHp-CPI from *H. polygyrus bakeri* is also capable to modulate the activation and differentiation of bone-marrow-derived CD11c+ DC (BMDC), and to interfere with antigen and MHC-II molecule processing and Toll-like receptor signalling pathway, resulting in functionally deficient dendritic cells that induce a suboptimal immune response in mouse models. (Sun et al., 2013).

In addition to individual molecules, the recent characterization of EVs secreted by different nematodes has highlighted their

**TABLE 1** | Immunomodulatory molecules expressed in the hookworm models *Heligmosomoides polygyrus bakeri* and *Nippostrongylus brasiliensis*.

| Molecule and description | Specie | Function | Reference |
|---|---|---|---|
| Hp-TGM | *H. polygyrus bakeri* | Ligation of TGF-β receptor on T cells leading to induction of Treg cells | (Grainger et al., 2010; Johnston et al., 2017) |
| HpARI | *H. polygyrus bakeri* | Blocks human and mouse IL-33 | (Osbourn et al., 2017; Chauché et al., 2020) |
| HpBARI | *H. polygyrus bakeri* | Blocks the receptor of IL-33 | (Vacca et al., 2020) |
| HpBARI_Hom2 | *H. polygyrus bakeri* | Blocks the receptor of IL-33 | (Vacca et al., 2020) |
| Calreticulin | *H. polygyrus bakeri* | Promotes Th2 cell-responses by interacting with scavenger receptor A | (Rzepecka et al., 2009) |
| EVs | *H. polygyrus bakeri* | Suppresses host macrophage activation and inhibits expression of the IL-33 receptor subunit ST2. | (Buck et al., 2014; Coakley et al., 2017) |
| EVs | *N. brasiliensis* | Suppresses inflammatory cytokines and increases expression of IL-10 | (Eichenberger et al., 2018a) |
| Cystatin (HpCPI) | *H. polygyrus bakeri* | Modulates differentiation and activation of BMDCs resulting in non-functional dendritic cells. | (Sun et al., 2013) |

*Hp-TGM, TGF-β mimic; HpARI, Alarmin release inhibitor; HpBARI, H. polygyrus Binds Alarmin Receptor and Inhibits; EVs, extracellular vesicles.*

potential role as immunomodulators. Administration of *H. polygyrus bakeri* EVs reduced lung immunopathology by modulating innate immunity *via* suppression of the early IL-33 and the later type 2 (specially ILC2) allergic responses (Buck et al., 2014). Furthermore, *N. brasiliensis* secreted EVs suppressed the production and secretion of proinflammatory cytokines and increased the expression of IL-10, protecting mice from T-cell-dependent induced colitis (Eichenberger et al., 2018a).

In conclusion, the latest advances have highlighted the similarities between several human nematodes and their respective murine models at a genomic and proteomic level. These results highlight the suitability of these models, not only for the study of the immune responses associated to infection with STHs, but also, in some cases, for the development of new vaccine candidates and immunomodulatory molecules. However, further research should aim at integrating the different available *omic* technologies (e.g. transcriptomic, proteomic, metabolomics and lipidomic, among others) to obtain a more comprehensive picture of the biology of these worms and confidently validate candidate molecules.

## AUTHOR CONTRIBUTIONS

KM, CC, and JS analyzed the data, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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PERSPECTIVE
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# Perspectives From Systems Biology to Improve Knowledge of *Leishmania* Drug Resistance

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Neglected Tropical Diseases include a broad range of pathogens, hosts, and vectors, which represent evolving complex systems. Leishmaniasis, caused by different *Leishmania* species and transmitted to humans by sandflies, are among such diseases. *Leishmania* and other Trypanosomatidae display some peculiar features, which make them a complex system to study. Leishmaniasis chemotherapy is limited due to high toxicity of available drugs, long-term treatment protocols, and occurrence of drug resistant parasite strains. Systems biology studies the interactions and behavior of complex biological processes and may improve knowledge of *Leishmania* drug resistance. System-level studies to understand *Leishmania* biology have been challenging mainly because of its unusual molecular features. Networks integrating the biochemical and biological pathways involved in drug resistance have been reported in literature. Antioxidant defense enzymes have been identified as potential drug targets against leishmaniasis. These and other biomarkers might be studied from the perspective of systems biology and systems parasitology opening new frontiers for drug development and treatment of leishmaniasis and other diseases. Our main goals include: 1) Summarize current advances in *Leishmania* research focused on chemotherapy and drug resistance. 2) Share our viewpoint on the application of systems biology to *Leishmania* studies. 3) Provide insights and directions for future investigation.

**Keywords: *Leishmania*, chemotherapy, drug resistance, systems biology, systems parasitology, molecular networks**

## INTRODUCTION

*Leishmania* is a complex biological system in itself. In the lack of an effective vaccine, human treatment relies on chemotherapy since the early 1920's. Drug resistance of parasite strains adds a layer of complexity to this public health issue. Systems biology, which access interactions and behavior of complex biological processes, may improve knowledge of *Leishmania* drug resistance. **Figure 1** shows the major components of *Leishmania* systems biology discussed in the present work.

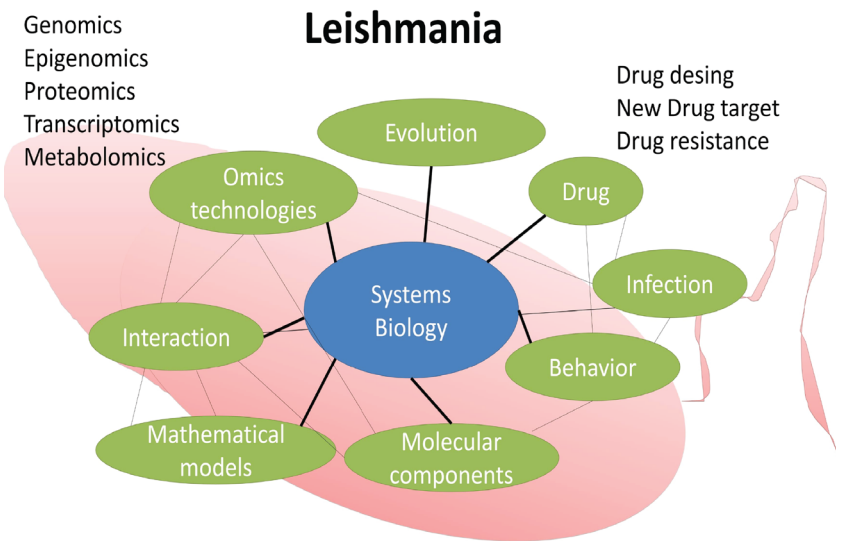


**FIGURE 1** | *Leishmania* systems biology. Some components of a systems biology study aiming at identifying molecular targets for drug design and development.

Here we present our perspective by providing a viewpoint on some specific areas of investigation as well as current advances and future directions. For this purpose, this article is organized into the following topics: *Leishmania* and leishmaniasis; Leishmaniasis treatment; Chemotherapy and antioxidant defense enzymes; Systems biology: concepts and applications; *Leishmania* systems biology; and Conclusions and future directions.

## *LEISHMANIA* AND LEISHMANIASIS

Leishmaniasis are among such diseases currently affecting 12 million people worldwide and presenting an incidence of 0.7-1.0 million new cases annually from nearly 100 endemic countries (WHO, 2021). Leishmaniasis are caused by over 21 different species of unicellular protozoan parasites of the genus *Leishmania* (Trypanosomatidae), which are transmitted to humans by infected female phlebotomine sandflies (Phlebotominae).

Some peculiar features are described for *Leishmania* and other Trypanosomatidae such as their kinetoplast, mitochondrial DNA editing (Simpson and Shaw, 1989; Ibrahim et al., 2008), glycosomes (Michels et al., 2006), polycistronic transcription (Martínez-Calvillo et al., 2003), trans-splicing (Boothroyd and Cross, 1982; Liang et al., 2003), GPI-anchored proteins (Mensa-Wilmot et al., 1999), and absence of promoter-mediated regulation of nuclear genes (Stefano et al., 2017). *Leishmania* species present a remarkable degree of conservation in gene content and architecture (synteny) according to their evolutionary divergence (Peacock, 2007; Lynn and McMaster, 2008; Real et al., 2013).

## LEISHMANIASIS TREATMENT

There is no human vaccine available against *Leishmania* infection and control is based mainly on chemotherapy using a few drugs currently available. Leishmaniasis chemotherapy presents several issues, such as high drug toxicity, long treatment protocols, and the occurrence of drug resistant parasite strains.

It is important to highlight that drug resistance and therapeutic failure are not synonymous. Therapeutic failure encompasses factors related to the host (e.g. patient immune system and genetic factors), infectious agent (e.g., drug resistance, virulence, and pathogenic profiles of parasite species or strains), drugs (e.g. pharmacodynamics/pharmacokinetics), chemotherapeutic protocol, etc. (Ponte-Sucre et al., 2017).

Nevertheless, isolate's drug resistance status is the first indication for therapeutic choice. *Leishmania* drug resistance threatens the prevention and treatment of infections. Literature shows that the mechanism of drug resistance in *Leishmania* involves different metabolic pathways including several molecular markers. However, little is known about the biochemical mechanisms underlying drug resistance in field isolates of this parasite. System biology approaches are very important to elucidate drug resistance mechanisms and identify new molecular markers and targets for drug development against leishmaniasis.

### Pentavalent Antimonials

Pentavalent antimonials (e.g. sodium stibogluconate and meglumine antimoniate) have been used as the first-line treatment in many countries (Croft et al., 2006). Their mode of action is still not completely understood. It has been reported that antimony inhibits macromolecule biosynthesis in amastigotes, possibly *via* the inhibition of glycolysis and fatty acid oxidation (Berman et al., 1987), changing the thiol redox potential (Wyllie et al., 2004), DNA fragmentation, and apoptosis (Sereno et al., 2001; Sudhandiran and Shaha, 2003).

Treatment failure with pentavalent antimony ($Sb^V$) has been reported in Bihar (India), where more than 60% of patients with visceral leishmaniasis (VL) are unresponsive to this drug

(Sundar, 2001). An epidemiological survey in this region suggested that arsenic-contaminated groundwater may also be associated with the treatment failure using $Sb^V$ (Perry et al., 2015). Different antimony-resistance mechanisms have been described including decreased antimony cellular entry, decreased drug reduction/activation, increased antimony efflux, and sequestration of the metal-thiol conjugate into vesicular membranes of *Leishmania* (Croft et al., 2006).

Some of these mechanisms were described in both experimental and clinical resistance to $Sb^V$. Several ATP-binding cassette (ABC) transporters have been involved in $Sb^V$ resistance. PGP/MRPA (ABCC3) was the first one described to be responsible for clinical resistance to $Sb^V$ in *L. donovani* (Mittal et al., 2007; Mukherjee et al., 2007). Other mechanisms involved in $Sb^V$ resistance in *L. donovani* include decreased drug uptake through inactivation of the aquaglyceroporin (AQP1) transporter (Mandal et al., 2010). AQP1 mutations are associated with a high level of antimony clinical resistance in *L. donovani* (Potvin et al., 2020).

Comparative proteomic and phosphoproteomic analyses of antimony trivalente ($Sb^{III}$)-resistant (R) susceptible (S) *L. braziliensis* lines identified several potential candidates for biochemical or signaling networks associated with the antimony resistance in this parasite (Matrangolo et al., 2013; Moreira et al., 2015). Proteomic and genomic analyses of $Sb^{III}$-resistant *L. infantum* mutants identified MRPA as a biomarker and suggested the involvement of chromosome number variations, specific gene amplifications, and SNPs as important features of antimony resistance (Brotherton et al., 2013). The transcriptomic profile showed that many pathways upregulated in *L. infantum* antimony-resistant lines are associated with protein phosphorylation, microtubule-based movement, protein ubiquitination, stress response, regulation of membrane lipid distribution, proteins involved in RNA metabolism, and other important metabolic pathways (Andrade et al., 2020). Together, these results show that the mechanism of antimony-resistance in *Leishmania* is complex and multifactorial, identifying several candidate genes that may be further evaluated as molecular targets for chemotherapy of leishmaniasis.

Several groups have used proteomic approaches for understanding the mechanisms of clinical resistance to antimony using $Sb^V$-resistant *L. donovani* isolates (Vergnes et al., 2007; Kumar et al., 2010; Biyani et al., 2011). These studies showed that the SbV-resistant *L. donovani* isolates have upregulated proteins of different metabolic pathways including glycolysis, gluconeogenesis, oxidative stress, and detoxification. Some of them include: ABC transporter, HSP-83, HSP-70, GPI protein transamidase, enolase, carboxypeptidase, among others.

Studies also demonstrated that the mechanism of antimony-resistance differs among the *Leishmania* species analyzed. A comparative proteomic analysis of $Sb^{III}$-susceptible and resistant lines of *L. braziliensis* (LbWTS and LbSbR) and *L. infantum* (LiWTS and LiSbR) showed that 71.4% of protein spots with differential abundance identified were different between both species (Matrangolo et al., 2013). Only 28.6% of protein spots were common between them. Western blotting analysis confirmed the proteomic data results. For instance, the expression of pteridine reductase was higher in the LbSbR line compared to its susceptible counterpart LbWTS. However, the expression level of the PTR1 protein was similar between both *L. infantum* lines (Matrangolo et al., 2013). Functional analysis confirmed that pteridine reductase is associated with the antimony-resistance phenotype in *L. braziliensis*, but not in *L. infantum* (Moreira et al., 2016).

### Amphotericin, Miltefosine, and Paromomycin

Amphotericin has shown efficacy for the VL treatment (Balasegaram et al., 2012). This is a polyene antibiotic that targets ergosterol, the major parasite membrane sterol. Liposomal amphotericin B shows lower toxicity compared to amphotericin B deoxycholate; however, it has a high cost. Amphotericin-resistant *L. donovani* lines selected *in vitro* displayed changes in drug-binding affinity to the plasma membrane as a result of a modified sterol composition (Mbongo et al., 1998). Treatment failure with amphotericin B has now been reported in India, where this drug has become the first-line option in areas where refractoriness to antimony is widespread (Purkait et al., 2012).

Miltefosine (hexadecylphosphocholine) is a phosphatidylcholine analogue initially developed as an antineoplastic drug shown to be very effective for the VL treatment in India (Sundar et al., 2002). This is the first and only drug administered orally against leishmaniasis. Miltefosine interferes in cell membrane composition by inhibiting phospholipid metabolism (Rakotomanga et al., 2007). The main mechanism of experimental resistance observed is associated with a significant reduction in miltefosine internalization (reduced uptake or increased efflux). Mutations or deletions in the miltefosine translocation process in *L. donovani* are associated with miltefosine resistance in both *in vitro* and *in vivo* assays (Perez-Victoria et al., 2006; Seifert et al., 2007). MT and/or Ros3 have also been associated with miltefosine-resistant phenotype in clinical isolates from leishmaniasis patients (Mondelaers et al., 2016; Srivastava et al., 2017).

Paromomycin is an aminoglycoside antibiotic that changes in the parasite protein synthesis, lipid metabolism, and mitochondrial activity (Maarouf et al., 1995; Maarouf et al., 1997). Clinical trials carried on in India indicated that paromomycin was effective in the VL treatment (Sundar et al., 2007). In contrast, a lower cure rate was not found in East Africa (Hailu et al., 2010). Paromomycin-resistant parasites selected *in vitro* showed a decreased drug accumulation (Bhandari et al., 2014). Differences in paromomycin susceptibility have been observed in different *Leishmania* species and clinical isolates (Prajapati et al., 2012).

## CHEMOTHERAPY AND ANTIOXIDANT DEFENSE ENZYMES

Trypanosomatidae antioxidant defense has been indicated as a potential target for chemotherapy based on their mechanism for trypanothione-dependent detoxification of peroxides, which

differs from vertebrates. In this system, the thiol trypanothione maintains the reduced intracellular environment by the action of a trypanothione reductase (Turrens, 2004). Other enzymes participate in the enzymatic cascade.

Superoxide dismutase removes the excess of superoxide radicals by converting them to oxygen and hydrogen peroxide. Besides, tryparedoxin peroxidase and ascorbate peroxidase metabolize hydrogen peroxide into water molecules (Turrens, 2004). In order to investigate these enzymes in the antimony-resistance phenotype, *L. braziliensis* and *L. infantum* mutant lines overexpressing them were obtained (Andrade and Murta, 2014; Tessarollo et al., 2015; Moreira et al., 2018).

Results showed that the overexpression of iron superoxide dismutase-A (Tessarollo et al., 2015), tryparedoxin peroxidase (Andrade and Murta, 2014), or ascorbate peroxidase (Moreira et al., 2018) are involved in the $Sb^{III}$-resistance phenotype in *L. braziliensis*. However, only iron superoxide dismutase-A plays a key function in maintaining the antimony resistance in the *L. infantum* line analyzed, while the other two enzymes are not directly associated with such phenotype. These results corroborate once again that the mechanism of antimony resistance differs among the *Leishmania* species.

Drug repositioning is an effective strategy to find new applications for existing drugs (Andrade-Neto et al., 2018; Silva et al., 2021). Thus, drugs and/or compounds that interact with different proteins involved in important metabolic pathways in *Leishmania* were searched. The ascorbate peroxidase sequence of *Leishmania* was used to seek possible drugs against this enzyme. This search returned the antibacterial agent Isoniazid, a synthetic derivative of isonicotinic acid used in tuberculosis treatment.

Results demonstrated that overexpression of ascorbate peroxidase confers resistance to Isoniazid (Moreira et al., 2018). Surprisingly, Isoniazid raised the antileishmanial effect of $Sb^{III}$, mainly against *L. braziliensis* clones overexpressing ascorbate peroxidase. Such drug combination might be a good strategy to be considered in leishmaniasis chemotherapy.

## SYSTEMS BIOLOGY: CONCEPTS AND APPLICATIONS

The origin of systems biology is still under debate among scientists, with some claiming that it was first applied by Norbert Wiener and Erwin Schrödinger or Claude Bernard around 90 and 150 years ago, respectively (cf. Saks et al., 2009). Despite different viewpoints, some agree that systems biology was first coined in the 1960s, when theoretical biologists began creating computer-run mathematical models of biological systems (Noble, 1960).

In our view, systems biology is the study of the interactions and behavior of complex biological processes based on their molecular constituents. The applied analytical approach focuses on the quantitative measurement of biological processes, mathematical modeling, and reconstruction with the aim of bringing to light the transfer of information resulting from the integration of biological data (Kirschner, 2005, Noble and Bernard, 2008, Breitling, 2010).

Systems biology is interdisciplinary and includes a wide range of data from *in vivo*, *in vitro*, *in situ*, and *in silico* studies. Ideally, *in silico* studies would be integrated and validated by other data sources especially when applicable outcomes are aimed (Butcher et al., 2004; Dunn et al., 2010; Bora and Jha, 2019).

Currently, mathematical models have been extensively used to understand biological processes in life sciences, including but not restricted to the analysis of genomics, proteomics, metabolomics, and epigenomics of a broad range of taxa (Zhao and Li, 2017; Cheng and Leung, 2018; Djordjevic et al., 2019).

Considering the complex parasite biology, the study criteria are crucial for choosing the dataset to be analyzed, taking into account the number of samples, amount of noise, experimental design, etc. Together, these criteria interfere in the network construction and downstream analyzes. The statistical network inference method, type of interaction structure (scale-free, random, and small-world), and error measurement (global and local) are also relevant.

Because of the complexity of biological systems, it is important to understand the interactions among genotype, phenotype, and environment. Systems biology addresses such aspects by applying quantitative measurement, mathematical modeling, and interdisciplinary studies including ecology and evolutionary biology (Kirschner, 2005; Medina, 2005).

By using a system biology approach, a large number of non-linear molecular interactions can be explored, such as post-transcriptional or post-translational modifications, metabolic effects, and protein recruitment dynamics in different cellular compartments. The idea is to go beyond the simplistic model of gene role determination and its phenotypic effect (Likić et al., 2010).

## *LEISHMANIA* SYSTEMS BIOLOGY

Efforts of drug repositioning and development of new drugs require systems biology approaches to understand the genetic basis of diseases including leishmaniasis. An essential aspect of systems biology in drug discovery is the identification of potential drug targets considering the presence of multiple genes and proteins involved (Kunkel, 2006; Chavali et al., 2008; Sharma et al., 2017). In parasites, this might be understood from signaling pathways in which essential proteins participate (Sharma et al., 2017). In addition to derive novel biological hypotheses about molecular interactions involved in drug resistance, such networks may provide information to support functional prediction of genes and proteins. Currently, a huge number of protein coding genes from sequencing projects are annotated with hypothetical, predicted, or unknown functions.

The so-called omics technologies have been the driving force behind systems biology (Silva et al., 2012; Moreira et al., 2015). These technologies include genomics, transcriptomics, proteomics, and among others applied to the study of a broad range of taxa including *Leishmania* (**Figure 1**). This multidirectional and interdisciplinary integration will certainly provide experimental outcomes with impact in public health.

The divide-and-conquer approach, in which a big problem is recursively breaking down into sub-problems of the same or related type simple enough to be solved, is a robust strategy to be implemented. Among the numerous fields (sub-problems) in which systems biology would play a crucial role, we highlight the issue of drug resistance in *Leishmania* treatment (Ponte-Sucre et al., 2017).

The inference of gene regulatory networks is just a "blueprint" in the discovery of new interactions among biological entities of the drug resistance in *Leishmania* and other taxa. Here we provide an overview of some components of a systems biology study aiming at identifying molecular targets for drug design and development in *Leishmania* (**Figure 1**).

The genome-scale metabolic model of *L. donovani* supported functional annotation for hypothetical or erroneously annotated genes by comparing results with experimental data (Rezende et al., 2012; Sharma et al., 2017). In addition to annotation, authors have predicted molecular networks for *Leishmania* and other Trypanosomatidae (Rezende et al., 2012; Vasconcelos et al., 2018).

System biology studies of pathway modeling may be able to identify pathways associated with mechanisms of drug resistance in *Leishmania* (Brito et al., 2017; Ponte-Sucre et al., 2017). Results of *in vitro* approaches for the identification of genes or proteins associated with drug resistance should be integrated with *in silico* studies and used for validation of the omics strategies (Kunkel, 2006). Combined drug and vaccine therapy can successfully treat leishmaniasis patients, but there are still several side effects and a high cost involved (Ghorbani and Farhoudi, 2017).

In the case of the pentavalent antimonials, a network integrating biochemical and biological pathways is reported (Ponte-Sucre et al., 2017). For instance, the ABC transport pathway is involved in drug efflux and therefore with drug resistance (Coelho and Cotrim, 2013). Aquaglyceroporin overexpression or deletion is also associated with resistance (Marquis et al., 2005). Gamma-glutamylcysteine synthetase may protect against oxidative stress and $Sb^{V}$ (Mukherjee et al., 2009). Reduction of $Sb^{V}$ to $Sb^{III}$ is involved in drug activity and internalization as well as glycolysis inhibition of fatty acid oxidation (Berman et al., 1987; Roychoudhury and Ali, 2008). Trypanothione and glutathione regulate the intracellular thiol redox balance and participate in the chemical and oxidative stress defense (Croft et al., 2006; Singh, 2006; Maltezou, 2010). Tryparedoxin peroxidase from a complex redox cascade and its overexpression is linked to resistance (Wyllie et al., 2008; Andrade and Murta, 2014). Zinc finger domains are associated with drug resistance due to the ability of $Sb^{III}$ to compete with $Zn^{II}$ and the modulation of the pharmacological action of antimonials (Frézard et al., 2012).

One possible approach is the integration of public available RNAseq data depicting the resistance phenomena in gene regulatory networks (Andrade et al., 2020). Such approach has demonstrated how genes interact with each other and how changes in their expression levels may result, for example, in different immunological responses promoting distinct disease outcomes including leishmaniasis (Mol et al., 2018).

The resulting association among specific transcriptional states of all genes involved in drug resistance will represent a key tool for the study and modeling of this complex biological process. Altogether, these studies have the potential to lead the identification of better drug targets and markers for pathogenesis.

## CONCLUSIONS AND FUTURE DIRECTIONS

Computational modeling of the molecular components of drug resistance in *Leishmania* through the biophysicochemical monitoring of genes and proteins involved in the processes is important. Integrating metabolic and signaling pathways is crucial to reveal the correlations among molecular functions and physiological processes shedding light on a broad understanding of the drug resistance phenomena.

We believe that in a near future, neither the understanding of Trypanosomatidae biology nor their drug resistance phenomena will be conceivable without studying molecular networks. In this context, protein-protein interactions and gene regulatory networks represent a practical embodiment of systems biology.

Biomarkers involved in drug resistance might be studied into more details from the systems biology perspective. Altogether, these studies could contribute to a better understanding of parasite biology and drug resistance mechanisms. Moreover, this approach will improve the knowledge of systems parasitology and open new frontiers in the identification of new molecular targets for drug development and treatment of leishmaniasis and other diseases.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

LN: designed and coordinated this work. JR, SM, and LN: wrote and revised the manuscript. EH and JH: collected data, wrote, revised the manuscript, and developed the artwork (figure). All authors contributed to the article and approved the submitted version.

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REVIEW
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# Autonomous Non Antioxidant Roles for *Fasciola hepatica* Secreted Thioredoxin-1 and Peroxiredoxin-1

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Trematode parasites of the genus *Fasciola* are the cause of liver fluke disease (fasciolosis) in humans and their livestock. Infection of the host involves invasion through the intestinal wall followed by migration in the liver that results in extensive damage, before the parasite settles as a mature egg-laying adult in the bile ducts. Genomic and transcriptomic studies revealed that increased metabolic stress during the rapid growth and development of *F. hepatica* is balanced with the up-regulation of the thiol-independent antioxidant system. In this cascade system thioredoxin/glutathione reductase (TGR) reduces thioredoxin (Trx), which then reduces and activates peroxiredoxin (Prx), whose major function is to protect cells against the damaging hydrogen peroxide free radicals. *F. hepatica* expresses a single TGR, three Trx and three Prx genes; however, the transcriptional expression of Trx1 and Prx1 far out-weighs (>50-fold) other members of their family, and both are major components of the parasite secretome. While Prx1 possesses a leader signal peptide that directs its secretion through the classical pathway and explains why this enzyme is found freely soluble in the secretome, Trx1 lacks a leader peptide and is secreted *via* an alternative pathway that packages the majority of this enzyme into extracellular vesicles (EVs). Here we propose that *F. hepatica* Prx1 and Trx1 do not function as part of the parasite's stress-inducible thiol-dependant cascade, but play autonomous roles in defence against the general anti-pathogen oxidative burst by innate immune cells, in the modulation of host immune responses and regulation of inflammation.

**Keywords: *Fasciola*, helminth, antioxidants, thioredoxin, thioredoxin peroxidase, peroxiredoxin, immunomodulation, inflammation**

## INTRODUCTION

Digenean trematodes are internal obligate parasites responsible for a plethora of foodborne zoonotic diseases in humans and their livestock. They have a complex life cycle that involves migration within multiple different intermediate and definitive host species. They can reside within their definitive mammalian host for years, and even decades. They include the liver flukes (*Fasciola* spp., *Opisthorchis* spp., and *Clonorchis* spp.), blood flukes (*Schistosoma* spp.) and lung flukes

(*Paragonimus* spp.) that, collectively, infect over 250 million people worldwide (Keiser and Utzinger, 2009; Furst et al., 2012).

Fasciolosis caused by infection with *Fasciola hepatica* is classically associated with livestock (sheep and cattle) on farms in temperate climates. Due to human migration and animal trade over the past few centuries the disease has one of the most widespread geographical distributions of any helminth (Robinson and Dalton, 2009). The spread of the disease has been enhanced by the superior adaptability of this parasite to its different hosts since it can infect, develop and produce off-spring in many mammals that it has only encountered in relatively recent times e.g., camelids, capybara and kangaroos. Fasciolosis caused by *Fasciola gigantica*, on the other hand, is most prevalent in tropical regions where it is most commonly found in cattle and water buffalo (Copeman and Copland, 2008; Mas-Coma et al., 2019). Where both *F. hepatica* and *F. gigantica* are sympatric, for example in China, Korea, and Southeast Asia, hybrids forms of the parasite have emerged (Calvani and Šlapeta, 2021).

Both *F. hepatica* and *F. gigantica* have a similar life cycle involving an intermediate snail and definitive mammalian host. The mammalian hosts become infected after they consume encysted parasites (metacercariae) attached to vegetation (grass, rice) or floating in water (Andrews, 1999). The metacercariae emerge from their cysts as newly excysted juveniles (NEJs) in the low-oxygen environment of the small intestine and, with the assistance of abundant protease secretion, traverse the intestinal wall within hours (Andrews, 1999; Cwiklinski et al., 2018). Aside from small tracks, the microscopic *F. hepatica* NEJs leave little clinical evidence of their travels through the intestinal wall that lack any signs of immune cellular infiltration to the vicinity of challenge in naïve animals (Van Milligen et al., 1998).

Serious damage begins after the parasite enters the liver and begins migration through the parenchymal tissues, again with the aid of secreted proteases, causing excessive haemorrhaging, which results in anaemia (Molina-Hernández et al., 2015). It is this damage that results in poor animal growth and loss of productivity (wool, meat and milk yields), the extent of which depends on the level of infection where large numbers of *F. hepatica* entering the liver around the same time can cause sudden death in sheep (Molina-Hernández et al., 2015; Nadis, 2020). After about 8-12 weeks *F. hepatica* migrates into the bile ducts where it matures and uses the nutrients from its obligate blood feeding activity to produce numerous progeny in the form of eggs. Eggs are passed in faeces, where they eventually embryonate on pasture before hatching to release miracidia that go on to infect the intermediate snail host. Within the snail host the parasites multiply *via* clonal expansion before emerging as cercariae that encyst as metacercariae on vegetation contaminating pastures, thus continuing the life cycle (Graczyk and Fried, 1999; Hodgkinson et al., 2018).

Similar to that observed in other helminth infections, the early immune response to the invading parasite is mixed or non-polarised (Espino and Rivera, 2009). However, within a week of infection the developing immune response in mice exhibits all the hallmarks of a strongly polarised Th2-driven response; including, the dominance of IgG1 antibody isotypes over IgG2 (Phiri et al., 2006), the recruitment and proliferation of eosinophils (Ruiz-Campillo et al., 2017), the differentiation of alternatively activated macrophages (M2s) (Donnelly et al., 2008), the secretion of IL-4/IL-5/IL-13 by T-cells (Espino and Rivera, 2009), as well as the suppression of Th1-associate cytokines (O'Neill et al., 2000). Studies in ruminants suggest that sheep and cattle also elicit Th2-driven responses to acute infection, which progresses into a hyporesponsive or immunosuppressive state as the disease becomes chronic (Escamilla et al., 2016; Sachdev et al., 2017). Several studies suggest that Th2-driven immune responses make the host susceptible to subsequent infection (Aitken et al., 1981; Chauvin et al., 1995; Brady et al., 1999; Cwiklinski et al., 2016), and both infection and vaccine studies suggest that it is necessary to induce Th1-mediated responses for protection to be achieved (Pleasance et al., 2011; Villa-Mancera et al., 2014; Noya et al., 2017).

## THE BEST FORM OF DEFENCE IS HAVING A GOOD OFFENCE

The ability of *F. hepatica* to survive and thrive in its varied mammalian hosts for such a long time is reflective of the parasites' capacity to evade, modulate or supress the host's immune responses (Molina-Hernández et al., 2015). The parasites immune evasion techniques include the continual sloughing of their exterior 'fuzzy' surface glycocalyx, along with bound host antibody, thus rendering it ineffective (Hanna, 1980a; Lammas and Duffus, 1983; Haçarız et al., 2011). Additionally, the antigenic and structural composition of the glycocalyx changes as the parasites migrate from intestine to liver to bile duct, leaving the successively-mounting host immune responses redundant (Hanna, 1980b). Histological observations of livers taken from infected hosts show that the damage caused by the aggressive and rapid tunnelling of the parasite becomes infiltrated with an immense amount of immune cells (eosinophils, lymphocytes, macrophages). However, rather than killing the parasite these cells appear to be playing the role of plugging the tracts left in the parasites' wake, preventing excessive blood loss and, most importantly, facilitating wound repair as the tracts gradually become fibrotic and sealed with collagen (Zafra et al., 2013a; Zafra et al., 2013b; Frigerio et al., 2020).

The best form of defence, however, is having a good offence, and helminth parasites achieve this largely by the excretion/secretion of a multitude of immune-impairing, -suppressive or -modulatory factors (Hewitson et al., 2009; Ryan et al., 2020). In the case of *F. hepatica*, these include proteases, protease inhibitors, antioxidants, cathelicidin-like helminth defence molecules (HDM) and glycolytic enzymes (Jefferies et al., 2001; Morphew et al., 2007; Ryan et al., 2020), many of which have been shown to influence different aspects of the host's immune response. For example, early studies showed that secreted cysteine proteases (now known as cathepsin B and cathepsin L proteases) can specifically cleave

immunoglobulins (Igs) at their hinge region, separating the antibody binding Fab fragment from the Fc domain and thus preventing the ability of bound Ig to attract Fc-binding innate immune cells (eosinophils, macrophages) (Chapman and Mitchell, 1982). The secreted *F. hepatica* HDMs abrogate NLP3-inflammasome-mediated inflammatory responses in innate immune cells by impairing lysosomal acidification (Robinson et al., 2012; Alvarado et al., 2017), while fatty acid binding proteins (FABP 12/15) are shown to induce alternatively activated macrophages that overexpress anti-inflammatory cytokines, thereby contributing to a hyporesponsive environment favoured by the parasite (Figueroa-Santiago and Espino, 2014; Ruiz-Jiménez and Espino, 2014; Ramos-Benítez et al., 2017). Several molecules, such as *F. hepatica* cathepsin L1 cysteine proteases, glutathione S- transferases (GST) and Kunitz-type molecules, reduce the capacity of dendritic cells to induce the robust T-cell responses required to effectively eliminate the parasite (Dowling et al., 2010; Falcon et al., 2014; Ryan et al., 2020). Glycosylated mucins and TGF-β mimics secreted by invading parasites may also play immunosuppressive or immunoregulatory roles by influencing DC or T-cell phenotype differentiation to block Th1 type responses developing (Musah-Eroje and Flynn, 2018). Metabolism-associated enzymatic factors liberated by the parasite and not classically associated with immunosuppression, such as fructose-bisphosphate aldolase and glyceraldehyde phosphate dehydrogenase, have recently been shown to bind to the host's immune mediating factors such as IFN-γ, IL-2 and IL-17 (Liu et al., 2017). However, the exact effect that the binding of *F. hepatica* products has on these factors remains to be elucidated.

Detailed genomic, transcriptomic, and proteomic (somatic and secretome) analyses that have emerged over the last few years have revealed that the liver fluke parasite tightly regulates the expression and secretion of many molecules during its migration in the mammalian host (Cwiklinski and Dalton, 2018). The growth from a microscopic organism to a mature egg-producing adult parasite (~2 cm x 1 cm) is correlated with the up-regulation and differential expression of a range of gene families critical for the stage-specific phases within the mammalian host (Cwiklinski et al., 2015). In particular, an increased transcription of >8000 transcripts, many of which encode pathways involved with intense signal transduction, protein production and neoblast development, is observed when the parasite invades and migrates through the liver parenchyma (Cwiklinski et al., 2021). Direct associations can also be made between transcript up-regulation and the secretion of molecules involved in the parasite-host inter-relationship e.g., the aforementioned secreted immune regulatory proteases, protease inhibitors and HDMs (Robinson et al., 2009; Cwiklinski et al., 2018; Cwiklinski et al., 2021).

An interesting observation emerged when the expression of various antioxidant enzymes of the migrating parasite were examined alongside the expression of homologous enzymes in the host liver, suggesting that metabolic stress occurs in both the parasite and liver (Cwiklinski et al., 2021). The mammalian liver is naturally a high-metabolising organ susceptible to oxidative stress in many infectious and non-infectious chronic diseases, including hepatitis C, alcoholic liver disease and liver fibroproliferative disease (Cichoż-Lach and Michalak, 2014). The elevation of metabolic stress in the liver during infection with *F. hepatica* is therefore not surprising since a major effort is required to recruit immune cells to damaged areas in order to minimise necrosis, repair perforated tissue and to induce fibrogenesis (Saleh, 2008; Bottari et al., 2015; Da Silva et al., 2017). In contrast, the metabolic stress exerted on the parasite arises from two sources; (1) internally by the increased cellular metabolic activity and associated generation of reactive oxygen species (ROS) driven primarily as a result of the aerobic respiration by the rapidly growing and developing parasite, and (2) externally by the parasite's need to respond to the general anti-pathogen ROS burst from the host's innate immune cells. Both of these metabolic pressures demand the up-regulation of transcripts encoding superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), as well as members of the thiol-dependent antioxidant system (**Figure 1A**). The thiol-dependent antioxidant system is the primary means by which cells and parasites defend against the major biological ROS hydrogen peroxide ($H_2O_2$). Recent studies have, however, shown that various players in this system are not only part of a defence mechanism but may also perform a range of functions that can be central to the parasite's ability to manipulate the host immune responses (Ishii et al., 2012; Perkins et al., 2014).

## ENZYMES IN THE *F. HEPATICA* THIOL-DEPENDENT ANTIOXIDANT CASCADE

The *F. hepatica* thiol-dependent antioxidant cascade includes the enzymes thioredoxin-glutathione reductase (TGR), thioredoxin (Trx) and thioredoxin peroxidase/peroxiredoxin (Prx). These proteins interact *via* a redox cascade event whereby TGR reduces Trx with the assistance of nicotinamide adenine dinucleotide phosphate (NADPH), which in turn reduces Prx to recharge its redox state and activates its antioxidant properties (**Figure 1A**).

TGR, the first protein involved in this redox antioxidant cascade, is an oxidoreductase enzyme that can reduce both Trx and glutathione disulfide (GSSG) in a 1:1 ratio, in much the same way as thioredoxin reductase (TrxR) and glutathione reductase (GR) separately perform this function in mammalian cells. It is important to note, however, that no independent TrxR or GR enzymes have been identified in *F. hepatica* (Guevara-Flores et al., 2011). Sequence analysis of TGR has identified binding domains suitable for both NADPH and FAD, a thiol-disulphide redox active centre that has been described for mammalian TR and GR, as well as a glutaredoxin (Grx) domain (Maggioli et al., 2011). TGR enzymes are atypical due to the presence of a selenocysteine insertion sequence (SECIS) element, encoded by a TGA codon that enables the incorporation of selenium into the TGR protein (Böck et al., 1991). A study of the structure of *F. gigantica* TGR demonstrated the requirement for the selenocysteine (Sec) element for both TrxR and GR activity (Kalita et al., 2018). The enzymes' high sensitivity to inhibition

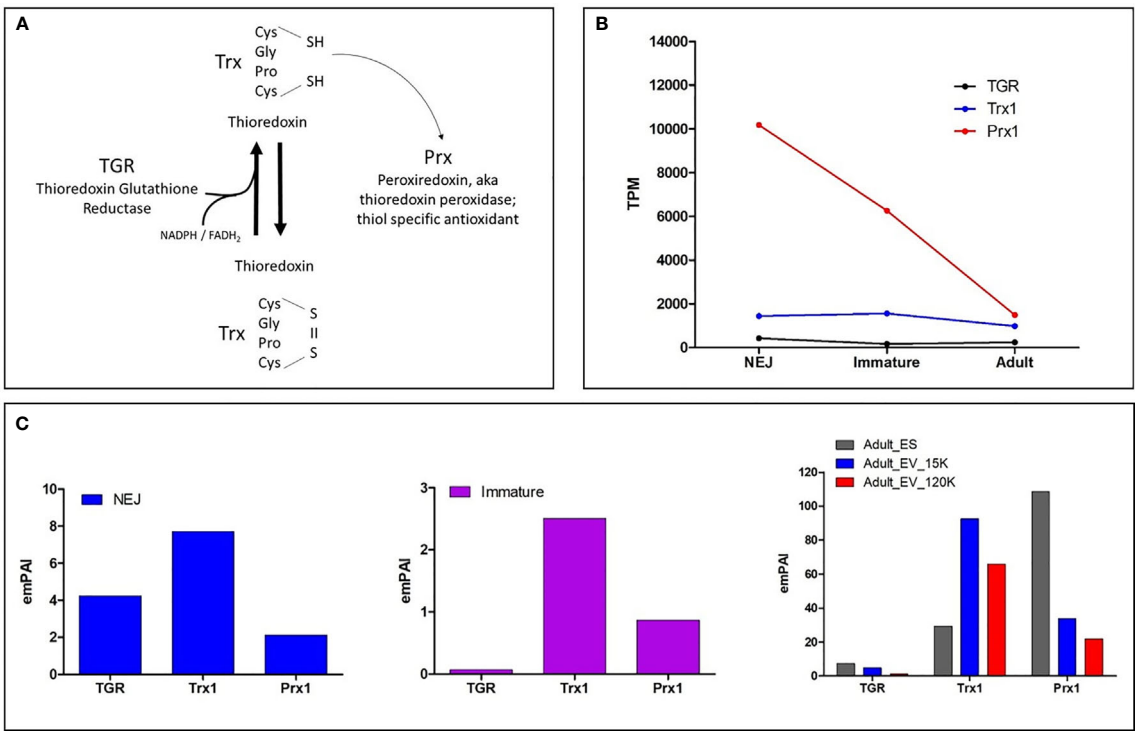


**FIGURE 1** | **(A)** Schematic of the generalised model of the thiol-dependent antioxidant cascade. TGR with the assistance of $FADH_2$ and NADPH converts oxidised Trx to reduced Trx which subsequently reduces Prx to its activated form. **(B)** Graphical representation of the most abundantly transcribed genes of the three enzymes in the *F. hepatica* thiol-dependent antioxidant cascade by the newly excysted juveniles (NEJ) that traverse the small intestinal wall, the liver stage immature parasites 21 days post infection (Immature) and the mature adult stage parasite that resides within the bile ducts (Adult). Data is displayed as transcripts per million (TPM) and is extrapolated from the transcriptome study by Cwiklinski et al. (2015). **(C)** Graphical representation of the protein abundance within the NEJ, Immature and Adult parasite secretomes (ES proteins), represented by Exponentially Modified Protein Abundance Index (emPAI). The Adult secretome data shows the protein abundance within the extracellular vesicles, specifically the microvesicles recovered following centrifugation at 15, 000 x g (Adult_EV_15K) and the exosomes isolated after centrifugation at 120, 000 x g (Adult_EV_120K), in addition to the EV-depleted ES proteins. The proteomic data for NEJ, immature and adult parasites is extrapolated from Cwiklinski et al. (2018), Cwiklinski et al. (2021) and Murphy et al. (2020), respectively.

by aurothioglucose confirmed it as a selenoenzyme (Maggioli et al., 2004). The inhibition of *Schistosoma mansoni* TGR by auroanofin, an antirheumatic compound (Kuntz et al., 2007), is facilitated by the binding of the compound between the catalytic cysteines of the FAD-binding site (Cys154-Cys159), preventing the donation of electrons to the enzyme by flavin adenine dinucleotide (FAD) (Angelucci et al., 2009). Similar investigations into the inhibition of *F. gigantica* TGR by auranofin determined that it is the interaction of the gold particle of auranofin with His571 of TGR that results in the inhibition of enzyme activity (Kalita et al., 2018). The *S. mansoni* TGR was proposed as a tractable drug target due to the parasites' inability to survive in the presence of auranofin (Feng et al., 2020).

Trx is a ~12 kDa oxidoreductase protein with a catalytically active dithiol site that reduces exposed disulfide bridges on Prx and other proteins. It has a conserved structure composed of a core that is formed from a four-stranded β-sheet, surrounded by three α-helices (Eklund et al., 1984; Martin, 1995). The tryptophan-cysteine-glycine-proline-cysteine (WCGPC) motif of the Trx active site protrudes from the 3-D structure of the molecule and is highly conserved (Shoda et al., 1999). The redox active cysteine pair (Cys31 and Cys34) (Ren et al., 2017) enables the enzyme to exist in either the oxidised disulphide state or the reduced dithiol state (Line et al., 2008). A proline situated between the two cysteines (Pro33) is essential to facilitate the reducing power of Trx (Collet and Messens, 2010). The substitution of this proline with histidine in *Escherichia coli* Trx resulted in a lower reducing potential compared to the wild-type enzyme (Krause et al., 1991). Similarly, in *Staphylococcus aureus* the substitution of the proline with either serine or threonine resulted in a seven-fold reduction in the reducing potential of the enzyme (Roos et al., 2007).

Prxs are found in both prokaryotes and eukaryotes and are well characterised in many protists and helminth parasites (see review by Angelucci et al., 2016). Their discovery in *F. hepatica* helped explain how helminth parasites deal with SOD-generated hydrogen peroxide since they lack the enzyme catalase that together with glutathione peroxidase (GPx) converts the toxic reactive oxygen molecules into water and hydrogen peroxide (McGonigle et al., 1997; McGonigle et al., 1998). Since the discovery of Prx, the enzyme has undergone several name

changes, first described as thiol specific antioxidants and then thioredoxin peroxidase (which often still appears in helminth proteomic studies) before being termed peroxiredoxin (**Figure 1**). Prx is a 25 kDa enzyme that when activated to its reduced form by Trx, provides protection to the parasite *via* the breakdown of hydrogen peroxide (McGonigle et al., 1997), a mechanism reliant upon a conserved cysteine residue in the enzyme's $NH_2$-terminal portion (Rhee et al., 2001). The enzyme found in *F. hepatica* shows high levels of homology to other peroxiredoxin enzymes, including those found in rodents, ruminants, and humans (Salazar-Calderón et al., 2000). The peroxiredoxin of *F. hepatica* is a 2-Cys enzyme, the most widely distributed subfamily of Prxs (Hall et al., 2009), that is characterised by two active cysteine residues at positions 47 and 170 (McGonigle et al., 1997). Incubation of recombinant *F. hepatica* peroxiredoxin, rFhePrx, with super-coiled plasmid DNA and DTT demonstrated the ability of the antioxidant to protect against oxidative stress (Sekiya et al., 2006), and supports the idea that *F. hepatica* produces Prx in order to protect itself from oxidative damage within the host environment. It has been shown that Prxs can form dimers and higher molecular size multimers depending on the redox status of the cell; at low oxidative stress they can act as peroxidases whereas at high levels of stress they act as holdases, enzymes that can assist the non-covalent folding of proteins and prevent protein aggregation (Jang et al., 2004; Teixeira et al., 2015). This interchange of oligomeric states has been shown for the 2-Cys peroxiredoxins of adult *S. mansoni* and provides an explanation of how a sensing mechanism for hydrogen peroxide concentration can be translated to a functional molecular switch (Saccoccia et al., 2012). At present, we can only assume a similar mechanism exists for *F. hepatica* Prx.

## WHAT DOES OUR -OMICS ANALYSIS REVEAL ABOUT THE ENZYMES IN THE *F. HEPATICA* THIOL-DEPENDENT ANTIOXIDANT CASCADE?

Our recent analysis of the *F. hepatica* genome published by Cwiklinski et al. (2015) revealed that TGR is encoded by a single copy gene, and transcriptomic analysis shows this is constitutively expressed during the stages that infect the mammalian host. The genome contains three genes that encode Trx enzymes, Trx1, 2 and 3, all of which are also constitutively expressed. However, Trx1 is expressed >50 times higher than both Trx2 and 3 (each less than 40 transcripts per million; TPM) (**Figure 1B**). There are also three Prx genes present within the *F. hepatica* genome (Prx1, 2 and 3) but, as in the case for the Trxs, the expression of one, Prx1, greatly outweighs the other two genes. Prx1 displays stage-specific transcription, with the highest expression observed during the NEJ stage at >250 times greater (~10,000 TPM) than the other two Prx genes and the other members of the thiol-dependent antioxidant cascade (**Figure 1B**). This disparity in the expression of Trx1 and Prx1 sets them apart from the other members of their family, implying that they play alternative roles.

Proteomic profiling of the secreted proteins of the infectious NEJs, immature 21-day post-infection and adult parasites identified TGR, Trx1 and Prx1 but no other members of the thiol-dependent antioxidant cascade (Cwiklinski et al., 2018; Murphy et al., 2020; Cwiklinski et al., 2021) (**Figure 1C**). TGR was detected in the NEJ secretome, but was only minimal in that of the immature and mature adult parasite. In contrast, both Trx1 and Prx1 were abundant in all secretomes, although Trx1 was more dominant in the NEJ stage while, conversely, Prx1 was most abundant in the adult parasite secreted products. We also found Trx1 and Prx1 enzymes within the contents of different sized extracellular vesicles/exosomes recovered by differential centrifugation (15K and 120K; Murphy et al., 2020) from adult worm secretory products. The majority of Trx1 observed was associated with the microvesicles (15K) while Prx1, although present within both the EV fractions, was predominantly in the soluble non-vesicle fraction and thus it seems unlikely that the two interact in a reducing cascade.

## LEADER (LP) AND LEADERLESS (LLP) PROTEIN SECRETORY PATHWAYS: TRX1 AND PRX1 GO THEIR SEPARATE WAYS

Since Trx1 and Prx1 are found in abundance in the secretory products of *F. hepatica*, it follows that they are readily released into the extracellular environment by the parasite and, by extension, likely to be released *in vivo* during infection (Murphy et al., 2020). Conventionally in eukaryotic organisms, proteins destined for secretion contain an N-terminal hydrophobic signal peptide that targets the protein for translocation and processing in the endoplasmic reticulum and Golgi apparatus. The proteins are subsequently packaged into secretory vesicles that fuse to the plasma membrane and release the proteins freely into the extracellular environment (Palade, 1966; Blobel and Dobberstein, 1975). Our recent analysis of the *F. hepatica* genome discovered that Prx1 is unique amongst the three-membered family in possessing a signal secretory peptide suggesting it is secreted *via* the classical leader pathway (LP). This would explain its predominance in the secretome and, more relevantly, in the freely-soluble fraction.

In contrast, Trx1 falls into the general category of leaderless secretory proteins (LLPs), of which the mechanism of cellular release remains less clearly understood but may occur through numerous unconventional processes (Sitia and Rubartelli, 2020). Direct translocation of LLPs through the plasma membrane *via* lipidic or proteinaceous pores is one proposed mechanism (Steringer et al., 2012; He et al., 2015). Packaging of these proteins into autophagosomes, multivesicular bodies, and secretory endolysosomes is another established mechanism involved in the translocation of LLPs across the plasma membrane (Dupont et al., 2011; Zhang et al., 2015). It is clear from our studies that Trx1 is packaged into extracellular vesicles/

exosomes before release from the surface tegument or from gastrodermal epithelial cells. Therefore, Trx1 is unlikely to be freely soluble but more probably delivered to host cells along with the total exosome cargo (de la Torre-Escudero et al., 2019; Murphy et al., 2020).

## PRX1 AND TRX1 – SPECIALISED IMMUNOMODULATORY PROTEINS?

The results of our analysis of the *F. hepatica* genome is in keeping with the idea that a functional thiol-dependent antioxidant cascade operates as a defence system against metabolic stress in this parasite. However, it is unclear how TGR, which is encoded by a single copy gene, interacts with the triple Trx and Prx members of this system. Perhaps the various Trxs and Prxs are expressed in different parasite tissues that are under varying levels of metabolic stress (e.g., tegument, reproductive system, gastrodermis etc) and/or duplication of the anti-oxidant genes have generated enzymes with enhanced or varied functions, a feature we have observed in the expanded families of cysteine proteases and protease inhibitors (Cwiklinski et al., 2019; De Marco Verissimo et al., 2020; Smith et al., 2020). Obviously, elucidation of this conundrum awaits more detailed biochemical and cellular studies of each antioxidant member. Notwithstanding, the abundant gene expression and secretion of Trx1 and Prx1 is at variance with a role for these two anti-oxidants alongside the other members in the general cellular metabolism of the parasite and is more in line with their involvement in specialised host-parasite interactions i.e., direct manipulation of host responses. Furthermore, the distinct secretory routes taken by Trx1 and Prx1 would suggest that these are not functional partners but act autonomously. So, what could the function of Trx1 and Prx1 be?

A secondary role for Prx, which we now know is Prx1, in host immune modulation was previously described by us after the antioxidant was discovered as a major component of a fraction of adult *F. hepatica* ES products that induced Th2-immune responses in mice (Donnelly et al., 2005; Donnelly et al., 2008). We subsequently found that addition of a functionally-active recombinant form of Prx1 to cultured macrophages induced their differentiation into M2s. Moreover, intraperitoneal injection of BALB/c mice with the same protein induced the recruitment into the peritoneal space of M2s that were not responsive to stimulation with LPS (Donnelly et al., 2008). Since M2s play a key role in maintaining the Th2 responses of the host immune system, as well as in suppressing the host inflammatory response (Donnelly et al., 2008), we proposed a role for Prx1 in the immunoregulation of the host response by *F. hepatica*. Importantly, the ability of Prx1 to induce M2s *in vitro* and *in vivo* was independent of its antioxidant properties since an inactive recombinant variant was equally immunoregulatory as the wild-type enzyme. We also demonstrated that host (mouse) Prx had similar M2 properties when injected intraperitoneally, prompting us to suggest that parasite Prx acts like a host damage-associated molecular pattern (DAMP).

Unlike Prx1, however, novel functions of *F. hepatica* Trx remain to be discovered. Sequence alignments and structural models of the various *F. hepatica* Trxs with those from other parasites (protozoan and helminth) and mammals reveal a fully conserved structure including the protruding five amino acid motif (WCGPC) at its active site and neighbouring residues vital for its functionality (Salazar-Calderón et al., 2001; Changklungmoa et al., 2014). This could infer that FhTrx1 exhibits some or all of the expanding assortment of endogenous and exogenous activities that are emerging for human Trx1 (hTrx). For example, besides reducing Prx intracellularly, Trx acts as a hydrogen donor for proteins involved in DNA synthesis (Zahedi Avval and Holmgren, 2009), is involved in the redox control of the inflammatory related transcription factors like NF-κB and AP-1 (Schenk et al., 1994), and prevents apoptosis *via* direct binding with apoptosis signal-regulating kinase (Liu and Min, 2002). Its extracellular activity is predominantly mediated enzymatically rather than through classical receptor-like binding (Bertini et al., 1999) and includes reduction and modulation of the activity of extracellular receptors (Schwertassek et al., 2007; Xu et al., 2008) and reduction of IL-1beta mRNA and protein synthesis through suppression of NF-kB activation (Billiet et al., 2005). Trx maintains extracellular cysteine in its reduced form, which is essential for the survival and expansion of activated T-cells (Angelini et al., 2002), and significantly enhances the production of IL-2 and IL-10 (Sido et al., 2005). Administration of recombinant hTrx abrogated the inflammatory progression of chronic pancreatitis, mediated in part by the inactivation of IL-4 *in vivo* (Plugis et al., 2018). It also reduced acute skin inflammatory reactions and lipopolysaccharide-induced infiltration by desensitising innate immune cells to the chemokines KC, RANTES and MCP-1 (Nakamura et al., 2001; Pagliei et al., 2002; Ono et al., 2013).

## CONCLUDING REMARKS

The growth and development of *F. hepatica* in the mammalian host places a major metabolic burden on the parasite as it migrates through host tissues, especially during the early invasive stages that rely on stored glycogen for energy (Bennett and Threadgold, 1973; Bennett, 1977). It is a vulnerable time for the parasites and, therefore, they must possess an effective antioxidant system to protect cells from stress-related oxidative damage. The parasites must contend with ROS-mediated attack from the host immune effector cells, such as macrophages and eosinophils, and a rapidly developing host inflammatory response. Turnover and antigenic changes to the surface tegument are effective mechanisms of immune avoidance but the secretion of molecules is a proactive way in which the parasite can penetrate and feed on its host, as well as manipulate its immune response to assist its survival.

The thiol-dependent antioxidant cascade is an important system for *F. hepatica* to cope with increasing metabolic-derived ROS, but expansion of members of this system by gene

duplication has freed up particular members, Trx1 and Prx1, to diverge in function and become part of the parasites' armoury in defence and, indeed, offense. The absolute function(s) of the secreted Trx1 and Prx1 remain uncertain but their abundance in parasite secretions and our few studies on their activity on immune cells encourage further studies on their potential in immune regulation. Recent studies in mammalian systems are unveiling a diverse range of novel functions for Trx and Prx independent of their antioxidants properties, particularly in the regulation of inflammation (Ishii et al., 2012; Perkins et al., 2014). Indeed, it is tempting to suggest that parasites are intervening in the host immune regulation by molecular and functional mimicry.

Our current -omics derived information provides a sound base to better understand the components of the *F. hepatica* thiol-dependent antioxidant cascade, and indeed other non-thiol dependent antioxidant systems, and their role in parasite physiology and parasite-host interaction. The pivotal role that these systems play in parasite survival makes them tractable targets to which anti-parasite drugs or vaccines could be targeted. Comparative studies by Piedrafita et al. (2000) on the susceptibility of *F. hepatica* and *F. gigantica* NEJs to killing by pro-inflammatory macrophages found a link between antioxidant expression and resistance. More recent studies have demonstrated the effectiveness of the TGR inhibitor gold (I) drug auranofin against several parasites, including schistosomes (Feng et al., 2020), that augurs well for the treatment of *F. hepatica* infection given that the single enzyme is pivotal to the whole cascade (**Figure 1**). The Trx1 and Prx1 enzymes may also be considered targets for vaccine-induced immune responses, either alone, together or in a cocktail with other antioxidants.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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# Lost and Found: Piwi and Argonaute Pathways in Flatworms

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Platyhelminthes comprise one of the major phyla of invertebrate animals, inhabiting a wide range of ecosystems, and one of the most successful in adapting to parasitic life. Small non-coding RNAs have been implicated in regulating complex developmental transitions in model parasitic species. Notably, parasitic flatworms have lost Piwi RNA pathways but gained a novel Argonaute gene. Herein, we analyzed, contrasted and compared the conservation of small RNA pathways among several free-living species (a paraphyletic group traditionally known as 'turbellarians') and parasitic species (organized in the monophyletic clade Neodermata) to disentangle possible adaptations during the transition to parasitism. Our findings showed that complete miRNA and RNAi pathways are present in all analyzed free-living flatworms. Remarkably, whilst all 'turbellarians' have Piwi proteins, these were lost in parasitic Neodermantans. Moreover, two clusters of Piwi class Argonaute genes are present in all 'turbellarians'. Interestingly, we identified a divergent Piwi class Argonaute in free living flatworms exclusively, which we named 'Fliwi'. In addition, other key proteins of the Piwi pathways were conserved in 'turbellarians', while none of them were detected in Neodermatans. Besides Piwi and the canonical Argonaute proteins, a flatworm-specific class of Argonautes (FL-Ago) was identified in the analyzed species confirming its ancestrallity to all Platyhelminthes. Remarkably, this clade was expanded in parasitic Neodermatans, but not in free-living species. These phyla-specific Argonautes showed lower sequence conservation compared to other Argonaute proteins, suggesting that they might have been subjected to high evolutionary rates. However, key residues involved in the interaction with the small RNA and mRNA cleavage in the canonical Argonautes were more conserved in the FL-Agos than in the Piwi Argonautes. Whether this is related to specialized functions and adaptations to parasitism in Neodermatans remains unclear. In conclusion, differences detected in gene conservation, sequence and structure of the Argonaute family suggest tentative biological and evolutionary diversifications that are unique to Platyhelminthes. The remarkable divergencies in the small RNA pathways between free-living and parasitic flatworms indicate that they may have been involved in the adaptation to parasitism of Neodermatans.

**Keywords: Piwi, Ago, Vasa, RNAi pathways, miRNA, piRNA, siRNA, flatworms**

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## INTRODUCTION

At the beginning of this century an unexpected and complex 'RNA world' started to be unveiled (Guil and Esteller, 2015), resulting in the discovery of novel layers of fine-tuned mechanisms for gene expression regulation, unimaginable until then. Regulatory activities were assigned to a growing range of new species of non-coding RNAs (Guil and Esteller, 2015). Single stranded non-coding RNAs of 20 to 30 nucleotides long are key mediators in small RNA pathways that underlie diverse biological processes. MicroRNAs (miRNAs) are post-transcriptional regulators involved in cell development and differentiation, metabolism, DNA methylation, neurological development, immune response, defense against viral infections and cancer (Huang and Zhang, 2014). Piwi-RNAs (piRNAs) are small non-coding RNAs specifically involved in the maintenance of genome stability by silencing transposable elements (TE) in germline cells (Weick and Miska, 2014). Finally, RNA interference (RNAi) is a pathway mediated by short-interfering RNAs (siRNAs) that might have originally emerged as response to double-strand RNA (dsRNA) generated during some virus infections (Ding and Voinnet, 2007). The presence of dsRNA molecules in the cell cytoplasm triggers a post-transcriptional degradation of complementary mRNA molecules. Consequently, this pathway has been exploited as a reverse genetic tool to silence specific genes (Han, 2018). Currently, more than two decades after it was first applied to a flatworm species (Sánchez Alvarado and Newmark, 1999), it is still the main tool to study gene function in worms (Mourão et al., 2012; Wang et al., 2020).

The regulatory pathways mediated by small RNAs have been extensively studied in the nematode *Caenorhabditis elegans*, first model species in which post-transcriptional gene-silencing mediated by dsRNA was described (Fire et al., 1998). In addition, *C. elegans* was the first organism from which a miRNA was isolated: lin-4, (Lee, 1993). On the other hand, *Schmidtea mediterranea* was the first free-living flatworm species to be silenced by RNAi (Sánchez Alvarado and Newmark, 1999). Planarians have long been models for tissue regeneration and stem cells homeostasis, and the emergence of RNAi as functional genomic tool has transformed the field (Reddien and Alvarado, 2004; Blythe et al., 2010; Sandmann et al., 2011).

Planarians are free living flatworms of the order Tricladida, phylum Platyhelminthes. Platyhelminthes are one of the major phyla of invertebrate animals, traditionally divided into four classes: the free living 'turbellarians', the ectoparasitic Monogenea, and the endoparasitic Trematoda (flukes) and Cestoda (tapeworms). All the parasitic classes are grouped in Neodermata given they all share the presence of a syncytial unciliated epidermis (the neodermis) that seem to be crucial for host immune system evasion and nutrient absorption (Caira and Littlewood, 2013). Studies based on rRNA (Larsson and Jondelius, 2008; Laumer and Giribet, 2014) and transcriptomic data (Egger et al., 2015; Laumer et al., 2015) showed that the 'turbellarians' constitute a paraphyletic group, splitting now the phylum Platyhelminthes into two clades; the ancestral Catenulida and the Rhabditophora, that contains several free-living orders and the parasitic neodermatans. More recently the Macrostomorpha was placed as the earliest diverging Rhabditophoran linage and the Tricladida as part of the later evolved 'turbellarians' (Egger et al., 2015; Laumer et al., 2015) (**Figure S1**).

The success of RNAi in planarians encouraged its use in parasitic species where genetic tools were desperately needed. RNAi has proven to be functional in other free-living and parasitic species (Orii et al., 2003; Rinaldi et al., 2008; Kuales et al., 2011; Dell'Oca et al., 2014; Moguel et al., 2015) and miRNAs have been detected in almost all flatworm lineages (Palakodeti et al., 2006; Cucher et al., 2011; Fromm et al., 2013; Fontenla et al., 2015; Cai et al., 2016; Protasio et al., 2017). Whilst piRNAs were early found in free living planarians (Palakodeti et al., 2008; Friedländer et al., 2009), remarkably, they have not been identified in parasitic species.

Our previous analysis of the small RNA pathways in parasitic flatworm genomes strongly indicated relevant gene losses within the neodermatans that may have been associated with the adaptation to parasitism (Fontenla et al., 2017). However, these observations were limited by the paucity of data from free-living species, represented only by *Macrostomum lignano* and the planaria *S. mediterranea*.

The availability of transcriptomic data from several early diverging free-living species (Laumer et al., 2015) allowed us now to expand our analysis and provide a complete picture of the phylum Platyhelminthes, including a representative set of 'turbellarian' species[1], adding also novel monogenean (Ilgová et al., 2017) and trematode genomes (Oey et al., 2018; Choi et al., 2020; Rosa et al., 2020). The emerging picture provides evidence of substantial differences in the distribution of small RNA pathways proteins suggestive of diverse regulatory possibilities in both free living and parasitic flatworms. Additionally, these findings shine a light into tentative relations between the divergency of small RNA pathways and mechanisms driving parasitism in organisms that are responsible for an enormous disease burden in both human and animals.

## METHODS

### Data Acquisition

Small RNA pathways proteins of *Macrostomum lignano*, *Schmidtea mediterranea*, *Gyrodactylus salaris, Schistosoma mansoni* and *Echinococcus multilocularis* together with other Neodermata species were characterized as described (Fontenla et al., 2017). The recently published genomes of the trematodes *Fasciola gigantica* and *Fasciolopsis buski* (Choi et al., 2020) and four species of the genus *Paragonimus* (Oey et al., 2018; Rosa et al., 2020) were also included. Transcriptomic data on several early diverging flatworms (Laumer et al., 2015) were obtained from public repository Data Dryad (doi: 10.5061/dryad.622q4). Transcriptomic data on *Eudiplozoon nipponicum* was

[1] Here and across the manuscript we used the old term 'turbellarian' as a proxy to refer to all the non-Neodermatan species, including the Catenulida and the early diverging Rhabdithopora taxons.

downloaded from GitHub repository (Ilgová et al., 2017). To study the quality of the transcriptomic data, BUSCO v4.1.4 (Seppey et al., 2019) was used with option –l metazoa to search for conserved Metazoan genes. Considering the levels of missing and fragmented transcripts we selected the seven best 'turbellarian' assemblies, comprising a reasonable overview of the 'turbellarian' clade diversity. The species analyzed were *Stenostomum leucops*, *Prostheceraeus vittatus*, *Geocentrophora applanata*, *Rhynchomesostoma rostratum*, *Monocelis fusca*, *Kronborgia cf. amphipodicola* and *Bothrioplana semperi*. TransDecoder.LongOrfs function of TransDecoder v4.1.0 software (available at https://github.com/TransDecoder/TransDecoder/) was used to predict longest open reading frames (ORFs) on transcripts. Detailed information and source of other sequences used in the construction of the gene trees can be found in **Table S10**.

## Identification of Small RNA Pathways Proteins

Flatworm small RNA pathways proteins previously identified by us (Fontenla et al., 2017), and *C. elegans* factors that we failed to detect in our previous search were used as query to interrogate with BLASTp the translated transcriptomes and genomes. We also inspected the presence of *D. melanogaster*'s Zuc and Vasa (Fontenla et al., 2017) using the same approach against all the species. Matched sequences were acquired and used to perform reverse BLASTp against the proteomes of *S. mansoni*, *M. lignano*, *C. elegans* and *D. melanogaster* retaining only the best hit. HMMScan (Johnson et al., 2010) was used to predict the functional domains in putative small RNA pathways proteins, and sequences with no functional domains were discarded from the analysis. HMMScan prediction was performed in the complete transcriptomes of *S. mediterranea* and *S. mansoni* as quality control, to confirm that distant homologous genes with the conserved function were not discarded in the BLAST search. The procedure did not show a different outcome to the blast results Detected sequences are available as **Supplementary Material** (**Folder S1**).

## Construction of Phylogenetic Trees

To avoid overestimating the number of genes in transcriptomic data or report entire genome duplications as gene expansions, CD-HIT (Huang et al., 2010) was used to cluster sequences with more than 90% similarity (option –c 0.9). MAFFT (Katoh and Standley, 2013) with local alignment option and structural information was used to align the selected sequences. Due to the fragmented nature of transcriptomic data, sequence alignments were manually curated using BioEdit (Hall, 1999), removing sequences that were too short (< 140 aa). Maximum Likelihood trees with statistical branch support (SH-like) were generated with PhyML (Guindon et al., 2010), with models inferred with Smart Model Selection (SMS) (Lefort et al., 2017). Trees were visualized with Evolview (He et al., 2016), and enriched by adding domain structure information. Argonaute unrooted tree (**Figure 1**) was visualized with MEGA version X (Kumar et al., 2018).

## Molecular Evolutionary Analysis of Argonaute Subfamilies

For the heatmap showing the sequence conservation of the Argonaute subfamilies, the consensus function of R package seqinR (Charif and Lobry, 2007) was used to build matrices with the residues count at each position of the alignments. Next, we applied a color scale to the most abundant residue at each

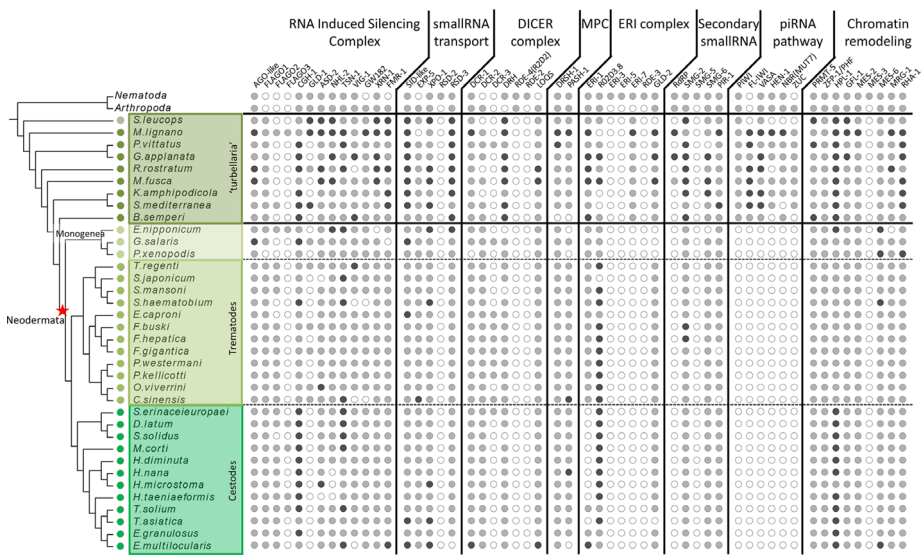


**FIGURE 1** | Distribution of miRNA, piRNA and RNAi proteins across flatworms, arthropods and nematodes. White circles indicate absence, grey circles indicate one homologue detected, black circles indicate two or more homologues detected. In arthropods and nematodes only presence/absence is indicated.

position. To calculate the percentage of conserved positions by domain, the positions with conserved residues in more than 50% of the sequences were added and corrected by the domain length.

For positive selection inference, Argonaute transcripts were codon aligned in MEGA version X (Kumar et al., 2018) with Muscle aligner. The alignment was edited with BioEdit (Hall, 1999) and MEGA version X Codon-based Z-test of Selection tool used to compute synonymous and nonsynonymous substitutions: the hypothesis tested was positive selection (dN>dS) in sequence pairs with the Nei-Gojobori method (Nei and Gojobori, 1986), with a *p-value* threshold ≤ 0.05.

Additionally, we tested for evidence of positive selected sites (PSSs) using the mixed effects model of evolution (MEME) (Murrell et al., 2012) method. MEME applies a branch-site random effects phylogenetic framework that allows the distribution of dN/dS to vary from site to site as well as from branch to branch, thereby identifying residues that have undergone episodic selection (i.e. positive selection that varies temporally throughout the tree). Only likelihood ratio test (LRT) with *p-value* ≤ 0.05 were considered as statistically significant evidence of PSSs.

# RESULTS

## While miRNA and siRNA Pathways Are Conserved Across Platyhelminths, the Complete Piwi Pathway Is Lost in Parasitic Flatworms

The presence of small RNA pathways was investigated on available transcriptomes from 25 early diverging flatworms (Laumer et al., 2015). We first evaluated the quality of the assemblies against a set of conserved metazoans genes using BUSCO v 4.1.4. Based on the level of fragmentation and number of missing orthologues, we selected assemblies from seven species, that added to *M. lignano*, and *S. mediterranea* capture the diversity of free-living flatworms. In addition, we included a novel dataset from underrepresented monogeneans and six novel trematode genomes (Oey et al., 2018; Choi et al., 2020; Rosa et al., 2020). Taken together this dataset provides a comprehensive phylogenetic view of the platyhelminth clade diversity (**Figure S1**).

The homology search with a curated set of proteins involved in small RNA pathways not only showed the presence of most of them in all free-living species, but also outstanding absences in neodermatans. While several proteins show differential distribution among diverse classes (**Figures S2**–**S6** and **Tables S1**–**S6**), a remarkable feature is the complete absence of all the piwi pathway components in all parasitic species (**Figure 1**).

The absence of piwi proteins in parasitic trematodes and cestodes have been previously proposed (Skinner et al., 2014; Fontenla et al., 2017), raising questions on how the parasitic species control the activity of repetitive mobile elements. Our extensive search of other piwi pathway genes across flatworms clearly shows that the complete pathway is missing in neodermatans while is conserved in free-living species.

## Amplifications in the Argonaute Family Show Differential Distributions Across Flatworms

Argonautes, small-RNA binding proteins, are central components of all the small RNA pathways (Niaz, 2018). Phylogenetic studies have traditionally classified the family into the Ago class (that is further subdivided in two subclasses comprising miRNA Agos and the siRNA associated proteins), the Piwi class, and the Wago clade, this latter, comprising nematode specific argonaute proteins (Wynant et al., 2017).

The comparison of Argonaute superfamily proteins from diverse metazoans, now including our extended sampling of flatworms, reveals interesting differences in their distribution, particularly between free-living and parasitic species.

All flatworms have putative orthologues to miRNA class proteins that constitute a well-defined clade.

Rather than grouping with siRNA class Agos from basal metazoans (poriferans and cnidarians), ecdysozoans (nematodes and arthropods) or other lophotrochozoa (mollusks, annelids, gastrotrichs and rotifers), all the other flatworm Ago sequences cluster together in a well-defined clade (**Figure 2**). This flatworm specific clade has been previously reported by us and others, and termed FLAgos (Zheng, 2012; Skinner et al., 2014; Fontenla et al., 2017).

Interestingly, while a single gene is found in all the free-living species (with the only exception of *S. mediterranea* that showed a gene duplication), the parasitic cestodes and trematodes have experienced gene amplifications leading to two or more genes (**Figures 2** and **3**). The two FLAgo genes from the model trematode *S. mansoni* (Smp_179320 and Smp_102690) are organized in tandem in chromosome 1, but show clear differential expression among developmental stages (**Figure S7**). While a similar tandem gene arrangement could be detected in *S. japonicum* genome, it is not possible to assess if this is a general trend in trematodes due to the still fragmentary nature of the assemblies for other species. Similarly, within cestodes three FLAgo genes are in tandem in *Echinococcus multilocularis* and a more complex array of amplified genes is evident in *Hymenolepis microstoma*. With the information available so far these amplifications appear as independent events in cestodes and trematodes (**Figure 3**). Since single genes are recovered in all free-living species, a more parsimonious hypothesis would be an initial duplication at the origin of neodermatans. However, more detailed analyses and better genome assemblies are needed, in particular considering that the gene trees suggest these are rapidly evolving Agos.

Two subgroups of piwi class genes have been previously described (Wynant et al., 2017; Jehn et al., 2018). Consistently within the free-living flatworms Piwis two sub-groups are evident. But while single genes from all free-living species cluster within the Piwi2 clade, a second separate clade is formed with all the remaining Piwi genes from free-living Rhabditophorans, here termed FLiwi (**Figures 2** and **4**). The complete absence of any piwi homologue in neodermantans is quite evident in monogeneans, trematodes and cestodes, and

this is confirmed based on the extensive sampling on available genomes.

The FLiwi cluster showed several independent gene duplications that have probably occurred after speciation, although we cannot rule out that gene duplications might be overestimated in those species in those species for which only transcriptomic data is available. We cannot rule out that transcripts from different genes were counted as one if the sequences were too similar due to very recent gene duplication events, as was the case for *S. mediterranea*'s FLiwis (Kim et al., 2020). Interestingly, *S. mediterranea* displayed the widest expansion of FLiwis (**Figure 4** dark green arch). Here, eight genes clustered in this group, some with identical sequences that can be collapsed to five quite similar genes (SmeT032534, SmeT030029, SmeT030034 and SmeT030033 present >99% identity). Whether these are functional genes or represent transcribed pseudogenes remains unknown (Kim et al., 2020).

Interestingly, single Ago (Sle67413) and Piwi (Sle58342) genes can be detected in the catenulid *S. leucops*, that generally is placed outside of the clades from other flatworms. It is not possible to assess if the absence of further genes is real or is due to partial sampling of the available transcriptome. In any case, it is quite interesting the placing outside of other flatworms since the inclusion of the group within platyhelminthes was in debate until recently (Larsson and Jondelius, 2008; Laumer and Giribet, 2014).

The phylogenetic tree shown in **Figure 2** suggests that FLAgo and FLiwi have probably experimented highly evolutionary rates, as indicated by their long branches. This may also explain the observed branching pattern inconsistent with the

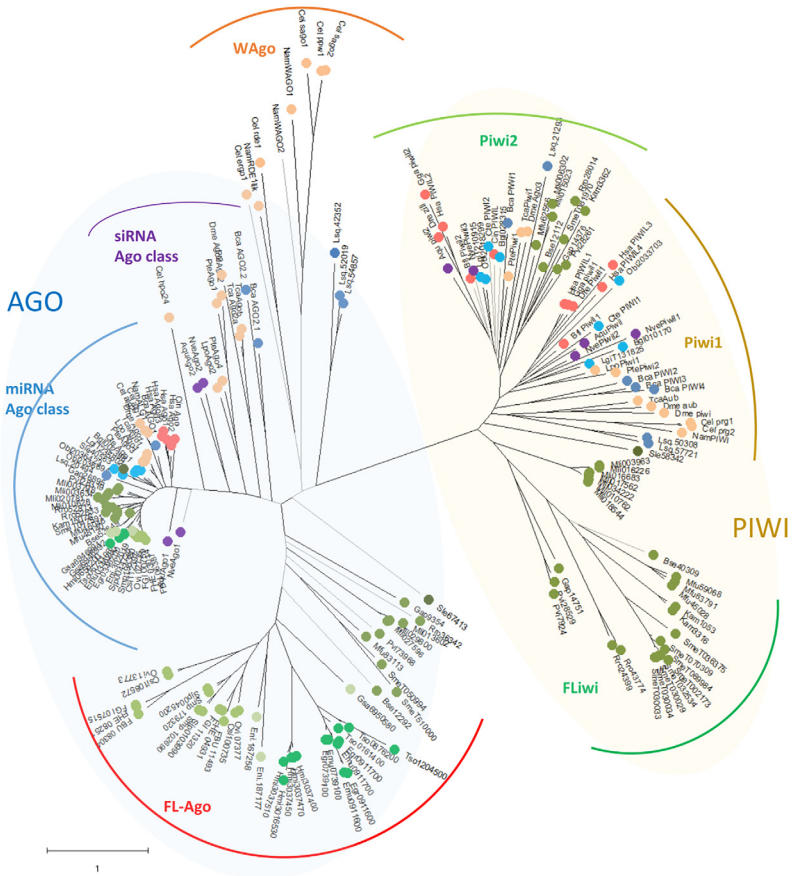


**FIGURE 2** | Unrooted maximum likelihood phylogenetic tree of Argonaute proteins belonging to Deuterostomia (•: Hsa, *Homo sapiens*; Gga, *Gallus gallus*; Dre, *Danio renio*; Bfl, *Branchiostoma floridae*; Cin, *Ciona intestinalis*), Ecdysozoa (•: Cel, *Caenorhabditis elegans*; Nam, *Necator americanus*; Dme, *Drosophila melanogaster*; Tca, *Tribolium casteneum*; Pte, *Parasteatoda tepidariorum*; Lpo, *Limulus polyphemus*), Mollusca & Annelida (•: Obi, *Octopus bimaculoides*; Bgl, *Biomphalaria glabrata*; Lgi, *Lottia gigantea*; Cte, *Capitella teleta*), Rotifera & Gastrotricha (•: Bca, *Brachionus calyciflorus*; Lsq, *Lepidermella squamata*) 'turbellaria' Catenulida (•: Sle, *Stenostomum leucops*), 'turbellaria' Rhabditophora (•: Mli, *Macrostomum lignano*; Pvi, *Prostheceraeus vittatus*; Gap, *Geocentrophora applanate*; Rro, *Rhynchomesostoma rostratum*; Mfu, *Monocelis fusca*; Kam, *Kronborgia amphipodicola*; Bse, *Bothrioplana semperi*), Monogenea (•: Eni, *Eudiplozoon nipponicum*; Gsa, *Gyrodactilus salaris*), Trematode (•: FBU, *Fasciolopsis buski*; FHE, *Fasciola hepatica*; FGI, *Fasciola gigantica*; Ovi, *Opisthorchis viverrini*; *Clonorchis sinensis*; Smp, *Schistosoma mansoni*; Sjp, *Schistosoma japonicum*), Cestode (•: Hmi, *Hymenolepis microstoma*; Tso, *Taenia solium*; Emu, *Echinococcus multilocularis*; Egr, *Echinococcus granulosus*), Cnidaria & Porifera (•: Nve, *Nemastella vectensis*; Aqu, *Amphimedon queenslandica*). Black branches indicate SH-like approximate likelihood ratios ≥ 90. Arches indicates the subgroups of Argonautes (blue shaded) and Piwi (green shaded) genes.

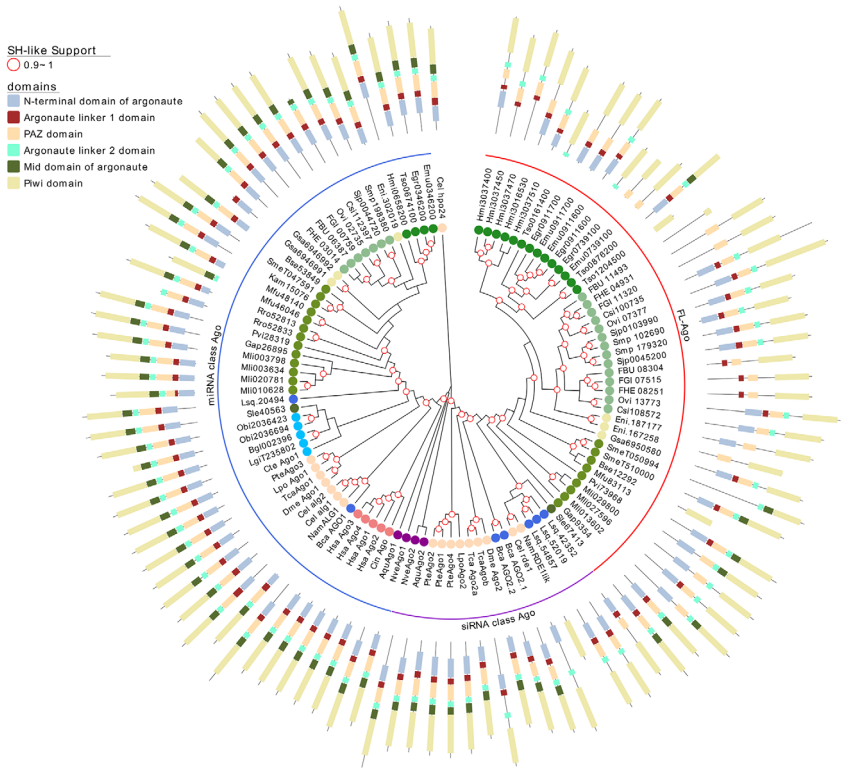


**FIGURE 3** | A maximum likelihood tree of canonical Argonaute proteins and FLAgos. SH-like approximate likelihood ratios are indicated. Conserved protein domains were predicted with HMMScan. Blue, purple and red arches correspond to miRNA Ago-class, siRNA Ago-class and FL-Agos, respectively. Abbreviations are as indicated in **Figure 2**.

species tree (**Figure 2**). In this sense, a parsimonious explanation would be that FLAgo might represent rapidly evolving siRNA class genes, while Fliwi could correspond to fast evolving piwi1 type proteins. Further evidence is needed to functionally validate these proteins.; therefore, we decided to investigate other aspects of these intriguing genes.

## FLAgos and FLiwis Structures Are More Variable Than Canonical Ago and Piwi Counterparts

Argonaute proteins consist of five distinct domains: the N-terminal, PAZ, Mid, PIWI and two linker regions, L1 and L2. When analyzing the domain conservation among the Argonaute proteins detected across flatworms, it was obvious that while canonical miAgo class genes are highly conserved in their structure, FL-Agos have a more variable structure with MID domain being poorly detected. Both Piwi subfamilies display PAZ and PIWI domains, and while the N-terminal and linker 1 domain are generally identified in the free-living Piwi 2 genes, they are devoid of the linker 2 domain (**Figures 3** and **4**). Conversely, although more structurally variable probably due to independent gene amplifications, most of the FLiwi proteins have linker 2 domain in addition to PAZ/PIWI domains, while the detection of the other domains is more scattered.

Next, we studied the conservation at sequence level of the flatworm Argonaute genes. The canonical miAgo family showed the highest overall conservation which reached up to 85% of conserved positions in the MID domain (**Figure 5A** and **Table S7** for complete list of conservation by domain). Piwi, FLiwi and FLAgos showed lower general sequence conservation. The most conserved domain across clades at amino acid level is the PIWI domain (**Figure 5A**). Remarkably, the less conserved domain in the canonical Ago (i.e. the N-terminal domain) showed more conservation than any of the domains in both Piwi clades and FL-Agos (**Figure 5A** and **Table S7**). This is suggestive of faster evolution rates of the FLAgo and FLiwi clades, consistent with previous reports (Wynant et al., 2017).

Since the differences in sequences conservation may be associated with selection, we measured the variation in the rate of non-synonymous/synonymous (dN/dS) substitutions of Argonaute family proteins. We found that within each subgroup of Argonautes, the FLAgos showed the highest number of statistically significant dN/dS substitutions when free-living to parasitic pairs of genes were considered (**Table S8**). The second group with dN>dS was the FLiwis followed by the miRNA class Agos and the Piwis. When considering variation between subfamilies the highest rate of dN/dS substitutions were observed between the Agos *vs* FLAgos, Ago/

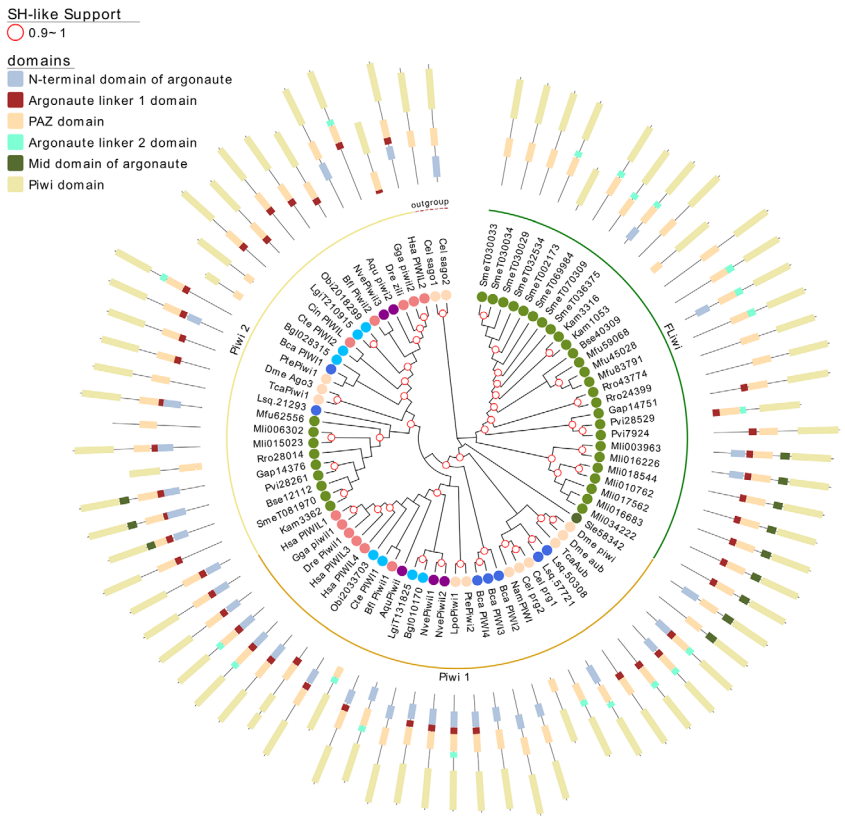


**FIGURE 4** | A maximum likelihood tree of Piwi proteins. Wagos were used as outgroup. SH-like approximate likelihood ratios are indicated. Conserved protein domains were predicted with HMMScan. Light green, yellow and green arches corresponds to the cluster of the Piwi 2, Piwi 1 and FLiwis, respectively. Abbreviations are as indicated in **Figure 2**.

Piwis, followed by Piwi/FLAgos and Ago/FLiwi. Using MEME, we aimed to detect sites that were subjected to positive or diversifying selection (see *Methods*). FLAgos showed the highest count of positions under positive diversifying selection, with a significant likelihood ratio test (LRT) of non-synonymous to synonymous substitutions (p-value ≤ 0.05) (dots in **Figure 5A**). Meanwhile, the Piwis showed 3 putative sites under positive selection, and the FLiwis and canonical Agos groups only had one position each (**Figure 5A**).

## Small RNA Binding and Catalytic Amino Acids Conservation in FLAgos and FLiwis

Structural studies have shined a light on the mechanisms of action of Argonautes. Human Ago2 bonded to miRNA has a bi-lobed architecture, with the guide miRNA threaded through a central cleft formed by the N-PAZ and MID-PIWI domains. Multitude interactions within this tight binding pocket were identified, involving mainly residues of the MID domain capped on the 5' side by PIWI domain residues (Elkayam et al., 2012; Kong et al., 2017). There, the target mRNA has access to mate with the miRNA and is cleaved by a RNAase H fold comprised by the Asp-Asp-His (DDH) triad in the PIWI domain (Tolia and Joshua-Tor, 2007). Consequently, we sought conservation of these active sites and functional residues in the different Ago classes (indicated by black and grey bars at **Figure 5A**).

Fourteen out of 51 functionally relevant positions were generally conserved in all the Argonaute proteins. Further fifteen positions were conserved between Ago-like and FLAgos, and a minor group of residues were shared between Piwi and FLiwi (**Figure S8**). These include four of the eight residues involved in positioning the guide RNA with respect to the active site to ensure that cleavage of targets occurs at a well-defined and predictable position. Three other positions are conserved only between canonical Agos and FLAgos (**Figure S8**). On the other hand, both Piwis and FLiwis present well conserved substitutions at positions 588 [K→ polar (Q)] and 845 [R→ hydrophobic (L, M or F)] (**Figure S8**), that could also be relevant in the binding to the small RNA.

The QSKN motif (positions 566 to 569) of the MID domain (blue bar in **Figures 5A** and **S8**) was reported to be involved in the binding to the seed region of the miRNA in the *Echinococcus canadensis* canonical Ago genes (Maldonado et al., 2017). Interestingly, while this QSKN motif is conserved in all Neodermatans, (**Figure S8**), the second position was occupied by a non-polar Alanine residue (QAKN), in 'turbellarians' as in *D. melanogaster* and *C. elegans*. In Deuterostomes, the same

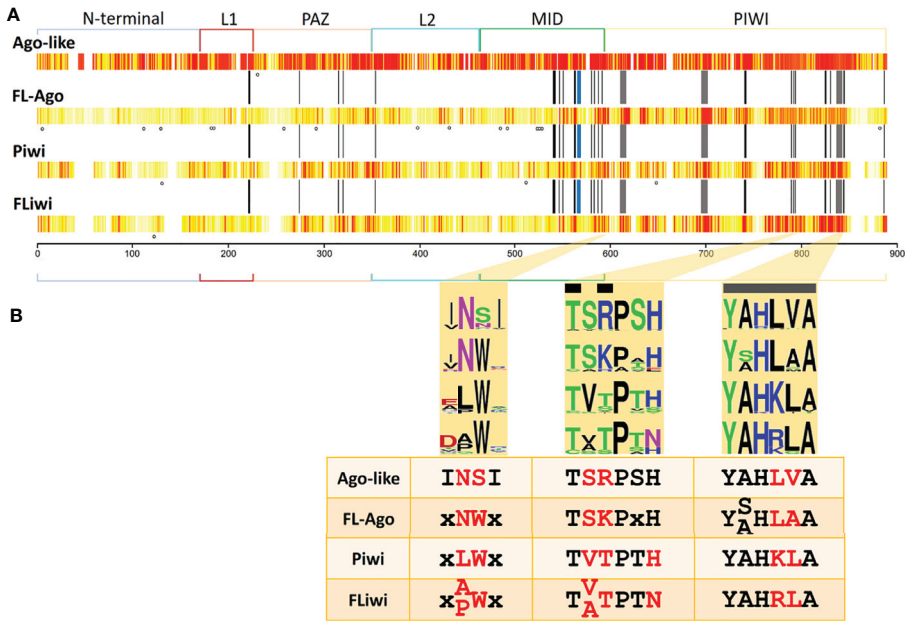


**FIGURE 5** | Sequence conservation among Argonaute subfamilies. **(A)** Conservation is shown in a white (less conserved) to red (most conserved) color scale. N-terminal, linker 1 (L1), PAZ, linker 2 (L2), MID and PIWI domains are indicated. Black bars indicate the positions that have been reported to interact with the miRNA (Elkayam et al., 2012). Grey bars indicate the positions of the catalytic DDH triad. Blue bar indicate the position that has been reported to interact with the seed region of miRNAs (Maldonado et al., 2017). Dots indicate positions with statistically significant LRT (p-value ≤ 0.05) detected with MEME tool. **(B)** Highlight of three regions of the MID and PIWI domains that produce a signature for each Ago subfamily. Black bars indicate the positions that have been reported to interact with the miRNA. Grey bars indicate the positions of the catalytic DDH triad.

position was occupied by the non-polar aliphatic residue Valine (QVKN) (or Methionine in Has_Ago2, QMKN). Although the functional implications of this substitution are not clear, the restricted conservation of this motif in the Ago-like genes suggests that it is relevant in this subfamily, and importantly, may be useful as a marker of linage in the future.

The RNase H activity associated with a conserved DDH triad (grey bars in **Figures 5A** and **S8**) is well conserved in all the miRNA class Ago genes of Platyhelminthes. In the case of the Piwi class subfamily only SmeT002173 possess substitutions in the catalytic triad. The absence of the DDH triad would not imply that the catalytic activity was lost, as Dme_piwi has been shown to possess "slicer" activity even though it contains a DDK active site (Tolia and Joshua-Tor, 2007). Furthermore, it was shown for Hsa Ago3 that irrespective of RNase triad conservation, the catalytic activity changes depending on the guide RNA that is loaded (Park et al., 2017). In any event, additional *in silico* approaches as structural homology modeling, ligand docking and molecular dynamics, as well as, experimental evidence involving site-specific mutagenesis and cleavage assays are needed to define the ligand-protein interaction and characterize the catalytic activity of the diverse flatworm Argonautes.

Based on the residue conservation we selected three short motifs with different conserved residues among the diverse flatworm Argonaute subfamilies that can be useful to differentiate them, classifying and assigning novel members (**Figure 5B**). The first motif consists of a duo located in the carboxi-terminal end of the MID domain and is an Asn (N) preferentially followed by Ser (S) in canonical Agos and Asn-Trp (NW) in FLAgos. The non-polar Asn is substituted by Leu (L) and Ala (A) or Pro (P) in Piwi and FLiwis, respectively. The second and third motifs are within the PIWI domain. Motif 2 is TSRPSH in miRNA class Agos, while is TSKPxH in FLAgos, TVTPTH in Piwi and TVTPTN or TATPTN in FLiwis. The third motif contains the Histidine residue of the DDH triad and corresponds to a sextet that has the sequence YALHVA in Ago-like, YSLHAA or YALHAA in FLAgos, YAHKLA in Piwis or YAHRLA in FLiwis. We suggest that the analysis of these motifs may be useful to classify Argonaute proteins in flatworms, and also might provide a means of rapidly identify members in other metazoan species.

## Trematodes Display a Shorter Dicer-2 Gene

Ribonuclease III family proteins represent central player in the small RNA pathways. Dicers belong to the ribonuclease III family with the ability to process dsRNA. Dicer (Dcr) is responsible for recognizing a hairpin (in pre-miRNA) or long dsRNA and processing them into 22-23 nt miRNA-miRNA* or siRNA duplexes (Jaronczyk et al., 2005). These small RNA duplexes are bound and processed by Ago proteins to form the RNA interference silencing complex (RISC). Like arthropods, flatworms have two Dcr genes, Dcr-1 and Dcr-2 (including a

putative *S. leucops* Dcr-2 placed in the root of Dcr-1 group) with a paralogue of Dcr-2 in some species we named Dcr-3 (**Figure 6A**) (Gao et al., 2014; Fontenla et al., 2017).

In Neodermatans, Dcr-2 is variable in number and organization (Fontenla et al., 2017). Strikingly, trematodes in contrast to cestodes have a shorter version of Dcr-2 with only the RNAse III domains and, in some cases, a PAZ domain (**Figure 6A**). Additionally, a duplication of Dcr-2 is evident in foodborne trematodes (FBT) compared to blood flukes (BF) (**Figure 6A** green arch). In FBT Dcr-2 and Dcr-3 genes are organized as inverted tandem repeats with the exception of *F. buski* and *Paragonimus spp*, where the fragmented nature of the assembled genomes does not allow to confirm or discard this gene arrangement.

The comparison of Dcr-2 genes from the cestode *E. multilocularis*, the planaria *S. mediterranea* and the trematode *S. mansoni* showed that the second exon of Sma033600 matches with exon 15 of the cestode or planarian counterparts. In contrast, exon 1 of cestode Dcr-2 matches with exon 1 in the planarian gene (**Figure 6B**). This observation suggests that the shortening of Dcr-2 is the result of a genomic reorganization that occurred ancestrally in trematodes, probably by an unequal crossover between different chromatids or an intra-chromatid recombination that resulted in the deletion of about half of the ancestral gene. Consequently, this may have led to the absence of the helicase and dsRNA binding domains. It is tempting to speculate that these proteins might only recognize ssRNA as substrates, but experimental evidence is missing.

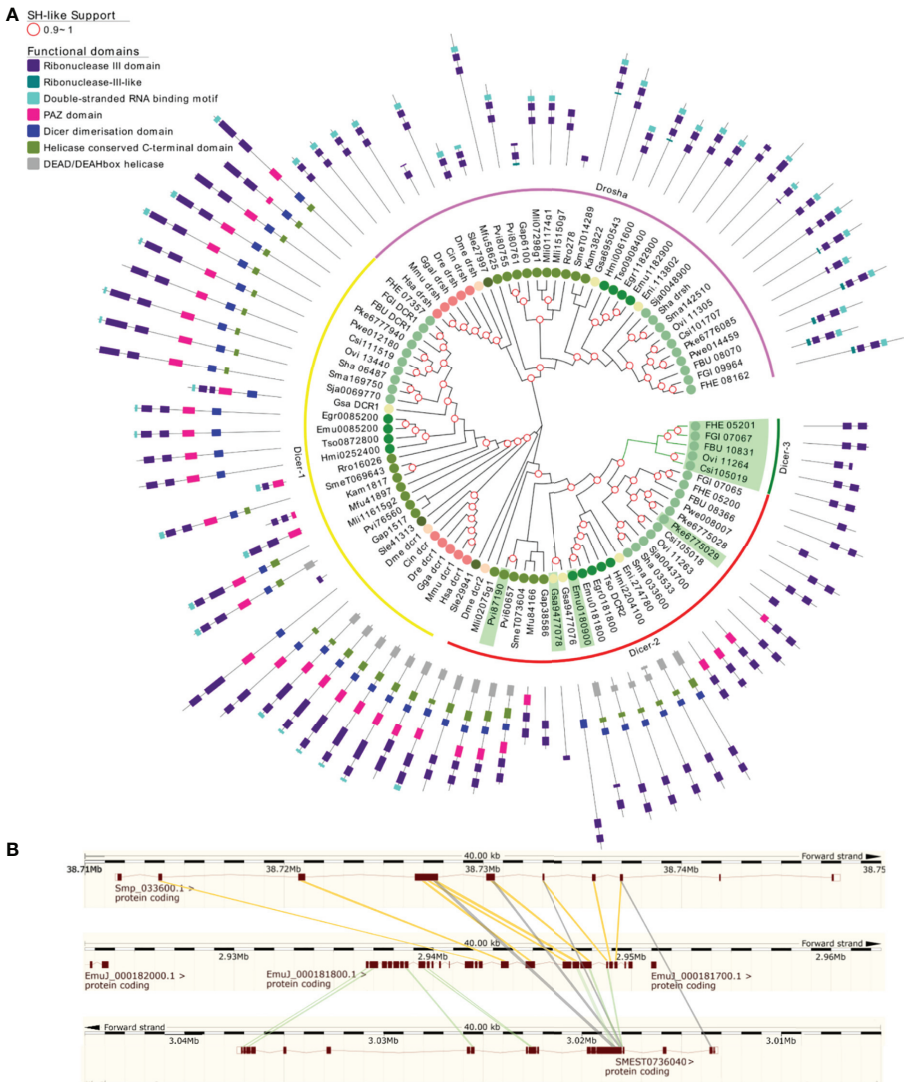


**FIGURE 6** | Ribonuclease III subfamilies of flatworms **(A)** A maximum likelihood tree of Ribonuclease III subfamilies. SH-like approximate likelihood ratios are indicated. Conserved protein domains were predicted with HMMScan. Dcr -2 (red arch) duplications are highlighted in green. Dcr-2 is ancestrally duplicated in FBT (dicer-3, green arch). Dcr-2 has missing domains in trematodes compared to cestodes, similar structural heterogeneity was observed between 'turbellarians'. Abbreviations are as indicated in **Figure 2**. **(B)** High scoring pairs between Dcr-2 genes of *S. mansoni*, *E. multilocularis* and *S. mediterranea*.

Although some variation in the structure of 'turbellarians' Dicers was also detected, we cannot rule out these may be artefactual due to the fragmented nature of these transcriptomes.

## Belle/PL10 Is Duplicated in Flatworms While Vasa Is Lost in Neodermatan Parasites

Since several other piRNA pathway genes, besides piwi itself, seem to be absent in neodermatans, we investigated in more detail other relevant members involved in the pathway. Vasa is a germline specific DEAD box RNA helicase and plays an essential role in regulating germ cell differentiation (Abdelhaleem, 2005). Taking advantage of our extended set of transcriptomes and genomes of free-living and parasitic flatworms, we analyzed the conservation of Vasa and its paralogous gene, Belle. We founded that while 'turbellarians' conserved homologue genes to Vasa and Belle (green and blue arches in **Figure 7**), Neodermatans have lost vasa orthologues while maintaining Belle/PL10 homologs (blue branches in **Figure 7**). The only exception is Ngvlg3 a Vasa orthologue detected in the monogenean *Neobenedenia girellae*. Interestingly, this gene was not found to be expressed in any tissue and its knockdown by RNAi produced no phenotypic effect on the worm (Ohashi et al., 2007). Thus, we speculate that given the basal position of *N. girellae* within the Neodermatans is possible that Ngvlg3 may be a non-functional pseudogene of the free-living ancestors.

Notably, Belle-like gene is duplicated in all Neodermatans and in *B. semperi*, the closest free-living ancestor of the Neodermatans (blue arch in **Figure 7**). We also found a third group of helicases that we classified as Belle related (light blue arch in **Figure 7**) given that they show a higher percentage of identity with Belle and/or laf-1 compared to other of the *D. melanogaster*/*C. elegans* helicases (**Table S9**). All genes considered within this family have a similar domain structure, with conserved DEAD/DEAH-box and C-terminal helicase domains (**Figure 7**). An amino terminal repeat of Zinc knuckle

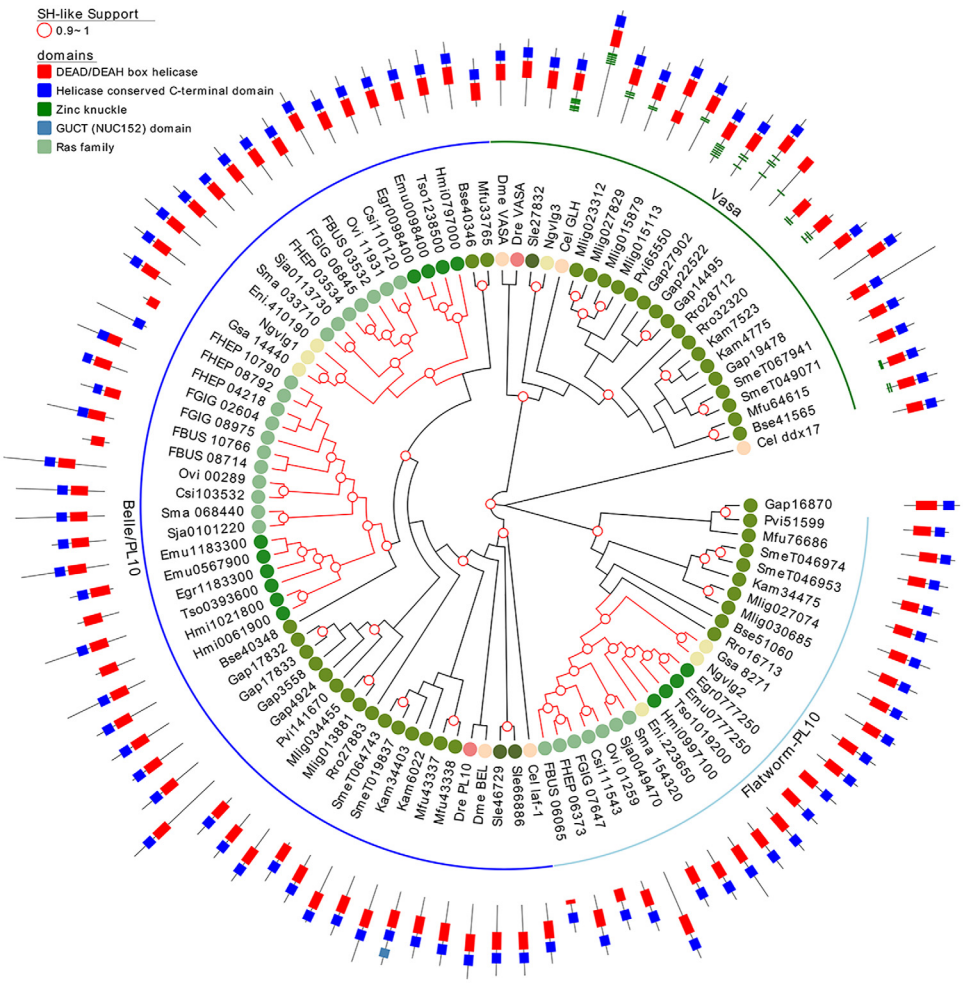


**FIGURE 7** | A maximum likelihood tree of Vasa and Vasa-like proteins. SH-like approximate likelihood ratios are indicated. Conserved protein domains were predicted with HMMScan. Green and blue arches correspond to Vasa and Belle/PL10, respectively. Light blue arch corresponds to flatworm PL10. Red branches correspond to Neodermatan species. Species abbreviations are indicated as in **Figure 2**.

was detected in several Vasa genes and in the *C. elegans* ortholog (GLH gene). Remarkably, Vasa homologous genes were amplified in several 'turbellaria' including *M. lignano* where 4 genes were detected after clustering highly similar sequences.

## Other PiwiRNA Pathway Proteins Are Conserved Only in Free Living Flatworms

An RNA dependent RNA polymerase (RdRP) amplifies the signal leading to the generation of secondary siRNAs in *C. elegans*. Two 'turbellarian' species (*G. applanata* and *M. fusca*) had sequences with RdRPs functional domains (**Figures 8B**, **S5** and **Table S6**) in addition to *M. lignano*, (Fontenla et al., 2017). RdRPs were not detected in *S. leucops* or *P. vittatus* the two other most ancestral species of Platyhelminthes analyzed here. Interestingly, we found RdRPs in the phylum Gastrotricha, sister phylum to all Platyhelminthes (Egger et al., 2015; Laumer et al., 2015) (**Figure S5B**), suggesting that RdRPs were conserved in the common ancestor to both phyla and were lost during the evolution of Platyhelminthes. While, it is clear that the piRNA pathway does not depend on RdRPs in *S. mediterreanea* and probably other species where RdRPs are not present, it might be possible to occur in species where RdRPs are conserved, as in *C. elegans* (**Figure 8B**).

We also found homologues to other piwi pathway genes like HEN1, Zuc or Mut7 in almost all 'turbellarian' species although they are completely absent in neodermatans. (**Figures 1**, **8A** and **Table S6**). MUT-7 is a protein that contains an exonuclease domain that, in complex with RDE-2, is required in the RNAi pathway in *C. elegans* (Tops et al., 2005)(**Figure S4**). However, in *D. melanogaster* RDE-2 is missing and the orthologue of MUT-7, Nibbler (Nbr), is involved in the maturation of piRNA 3' ends (Wang et al., 2016). As in insects, RDE-2 is missing in flatworms suggesting that the MUT-7/Nbr homolog in 'turbellarians' is involved in piRNA biogenesis but not in the RNAi pathway in free living flatworms (**Figures 1**, **8A** and **Table S6**).

# DISCUSSION

Platyhelminthes comprise one of the early diverging phyla of bilateral metazoans, originated approximately 839 million years ago (Hedges et al., 2015), inhabiting a wide range of ecosystems and particularly successful in adapting to parasitic life. Since small non-coding RNAs have been implicated in regulating developmental transitions, we reasoned that they might be involved in the adaptation to parasitism way of life. Therefore, comparing the conservation of small RNA pathways among the paraphyletic group of free-living ('turbellarians') and the monophyletic parasitic Neodermatans may provide evolutionary clues to disentangle possible adaptations to parasitism.

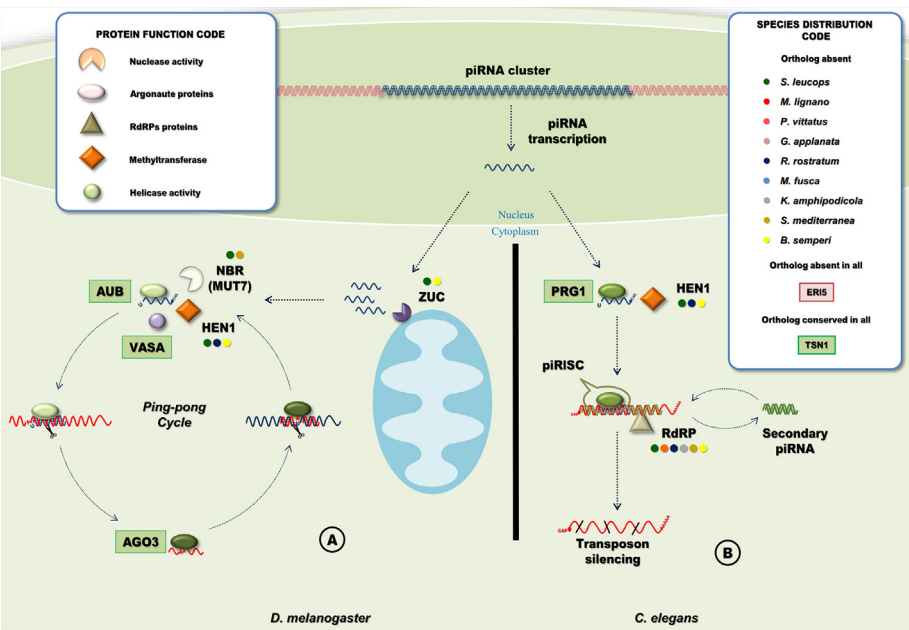


**FIGURE 8** | Piwi-interacting RNA pathways. **(A)** PiRNA pathway in *D. melanogaster*. PiRNA precursors are cleaved by a protein of the mitochondrial membrane, Zucchini (Zuc) producing the primary piRNAs that are loaded into Aub, maturation of piRNA requires the 2-O methylation and cleavage of the piRNA 3' end by HEN1 and NBR (MUT7), respectively. The primary piRNA and Aub form the piRISC, secondary piRNAs are generated in a slicer-dependent amplification loop that silence cytoplasmic TE transcripts named "Ping-Pong" cycle (Tóth et al., 2016). **(B)** PiRNA pathway in *C. elegans*. In the cytoplasm, piRNAs are bounded by a PRG protein and methylated at the 3' end by HEN1. The processed piRNA with PRG form the piRISC that will cleavage target RNA, target recognition is followed by the generation of secondary siRNAs mediated by RdRPs (Weick and Miska, 2014). Circles indicate species with missing homologous genes ("Species Distribution Code" box). A 'shape' code was used to indicate predicted function of factors ("Protein Function Code" box).

## The Argonaute Gene Family

We have previously shown (Fontenla et al., 2017) that key proteins involved in small RNA pathways are conserved in all major clades of Platyhelminthes; however, clear differences between clades are evident, particularly the complete absence of the Piwi pathway genes in Neodermatans.

While miRNA class Ago proteins are conserved in all flatworms, a platyhelminth-specific family that we named FLAgos (Fontenla et al., 2017), showed independent gene amplifications in Trematodes and Cestodes but not in 'turbellarians', with the only exception of a duplication in *S. mediterranea* (**Figure 3**). This group might have originated as a highly divergent siRNA class Ago, that was further amplified and diversified. On the other hand, while the parasitic Neodermatans lack Piwi-like proteins, these are amplified in free-living flatworms, including the canonical Piwi 2 class, and a new group, the FLiwis, specific of free living Rhabditophora (**Figure 4**). As in FLAgos, since Piwi1 class homologues are missing, is possible that the Fliwi group represents a fast-evolving piwi1 class that diverged within flatworms. Interestingly, neither FLAgos, nor FLiwi are shared by other lophotrochozoans lineages analyzed in this study, despite amplifications can also been detected within them.

Piwi proteins are involved in the biogenesis and activities of piRNAs, being crucial at silencing transposable elements. In *S. mediterranea*, Piwi genes were reported to be essential in the regeneration and homeostasis of neoblasts, the pluripotent stem cells of Platyhelminthes (Reddien et al., 2005; Palakodeti et al., 2008). Additionally, *S. mediterranea* piwi-1 (SmeT036375) is highly expressed in blastomeres and is critical during embryogenesis and organogenesis (Davies et al., 2017). The highest levels of piwi-1 are found in epidermal progenitors and tetraspanin-1-positive neoblasts. Differentiation of pluripotent neoblasts into fate-determined progenitors and terminally differentiated cells is accompanied by a successive reduction of piwi-1 levels (Kim et al., 2020). In *M. lignano*, piwi-1 (Mli034222) but not piwi-2 (Mli016226) was found to be involved in the piRNA pathway in both germline and somatic cells, as well as in the maintenance of stem cells (Zhou et al., 2015). It is of interest to decipher if similar functional restrictions or labor division is found in other 'turbellarians', now that we show that the key genes are conserved.

FLAgo proteins show less sequence conservation, due to a higher substitution rate, a feature generally associated with acquiring novel functions. We show evidence of site-specific diversifying selection mainly in FLAgos compared to other Argonaute groups. We hypothesize that the sequence variation observed for FLAgos might have been associated to specialization in the gene function within this cluster. Further experimental evidence is needed in to validate this hypothesis, for instance evaluating if the substitutions detected are associated with changes in functional domains or protein conformations. Even more, flatworm-specific proteins like the FLAgos (not conserved in the host) could be targets for new drugs or vaccines. In that sense, a drug that specifically target this highly diverting subgroup could be a potential innovation in the treatment of helminthiasis. Such drug has already been proposed in *in silico* modeling to target Hsa Ago-2 (Schmidt et al., 2013).

## The Dicer Family

In flatworms, the Dicer family is organized in two subgroups. While the Dcr-1 group is invariable with only a single gene per species, the Dcr-2 group is heterogenous both in number of copies and structure (**Figure 6A**). The shortening of Dcr-2 is probably the consequence of a genomic reorganization that eliminated the first 14 exons of the gene in the ancestor of all trematodes (**Figure 6B**). Transposable elements (TE) are recognized as contributors to genomic innovation as well as genomic instability across a wide variety of species (Klein and O'Neill, 2018). We have previously reported the accumulation of TEs in the genomes of trematodes, especially FBT, as an extreme example, more than 50% of the genomes of *Fasciola* spp. corresponds to repetitive elements (Choi et al., 2020). It is tempting to speculate that the accumulation of TEs in the ancestor of the trematode class has driven the Dcr2 reorganization and, possibly, further accumulation of TEs in the FBTs contributed to generate a duplication of Dcr2 (Dcr3) in that linage. Whether these shorter Dcr 2/3 are functional or represent a pseudogene awaits confirmation. However, while Dcr1 and Dcr2 are express across different stages, Dcr3 seems to be limited to eggs (Fontenla et al., 2017).

The detection of Dcr-2 proteins in *G. applanate* and *M. fusca* with very similar structures to the ones in trematodes, may indicate that the genomic reorganization detected in trematodes may have occurred more than once during the evolution of flatworms. However, given the fragmentation of the 'turbellarian' transcriptomes, further genomic data are needed to verify these observations.

## The Vasa and Vasa-Like Genes

Vasa genes in planarians are expressed in ovary and testis of sexual worms and in the totipotent cells (neoblasts) of asexual ones (Shibata et al., 1999). Increase in the expression of Vasa was detected in growing blastema of regenerating planarians and lost in irradiated organisms (Shibata et al., 1999). Belle (also known as PL10), on the other hand, is a Vasa-related protein, that has conserved roles in fertility and development, and co-localizes with Vasa to the germline (Johnstone et al., 2005). Vasa was proposed to be part of the biogenesis of piRNAs and to be differentially conserved between 'turbellarians' and Neodermatans (Skinner et al., 2014). Like the absence of Piwi, the absence of Vasa in Neodermatans results puzzling. Piwi and Vasa are widely known among developmental biologists as germline markers. Vasa was even found to be expressed in the germline of early branching Metazoans like the ctenophore clade, suggesting a central role in the development of all Metazoans (Mochizuki et al., 2001; Rebscher et al., 2007; Alié et al., 2011). Even more, vasa mutants in *D. melanogaster* fail to form pole cells, the precursor of the gonadal germ cell population, and show deletions of abdominal segments (Schupbach and Wieschaus, 1986). It has been proposed that Vasa genes arose by duplication of an ancestral PL-10-related gene before

appearance of sponges but after the diversion of fungi and plants (Mochizuki et al., 2001). Our data suggest that a second duplication of PL-10 took place in an early ancestor of the Platyhelminthes producing a flatworm specific family of PL-10-related genes. If that is the case, we speculate that a redundant role of Vasa, PL-10-related and flatworm PL-10 in 'turbellarians' was further simplified in Neodermatans with the loss of Vasa. Why the loss of Vasa was evolutionary favored in Neodermatans is still unknown; however, the germline expression and role in the gametogenesis of flatworm specific PL-10 has been reported in Neodermatans by RNAi assays (Ohashi et al., 2007). Moreover, vasa-like genes, i.e. PL-10 are strongly expressed in the ovary of *Schistosoma mansoni* female adult worms and showed high expression in female adults and eggs laid *in vitro* by worms in culture (Skinner et al., 2012). More recently, RNAi against vasa/PL10-like gene -1 in *S. mansoni* adult female worms resulted in smaller ovaries and a reduced number of ovarian dividing cells (Skinner et al., 2020). Similarly, the knockdown of vasa-like genes in *S. japonicum* induced changes in the morphology of the reproductive organs, especially in the female ovary, vitellarium and the male testes. In addition, a significant reduction in egg production in knocked-down parasites was evident (He et al., 2018).

## PiRNA Biogenesis in 'Turbellarians'

The piwiRNAs (piRNAs) are generated either from RNA transcripts of active transposable element (TE) copies or from transcripts originated from specialized loci in the genome called piRNA clusters. In general, piRNAs generated from piRNA clusters are mostly antisense to TE mRNA sequences (Tóth et al., 2016). However, these regulatory non-coding RNAs are also originated from different biogenesis pathways depending on the species. In *D. melanogaster* and vertebrates, piRNAs are 26-30 nt in length. These are derived from single-stranded piRNA precursors and processed in the cytoplasm by Zucchini (Zuc). This is a protein with endonuclease activity for single-stranded RNA and expressed in the mitochondrial surface with a predicted phospholipase D-like domain (**Figure 8A**). The piRNAs generated this way preferentially have a 5'-end uracil. HEN1 is required for 2'-O-methylation of maturing piRNAs (Horwich et al., 2007; Montgomery et al., 2012). Mature piRNAs are bounded by the Piwi protein Aubergine (Aub), to form the piRISC that targets and degrades TE mRNAs. Ago3, on the other hand, binds to TE mRNA cleaved sequences that contains an adenosine residue at position 10 and will target piRNA sequences resulting in an amplification loop named ping-pong cycle (Weick and Miska, 2014). The ping-pong cycle includes not only Aub and Ago3, but also Vasa, that has two proposed roles in piRNA processing. First, Vasa participates in the assembly of the ping-pong complex (Xiol et al., 2014). Second, the RNA-unwinding activity of Vasa helps to release cleaved products from the piRNA-protein complex to facilitate the ping-pong cycle (Nishida et al., 2015).

*C. elegans* piRNAs are shorter (21 nt long), also with a 5' uracil, and require methylation by HEN1 for maturation. Mature piRNAs bound to the Piwi orthologue PRG1, form the piRISC complex that targets and silence TE mRNAs. However, instead of the ping pong mechanism, the amplification of the silencing signal relies on RNA dependent RNA polymerases (RdRPs) (**Figure 8B**) (Weick and Miska, 2014).

The conservation of Zuc, Nbr, Vasa and RdRPs in some 'turbellarian' species rises a question about the biogenesis of piRNAs in the free-living clades. In *M. lignano*, it has been reported that the knock down of vasa produce a severe reduction in the piRNA fraction (Zhou et al., 2015). Additionally, in *M. lignano*, *S. mediterranea* and *Dugesia japonica* where the small RNA population has been sequenced, piRNAs are ~32 nt in length preferentially displaying U at the 5' end (Palakodeti et al., 2008; Friedländer et al., 2009; Qin et al., 2012; Zhou et al., 2015) like the ones described in *D. melanogaster*. Besides, a preference for A at position 10 and the overlap of reads by 10 nt suggest that a ping-pong cycle occur in these species with no evidence of any other mechanism of amplification. Therefore, it is possible to speculate that the RdRPs detected in *M. lignano* (and some other 'turbellarians') might not be involved primarily in the amplification of piRNAs. In any event, additional experimental evidence, possibly involving RNAi against RdRPs genes and sequencing of the small RNA population, is needed to test this hypothesis.

## Alternative Solutions to Piwi Absence in Parasites

The absence of Piwi in addition to the amplification of the FLAgos in Neodermatans raise the hypothesis that some of the FLAgos substitute the role of the Piwi proteins in this clade (Skinner et al., 2014). In this regard, Cai et al. (2012) sequenced the population of small RNAs associated to the FL-Ago SjAgo2 (Sja_0045200) and found that it was preferentially associated with siRNAs derived from LINE and LTR retrotransposons, the main targets of Piwi proteins (see below). This observation suggests that the FLAgos could be at least partially mimicking the role of the lost Piwi genes in Neodermatans. Furthermore, the silencing of SmAgo2 resulted in a moderate increased expression of transposable elements, suggesting that this protein might be involved in regulating transposons in *Schistosoma mansoni* (Protasio et al., 2020).

The absence of piwi pathway proteins in parasitic species seems to be a consistent trend. We here provide strong evidence of the complete absence of the pathway across all parasitic flatworms. Similarly, the absence of Piwi have been reported in all nematode clades, except clade V, the one containing the model species *C. elegans*, and some animal parasites as *Haemonchus contortus* and *Pristionchus pacificus* (Sarkies et al., 2015). Other proteins of the piwi pathway were also absent in non-clade V nematode, confirming the absence of a functional pathway. Furthermore, the piwi pathway was also found absent in the dust and scabies mite genomes (Mondal et al., 2018). Both in nematodes and in dust mites there is evidence that alternative siRNA related mechanisms are involved in controlling TEs. The amplification of genes associated with the main small RNA

pathways in parasitic flatworms is suggestive of a similar cooption of functions. Furthermore, as already mentioned primary evidence show that this might be the case in parasitic flatworms (Cai et al., 2012; Protasio et al., 2020). Since TEs are recognized as contributors to genomic innovation as well as genomic instability across a wide variety of species (Klein and O'Neill, 2018), is tempting to speculate that piwi loss might be associated to rapid genomic reorganization leading to adopt a parasitic way of life.

## CONCLUSIONS

We provide a strong bioinformatic support for the presence and absence of key proteins involved in small RNA pathways in early diverging free-living flatworms, suggesting that miRNA regulation, piRNA mediated silencing and RNAi are ancestral regulatory mechanisms in flatworms. In addition, differences observed in later evolving parasitic species strongly suggest that small RNA mediated mechanisms might have been also relevant during the transition to parasitism.

A long and still unsettled discussion has taken place regarding the biological simplification occurred in flatworms, especially in the parasitic Neodermatans. It is unclear, yet, if the process of loss of redundancy is the product of an adaptive mechanism to parasitism (Tsai et al., 2013; McNulty et al., 2017) or is an ancestral characteristic acquired by the Neodermata clade (Hahn et al., 2014). Interestingly, in the present study we found that the 'turbellarian' parasite *K. amphipodicola* showed no major differences in the conservation of small RNA pathway factors respect to other phylogenetically related free-living 'turbellarians', like *S. mediterranea*, including the conservation of a putative functional piRNA pathway that has been lost in Neodermatans (Skinner et al., 2014). Then, it is possible to hypothesize that the overall simplification associated to the Neodermatans is not a characteristic needed for parasitism in the phylum Platyhelminthes. However, the complete absence of the piwi pathway mediators in all trematodes and cestodes is suggestive of an early loss in an ancestor of the Neodermatans. This single loss might have had dramatic evolutionary consequences, since transposable elements might have driven genome instability that led to biochemical, morphological and functional transformations, for instance the origin of the neodermis (Caira and Littlewood, 2013), and other changes that favored the adaptation to a novel lifestyle. Along these lines, it is tempting to think that the later independent amplification of FLAgos, and Belle/PL10 in trematodes and cestodes might have resulted from an adaptation to these changes, either for control of transposons, and/or generating novel regulatory mechanisms mediated by small non-coding RNAs.

Different experimental approaches can be considered to test this hypothesis. Functional genomic tools tested in flatworms like RNAi (Dell'Oca et al., 2014), transgenesis (Rinaldi et al., 2012; Suttiprapa et al., 2016), genome-editing by CRISPR-Cas9 (Lok et al., 2017; Ittiprasert et al., 2019; Sankaranarayanan et al., 2020) and immunoprecipitation assays (Free et al., 2009) could be used to define the function of the factors reported here or to detect novel ones. Additionally, experimental evidence involving chromatin immunoprecipitation of nucleosome core particles followed by high throughput sequencing has proven to be useful to detect chromatin modification triggered by dsRNA (Gu et al., 2012).

To conclude, these findings together with our previous report (Fontenla et al., 2017), describe novel features of the biology and evolution that are unique to Platyhelminthes, implying that subtle mechanisms involved in the small RNA pathways of flatworms are different to the ones described in model organisms like mammals, *C. elegans* or *D. melanogaster*.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

SF performed the acquisition, bioinformatics analysis and interpretation of data and contributed in writing the manuscript. GR was involved in drafting the manuscript and critical revision of its content. JT participated in the design of the study and the interpretation of data, drafting the manuscript and critical revision of its content. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.653695/full#supplementary-material

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MINI REVIEW
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# Deciphering Human Leukocyte Antigen Susceptibility Maps From Immunopeptidomics Characterization in Oncology and Infections

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Genetic variability across the three major histocompatibility complex (MHC) class I genes (human leukocyte antigen [HLA] A, B, and C) may affect susceptibility to many diseases such as cancer, auto-immune or infectious diseases. Individual genetic variation may help to explain different immune responses to microorganisms across a population. HLA typing can be fast and inexpensive; however, deciphering peptides loaded on MHC-I and II which are presented to T cells, require the design and development of high-sensitivity methodological approaches and subsequently databases. Hence, these novel strategies and databases could help in the generation of vaccines using these potential immunogenic peptides and in identifying high-risk HLA types to be prioritized for vaccination programs. Herein, the recent developments and approaches, in this field, focusing on the identification of immunogenic peptides have been reviewed and the next steps to promote their translation into biomedical and clinical practice are discussed.

**Keywords: immunopeptidomics, human leukocyte antigen, immunochromatography, proteomics, vaccines**

## INTRODUCTION

Immunopeptidome is known as the list of peptides (independent of length, short-large list) presented on the surface of the cell by class I and class II human leukocyte antigen (HLA) molecules, which activate the immune response through selective and specific recognition by T cells. Currently, immunopeptidomes is gaining high significance in basic and translational biomedical science. Regarding basic research, to understand the immune system and specific responses to tolerogenic and non-tolerogenic antigenic stimulus, an exhaustive analysis of the immunopeptidome could be highly relevant and a key point to understand the mechanisms of immune response to be able to manipulate the specific immune responses. About translational biomedical research, the accurate knowledge of the immunopeptidome could improve immunotherapies and help in the next generation vaccine development against cancer,

autoimmune or infectious diseases (Human Immuno-Peptidome Project, 2015; Mahdi, 2019). Bearing these concepts in mind, deciphering the immunopeptidome is becoming of great interest.

The HLA system is a group of proteins encoded by the major histocompatibility complex (MHC) genes and they present peptides (antigens) to T lymphocytes. They are expressed on the cell membrane, and Class I molecules are displayed on all human cells (except for red blood cells). The principal function of these cell surface proteins is the regulation of the adaptive immune responses by engaging with the cognate T cell receptor. They are also important for the self vs. nonself discrimination by the immune system for distinguishing between the body's own proteins and foreign proteins from invaders (such as viruses, bacteria, or any type of pathogen). Moreover, the HLA system is also involved in the immunopathogenesis of many diseases, such as oncology and autoimmune pathologies, among others (Human Immuno-Peptidome Project, 2015; Mahdi, 2019).

The HLA system is composed of genes (all of them are encoded in chromosome 6) that are co-dominantly expressed and highly polymorphic. The HLA molecules are classified into two main classes, which are: *i.*-MHC class I complex is composed of major genes (HLA-A, HLA-B, HLA-C) and non-classical genes (HLA-E, HLA-F, HLA-G). The function of the MHC class I complex is the presentation of intracellular peptides to CD8 + cytotoxic T lymphocytes. *ii.*- MHC class II complex is composed of major genes (HLA-DP, HLA-DQ, HLA-DR) and non-classical genes (HLA-DM, HLA-DO). The function of the MHC class II complex is to present extracellular processed antigenic peptides to CD4+ helper T cells (Human Immuno-Peptidome Project, 2015; Mahdi, 2019).

As the source of the peptide is different between these two classes, MHC class I and II molecules have different intracellular pathways for antigen processing. This difference ranges from the HLA complex formation to the peptide loading and HLA migration to cell membrane. Therefore, the structural properties of the epitopes presented on these classes differ from each other. In HLA Class I antigen processing, the first step takes place in the cytosol where intracellular antigens are degraded, mainly by proteolysis. Then, the antigen precursor peptides are transported to the endoplasmic reticulum, where they are further modified into short linear peptides, 8 to 11 amino acids (aa′) which are assembled with HLA class I. This class I HLA-peptide complex go out to the surface of the cell and is carefully checked by CD8 + T cells for foreign antigens that differ from healthy or normal ones (Human Immuno-Peptidome Project, 2015; Mahdi, 2019; Purcell et al., 2019). When CD8+ T cells recognize the presented antigens through their T cell receptors, they ultimately eliminate the affected cells through the cytotoxic arsenal of these effector immune cells. On the other hand, the exogenous antigen will be recognized and captured by a professional antigen presenting cell (APC). Then, exogenous antigens are degraded at the endosomal compartment of the APC and the resulting peptides, larger than others, from 10 to 24 aa′, could be assembled by HLA class II molecules to be presented to CD4+ T cells (**Figure 1**) (Human Immuno-Peptidome Project, 2015; Mahdi, 2019; Purcell et al., 2019).

One of the concepts that antigen processing and subsequent presentation has a main effect on is the process named immune surveillance, which consist of the interaction of immune system with expressed intracellular and extracellular proteins. In this process, dendritic cells (DC), one of the most important APCs, are involved in scanning antigens on the surrounding tissues. After the antigen is recognized and internalized, the DCs are activated and migrate to the draining lymph nodes, where they can induce an adaptive immune response (Reis e Sousa, 2004). The antigen presentation pathway is completed when APCs process the internalized antigens and load their derived peptides onto MHC molecules (Embgenbroich and Burgdorf, 2018). Then, it is required to check endogenous expressed proteins (CD8 + T cells-HLA class I) or exogenous antigen presentation (CD8 + T cells-HLA class I for cross-presenting antigens and CD4 + T cells-HLA class II), due to constantly changes in the cell proteome that may trigger an immune response (Grabowska et al., 2018; Purcell et al., 2019).

Differences in the HLA subtypes of each class as well as differing antigen processing between the two classes lead to different peptides presented on these molecules. The nature of peptides also changes with the source of protein it is derived from. In recent years, HLA binding peptides have been analyzed to for databases (Altman et al., 1996; McHeyzer-Williams et al., 1996; Rodenko et al., 2006; Mommen et al., 2014; Bassani-Sternberg et al., 2015; Caron et al., 2015; Giam et al., 2015; Schittenhelm et al., 2015; Ternette et al., 2015; Bassani-Sternberg et al., 2016; Liepe et al., 2016; Mommen et al., 2016; Ternette et al., 2016; Abelin et al., 2017; Khodadoust et al., 2017; Shao et al., 2018), and the accuracy of the process as well as the role of antigen abundance, peptide length and post-translational modification have been investigated, to generate the complete immunopeptidome. Once peptides in the immunopeptidome is determined and characterized, these specific peptides can provide useful information for the development and design of several treatments like peptide-based vaccines as well as having a potential to be used as biomarkers in functional assays or enumeration of antigen-specific T cells (Altman et al., 1996; McHeyzer-Williams et al., 1996; Rodenko et al., 2006). Peptide-based vaccines are gaining attention in recent years, especially in the field of general vaccines against conserved regions of infectious pathogens (such as HIV, influenza and Plasmodium, among others) (Nardin et al., 2001; Parra-López et al., 2006; Purcell et al., 2007; Assarsson et al., 2008; Clemens et al., 2016; Sheikh et al., 2016). Also, this type of vaccines have a huge therapeutic potential in cancer with the discovery of many related novel epitopes, targeted by tumor-specific T cells after checkpoint suppression (Brennick et al., 2017; Verdegaal and van der Burg, 2017). For this goal, Human Immunopeptidome Project (HIPP) was been recently launched (https://www.hupo.org/Human-Immuno-Peptidome-Project), to provide a complete map of the human immunopeptidome and making the technology easy accessible, more robust and reproducible so that information will become available faster in translational clinical research (Vizcaíno et al., 2020).

In this mini-review, most relevant aspects about HLA-bound immunogenic peptides, the novel therapeutics approaches, the methodological strategies to identify MHC-I and II loaded

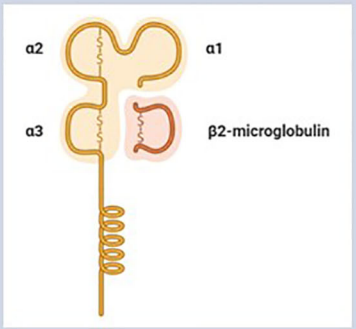
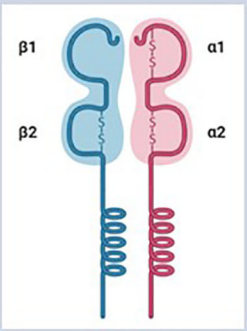
| | MHC Class I | MHC Class II |
|---|---|---|
| |   |   |
| **Structure** | •Heterodimer polypeptide: α and β2-microglobulin.<br>•Non-covalently interaction of β-2 microglobulin with α3.<br>•Antigen binding groove: α1 and α2 domains.<br>•Membrane domain: α3. | •Homogenous polypeptides: α and β polypeptide chains (immunoglobulin-like domain).<br>•Antigen binding groove: α1 and β1.<br>•Membrane domain: α2 and β2 domains. |
| **Antigen processing** | Endogenous (through proteasome) | Exogenous (through endosome) |
| **Lenght peptides** | 8-11 amino acids | 10-24 amino acids |
| **T cell recognition** | CD8 | CD4 |

**FIGURE 1** | Schematic summary of MHC characteristics. Structure, antigen processing, peptides length, T-cell recognition.

peptides and the bioinformatic open-access databases in order to perform an *in silico* prediction of the potential target peptides with therapeutical interest it is detailed described.

## IMMUNOPEPTIDOMICS: CONCEPT, APPLICATIONS, AND METHODOLOGICAL STRATEGIES

Immunopeptidomics is the large-scale study of peptides presented on HLA molecules. Thanks to the complete study of the endogenous peptides contained within a biological sample under defined conditions, the multitude of native peptides in a biological compartment can be exhaustively described with detailed features (ie. amino acid sequence, PTMs, peptide length, proteolysis processes) (Sirois et al., 2020).

In recent years, the study of immunopeptidomics has been of great interest to researchers in many scientific areas, from infectious and auto-immune diseases to oncology (Caron et al., 2017). In fact, Immunopeptidomics, based on mass spectrometry, currently is helping in the discovery of T cell targets against tumors, against autoimmune diseases and, more recently, against infectious pathogens for their application in the pandemic effects (ie. accelerate vaccine design and development, immune monitoring, engineering T cells (Caron et al., 2015).

Herein, the main features (in addition to advantages/disadvantages) of several currently developed approaches to decipher the peptides assembled in HLA molecules are critically discussed, as well as their applications in different biomedical research areas, noting that immunological knowledge and clinical translation might be highly similar regardless of the area of study, such as for cancer and infectious diseases.

### Infectious Diseases and Cancer Immunopeptidomics

Nowadays, it is well-known that many human health challenges are accompanied by disruptions in immune system and immune response. As the altered immune response is the key factor in the

origin of many diseases (ie. auto-immune diseases, infectious diseases, chronic inflammation) and also innate and specific immune responses are involved in other pathologies with different ontogeny (ie. cancer, neurodegenerative,…); then, it is expected than multiple of methodological approaches for immunopeptidomics might be similar and commonly applied to identify antigen peptides in the pathological situations (Marko-Varga and LaBaer, 2017). Therefore, it seems that it is highly interesting to bring together an overview of complementary immunopeptidomics research in order to advance in the development of novel therapeutic approaches (ie. peptides vaccines,…) or to provide novel knowledge into the disease (Vance et al., 2017).

Given also the activation of immune response in infectious diseases is due to the presence of a pathogen; recently it is also been well-described that 20% of cancers could be caused by infectious agents, such as Helicobacter pylori, hepatitis C virus (HCV), Rouse Sarcoma Virus (RSV), Kaposi´s sarcoma-associated herpesvirus (KSHV), … as solid tumors and also in onco-hematological pathologies (such as chronic lymphocytic leukemia (CLL),…) (Mantovani et al., 2008; Kowalewski et al., 2015; Vance et al., 2017).

In addition, as the immune tolerance mechanism require a continuous steady-state between self- and non-self components, it is expected that the characterization of cell-cell communication, cellular microenviroment, cell migration, immune evasion and suppression, endothelial activation, inflammation initiation and evolution, phagocytosis, cell death mechanism, … are also critical on the immune tolerance (Vance et al., 2017). Thus, infectious disease studies influence cancer studies, and vice versa. Moreover, nowadays, due to the enormous success in cancer immunotherapy, it is becoming essential to understand the immunopeptidome because it is opening novel therapeutical opportunities to treat and prevent both, infectious and cancer diseases; among that infectious disease might be a risk factor to considered in the efficiency of multiple onco-immunotherapies (Acebes-Fernández et al., 2020).

In the last decade, many studies have been focused on the relationship between infectious diseases and cancers. One of these studies described the direct relationship between *Helicobacter pylori* infection and the cause of gastric cancer by evaluating the humoral immune response. In a previous study by L. Song et al. (2020), the humoral response to 1527 proteins (almost the entire immunoproteome of *Helicobacter pylori*) in 50 cases of gastric cancer was characterized, highlighting that decreased immune response to several proteins in gastric cancer which can reflect mucosal damage and low bacterial load. Among this, there is also evidence that 8% to 10% of gastric cancers are related to Epstein-Barr virus (EBV). Another study performed by L. Song et al. (2021), screened the humoral response to this virus in gastric cancer patients. As a consequence, of this screening, it is reported that Epstein-Barr virus-positive cancer can be detected by specific antibodies that can also be used for the diagnosis and treatment of the disease (Song et al., 2021).

Furthermore, the relation between cancer and infections has been also explored for the HLA-I and II molecules. In fact, several studies have found that protein fragments from bacteria invading tumor cells can be presented by HLA molecules on the surface of tumor cells and consequently these peptides are recognized by T-cells (Kalaora et al., 2021). Also immunopeptidomics showed that both antigen-presenting cells and tumor cells display bacterial and/or virus peptides on their cell surface by HLA molecules, which are specifically recognized by CD4+ and/or CD8+ T cells. Hence, these results could help in the selection of suitable bacterial or virus targets for cancer immunotherapy (Riemer, 2021). And more recently, in the last year, immunopeptidomics has also been successfully used to identify SARS-CoV-2 peptides; so then, the relationship between the binding capacity of viral peptides to 52 common MHC-I alleles and the mortality rate has been assessed, resulting in a high inverse relationship between peptides identified from the virus using a personal workflow called Ensemble-MHC (a consensus algorithm for the prediction of MHC-I peptides) and the mortality rate (Wilson et al., 2021).

Among these aspects, there is also a close relationship between onco-immunotherapies and infectious disease. Immune checkpoint inhibitors (ICI), one of the successful therapies in immunoncology, are under continuous investigation to be applied against T cell dysfunction in chronic viral infections (Barber et al., 2006). Similarly, CAR T-cell therapies being used in cancer therapy is also being repurposed for infectious diseases (Parida et al., 2015), mainly in design and develop CAR-T cells targeting pathogens infections. Since immunopeptidome could provide information about the peptides presented by the HLA system, which could be used to design tailored chimeric antigen receptors and vaccines that induce CD8+ T cells, in order to control infectious pathogens or fight tumors. An example of these approaches is the study of peptides presented by the human immunodeficiency virus type 1 (HIV-1) (Partridge et al., 2018) in which they found viral peptides specifically bound to HLA I and II molecules but did not elicit CD8+ T-cell responses. In a similar manner, in another immunopeptidomics and infection study, an attempt is being made to block the progression of pre-erythrocytic malaria, the asymptomatic stage of the disease, by means of a vaccine. So far, using live sporozoite-based vaccines is not feasible due to the great challenges. For this purpose, the identification of *Plasmodium falciparum* antigens expressed during this stage of the disease may be useful as vaccine candidates and improve the current state of treatment (Bettencourt, 2020).

Overall, immunopeptidome characterization, both for (and sometimes together) cancer and infectious diseases help to face challenges in vaccine development, overcome drawbacks and resistances to treatments, and help to increase the efficiency and efficacy of other already implemented onco-immunotherapies.

## Personalized Vaccines

The principle of producing personalized vaccines seems simple, but accurate and selective prediction of selective & specific disease peptide antigens for each patient remains as one of the

major obstacles (Creech et al., 2018) It is estimated that each HLA heterodimer binds to thousands of peptides of allele-specific binding preference (Hunt et al., 1992; Rammensee et al., 1995; Rammensee et al., 1999; Bassani-Sternberg et al., 2015; Vita et al., 2015). Realizing the binding preference of each HLA heterodimer is the clue to successfully predict which antigens may cause specific T cell responses. For this reason, during the last decade, many efforts have been made in order to generate robust and reproducible methodological approaches for the specific identification of HLA loaded peptides which could be potential candidates for developing personalized peptide vaccines.

One of the promising therapeutic strategy in biomedicine are vaccines based on immunotherapy, especially active immunotherapy, which aims to activate the immune system *in vivo* and induce it to develop a high-specific response against exogenous and endogenous antigens. Following this way, therapeutic vaccines are divided in three types according to their content: cell vaccines, protein or peptide vaccines and genetic vaccines (made with DNA, RNA and viruses) (Acebes-Fernández et al., 2020). Here, the principal characteristics of each vaccine types will be revised from view of the role of HLA loaded peptides in the development of personalized peptide vaccines.

In cell vaccines, it is highlighted the Dendritic Cell (DC) vaccines, which are based on the intrinsic main features of DCs, commonly called professional APC. DCs, as professional antigen presenting cells, work in surrounding tissues where absorbs, process and present the pathogen and/or host antigenic peptides to primitive T lymphocytes in lymphatic organs through HLA. Therefore, although DC has a fundamental role in connecting innate and adaptive immunity, the functional characterization in DC determines that three signals are required to reach a complete and full activation. The first one is that for priming of T cells is necessary to proper loading MHC-peptide complexes. The second one, there must be an upregulation of costimulatory molecules (CD40, CD80, and CD86, for example). And the last one is the polarization of the immune response through the production of cytokines (Guo et al., 2013; Acebes-Fernández et al., 2020). There are numerous examples of these vaccines in difficult-to-treat diseases such as cancer, where DCs produced *in vitro* are used as tumoral vaccines. Mechanistically, human DC can be produced in culture from CD34+ hematopoietic progenitor cells or peripheral blood monocytes (Banchereau and Palucka, 2005). Thus, a DC vaccine is obtained by loading Tumor Associated Antigens (TAAs) onto the patient's own DC, and then treating them with adjuvant. For example, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is essential for *in vitro* production of monocyte-derived DC (Banchereau and Palucka, 2005). These cells require a maturation process, which is related to changes in the morphology and function of DCs. These procedures can improve the expression of MHC class I and II and co-stimulatory molecules, as well as increase the production of cytokines (Inaba et al., 1992). Then, these DCs are administered to patients to induce anti-tumor immunity. The first therapeutic cancer vaccine approved by the FDA was the DC vaccine Sipuleucel-T (Provenge™). It has successfully improved the survival rate of patients with a favorable toxicity profile in prostate cancer, opening a new paradigm for cancer treatment (Murphy et al., 1996; Small et al., 2006; Kantoff et al., 2010; Guo et al., 2013). Although there are more vaccines that have been used in clinical trials to treat other types of cancer such as melanoma, renal cell carcinoma and glioma (Nestle et al., 1998; HOLTL et al., 1999; Thurner et al., 1999; John et al., 2004; Small et al., 2006; Kantoff et al., 2010; Romano et al., 2011), further research is needed to prove its clinical efficacy and survival of patients with these types of cancers.

Another group of vaccines are the protein or peptide-based vaccines. Although initially these injections have been based on Tumor Associated Antigens (TAA), Cancer Germline Antigens (CGA), or Tumor Specific Antigens (TSA), together with some adjuvants, they could also be useful against infectious disease antigens. Protein or peptide-based vaccines include synthetic peptides with 20 to 30 amino acids from specific epitopes of tumor or infectious antigens. In these vaccines, the antigen could be adjusted to bind to immunogenic peptides, cytokines or antibodies (Pan et al., 2018). This type of vaccine is stable and not very expensive but has a major limitation which is the need to decipher the peptide epitopes to be used in these vaccines. Also immunosuppression present in the disease settings,as well as the weak immunogenicity of these antigens could be some disadvantages (Mocellin et al., 2009).

And the last type of vaccines is DNA vaccines: These are gene-based vaccines that use DNA (such as plasmids) or RNA (such as mRNA) (Mocellin et al., 2009). Viral DNA vectors can be used to deliver the cargo to the infiltrating somatic cells or DC (Guo et al., 2013). APC absorbs genetic material and translates them into cancer-specific antigens, thereby stimulating the immune system (Mocellin et al., 2009). Peptide or protein transcription and antigen presentation might be limited by the DNA/RNA delivery method for transfection efficiency and targeting (Mocellin et al., 2009). To administer the vaccines there are two methods: using viral vectors or by electroporation. Despite its effectiveness, it remains difficult to apply in routine clinical studies (Osada et al., 2012; Lee et al., 2015). Also it is necessary to report that the injection of live viruses can cause side effects and reduce the success of the vaccination due to clearance by antiviral antibodies in patients (Osada et al., 2012).

Considering the peptides and proteins used in cancer vaccines, in recent years, the NY-ESO-1 protein has been found to be a potential cancer vaccine antigen because of its high capacity to induce both humoral and cellular immune responses. In the study by Anna Pavlick et al. a phase I/II adjuvant clinical in resected high-risk melanomas was completed to improve the delivery of poly-ICLC as a constituent of the vaccine development. Poly-ICLC is a synthetic, stabilized, double-stranded RNA viral mimic capable of activating multiple innate immune receptors, activating CD4 and CD8 T cells and making it the optimal adjuvant for inducing *de novo* immune responses against tumor neoantigens (Pavlick et al., 2020).

The success of these vaccines depend on the right target selection and therefore, understanding peptide profiles presented on HLAs is essential for advancing the study of immunology, active immunotherapy, vaccine research and for further treatment development for any disease.

## Deciphering MHC-I and MHC-II Loaded Peptides

Recently, it has emerged of high clinical relevance to investigate how the HLA systems can affect susceptibility to infections, to immunotherapy response in oncology or in auto-immune diseases. For example, individual genetic variation will give different immune responses to a particular antigen, such as a microorganism, in a particular population. Bearing this in mind, the latest advances in this area has lead to specific identification of the peptides assembled in HLA molecules; for this purpose, several different strategies have been designed and developed which are briefly described in this review.

### Systematic Isolation of HLA Molecules

By proteomics approaches, the identification of HLA loaded peptides require multiple sequential steps because of the relative low abundance of HLA molecules (except in APCs) and a low abundance of loaded peptides; among the particular features (such as size, fixed positions of hydrophobic/hydrophilic moieties, PTMs,…) of the loaded peptides derived from protein degradation (proteosome,…) and assembled in HLA groove…. Hence, it seems that the separation and enrichment of HLA molecules is critical, and the selective elution of loaded peptides is also a key point. Thus, several methodological strategies have been performed and here it is discussed a few of them; despite of it is still an area in continuous change, progress and evolution (**Figure 2**).

Most of the performed approaches are based on the specific and selective enrichment of HLA complexes by immuno-chromatography. Then, the HLA complex is immunopurified (IP) from the cell lysate (in presence of mild detergents), and

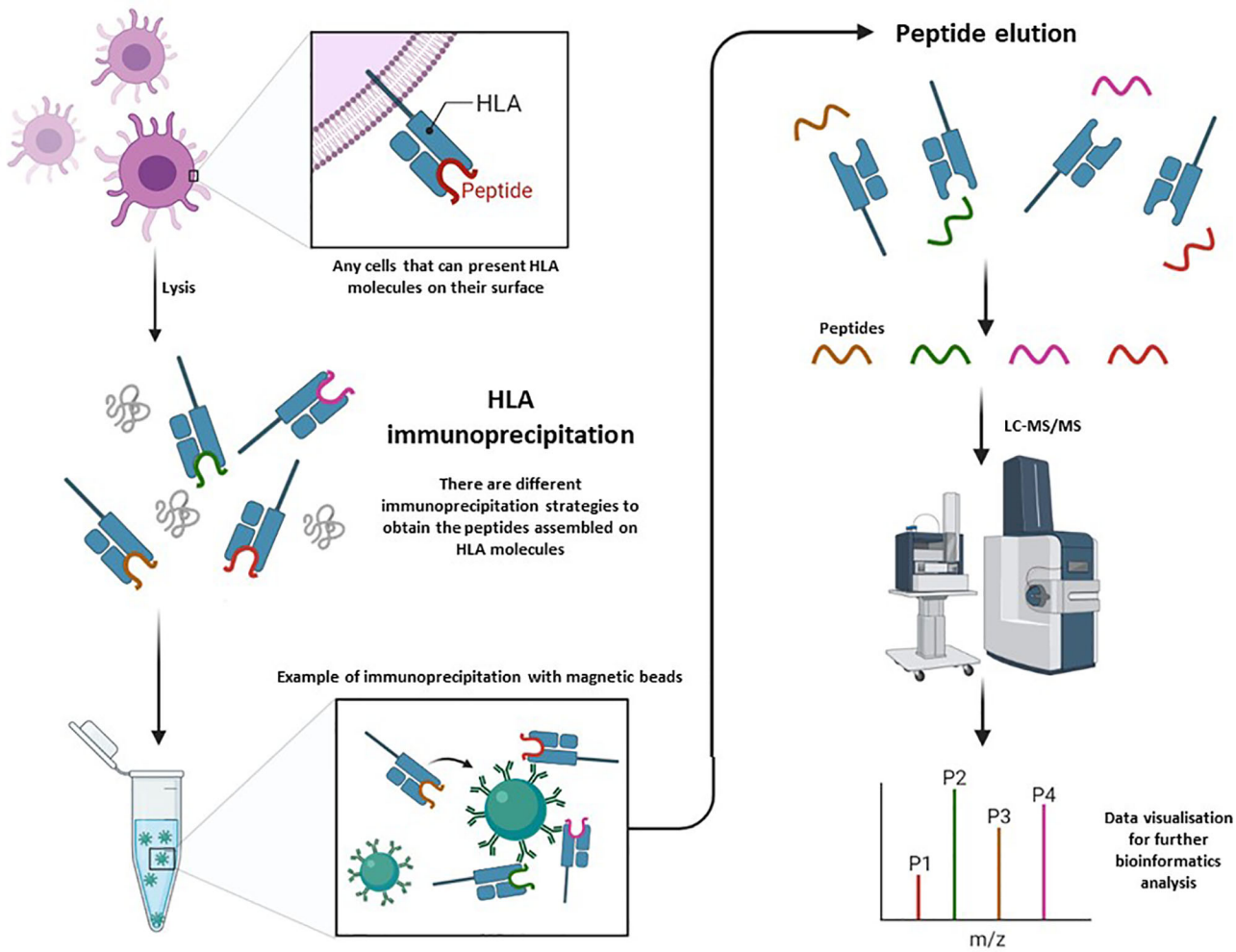



**FIGURE 2** | Global overview of the workflow for specific isolation of peptides from HLA molecules by immunochromatography and their further systematic characterization by LC-MS/MS.

further elution of peptides from the captured HLA complexes, which could be further analyzed, at high-resolution conditions, by liquid chromatography–mass spectrometry/mass spectrometry(LC-MS/MS). For this process, it is absolutely necessary to use optimal antibodies. Here, the most used are pan-HLA-I (Anti-Human HLA A, B, C: clone W6/32) and pan-HLA-II (Anti-Human HLA DR, DP, DQ: clone Tü39) (Bassani-Sternberg et al., 2010; Chong et al., 2018).

Regarding immunopurification, Chloe Chong et al. (2018) described a method based on sequential HLA purification by a chromatographic combination, starting by a pro-A beads (to capture endogenous antibodies), followed by an anti-HLA-I and/or anti-HLA-II antibodies coupled to pro-A beads to capture HLA class I and II, respectively. Then, HLA-I and HLA-II complexes are eluted and collected on a hydrophobic resin (C18) in order to selectively enrich loaded HLA peptides. The principal advantage of this strategy is that there are no intermediate steps, so it can continuously depleted endogenous antibodies and immunoaffinity purified class I and class II HLA complexes. Following this strategy, Chloe Chong et al. (2018) used B and T human cell lines and identified a total of 42,556 singular HLA class I peptides correlated to 8,975 proteins and 43,702 unrepeated HLA class II peptides from 4,501 proteins with a 1% false discovery rate (FDR). In both types of cell lines, the number of distinctive peptides changed from 3,293 to 13,696 for HLA I peptides and from 7,210 to 10,060 peptides for HLA II.

Another method is the direct immunoprecipitation using magnetic microspheres conjugated with anti-pan HLA I and II antibodies, respectively (Bassani-Sternberg et al., 2010; Chong et al., 2018). This procedure has the advantage of concentrating the cleft peptides of the HLA system thanks to the employment of super-paramagnetic microspheres conjugated to these highlighted antibodies. After separation of the peptides from the HLA molecules, the samples are eluted with an acid buffer and then the peptides are sequenced by mass spectrometry. If the whole exome or genome of the sample of interest is previously analysed to identify somatic mutations, then the results could be compared with the complete proteome (Chong et al., 2018; Kalaora and Samuels, 2019). In these studies, they compared several cancer cell lines with healthy ones, identified thousands of soluble HLA peptides, including some cancer specific peptides, shared among multiple several cell lines.

Recently, a novel procedure has been developed based on a mild acidic elution (MAE) of the HLA loaded peptides directly, in one single step, from the cells without any previous cell lysis and HLA selective enrichment. Here, thanks to the elution buffer thrown on the cells, the peptides carried by the cells of interest could be detected by further LC-MS/MS analysis. MAE strategy may be a cheap alternative to the other methods of immunoaffinity but is hindered by a large number of contaminating peptides not related with HLA molecules. By this strategy, Sturm T. et al., has identified the 50% common peptides between both approaches (MAE and immunoaffinity chromatography) as well as 22% of peptides identified only with MAE strategy (Sturm et al., 2020).

## *In Silico* Prediction of HLA-I and HLA-II Loaded Peptides

Having in mind that all human cells present HLA complexes and the critical role of HLA complexes in pathogen response and pathologies he precise and deep characterization of the immunopeptidome is highly important; hence, a synergically combination with multi-omics analysis (ie. metabolomics, genomics, proteomics, transcriptomics, epigenomics, among others) seems to be a powerful strategy for systematic determinations of immunopeptidomes. In addition to all of them, *in silico* prediction is a fundamental initial step to identify potential target neoantigens. For this purpose, it is crucial to carry out bioinformatic analysis that correlated the different databases and repositories of interest and integrate information from the immunopeptidome characterization (**Figure 3**).

Currently, several open-access bioinformatics tools are available for the prediction and selection of neoantigens by personalized proteogenomic workflows. One of them is called ProGeo-Neo (https://github.com/kbvstmd/ProGeo-neo) that allows neoantigen prediction and selection based on a customized proteogenomic pipeline (Li et al., 2020). ProGeo-Neo is based on the integration of three dataset packages: i.- RNA-seq data analysis, which could generate variant peptides; ii- HLA alleles are inferred from RNA-seq data; then it is possible to work only on selected HLA alleles; iii.- Neoantigen prediction is based on genomic and proteomics information which allows the screening of new antigens by LC-MS/MS and a neoantigen filtering through RNA expression and T cell receptor recognition (epitope). This novel pipeline is already being used as in Xiaoxiu Tan's study, where a platform was developed to facilitate the screening and confirmation of potential neoantigens in cancer immunotherapy (Tan et al., 2020).

In a similar manner, several databases have been recently created for identified peptides assembled in HLA complexes; mainly based on the employed methodology (commonly LC-MS/MS) for the identification of the peptides. Herein, it is briefly described some relevant ones with relevance to oncology and infections (**Table 1**).

**TRON Cell Line Portal** or TCLP (http://celllines.tron-mainz.de/) (Scholtalbers et al., 2015) is a database that integrated the public RNA-Seq datasets of different exposed cell lines available in two repositories: The first set of data collected by Klijn et al. (2015) and the second one in The Cancer Cell Line Encyclopedia (CCLE). This database has been able to re-analyze accessible raw RNA-Seq datasets, determined the abundance and the type of HLA molecules as well as recognized virus and quantify the gene expression of 1,082 human cancer cell lines. Using all these available datasets of established HLA isotypes, cell lines-mutations and HLA prediction algorithms, Tron Cell Line Portal allows to predict the antigenic mutations in each analyzed human cell line. There are several studies in which they did typing of Human Leukocyte Antigens by High Throughput DNA and RNA Sequencing using TCLP, which includes an overview of approaches using high-throughput sequencing for HLA typing, as well as providing

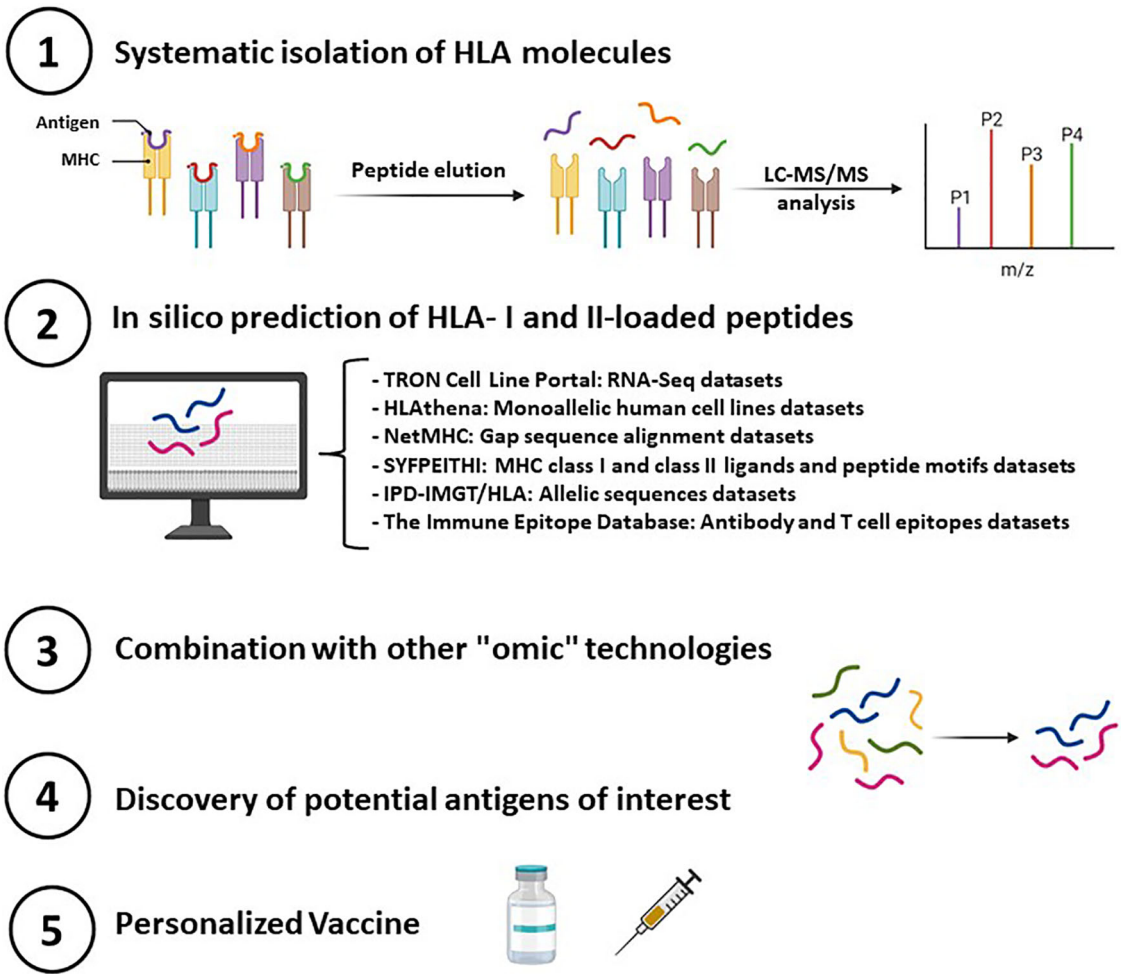



**FIGURE 3** | Essential steps in deciphering immunopeptides that elicit a T-cell response and therefore serve to design personalized peptide vaccines.

**TABLE 1** | Main features related to current available databases focused on immunopeptidome characterization.

| Database | Source Data | Info |
|---|---|---|
| TRON Cell Line Portal (http://celllines.tron-mainz.de/) | RNA-Seq datasets | Sample-details; Mutation-Data; Neo-Epitope-Data; Expression Data; |
| HLAthena (http://hlathena.tools/) | Monoallelic human cell lines datasets | Explore alleles; Select peptide length; Similarity based on allele motifs; Prediction peptides |
| NetMHC 4.1 (http://www.cbs.dtu.dk/services/NetMHC/) | Gap sequence alignment datasets | Alignment-based prediction algorithm; Peptide-MHC binding pattern; Length distribution of different HLA molecules |
| SYFPEITHI (http://www.syfpeithi.de/) | MHC class I and class II ligands and peptides motifs datasets | Ligand prediction; Correlates the prediction of T cell epitopes and HLA-loaded peptides |
| IPD-IMGT/HLA (https://www.ebi.ac.uk/ipd/imgt/hla/) | Allelic sequences datasets | Sequence alignment; Allele query; Sequence search tool; Cell query |
| The Immune Epitope Database (https://www.iedb.org/home_v3.php) | Antibody and T cell epitopes datasets | Prediction epitopes algorithm; Analysis epitopes tool |

supplementary wet-lab protocols and *in silico* screening tools (Bukur, 2017; Boegel and Castle, 2019).

In this database, seq2HLA v2.2 is useful to identify the HLA isotypes (Boegel et al., 2014) which is able to calculate the four-digit HLA type from the RNA-Seq reads. It also generates two- and four-digit calls (Boegel et al., 2013; Boegel et al., 2014) with high precision. This public data includes HLA type data investigated by Adams et al. (2005), where sequence-based typing method (SBT) is used for HLA typing to determine the HLA class I and class II genotypes of the 60 cell lines used by

National Cancer Institute (NCI-60). Using established HLA class I and II types in combination with systematic mutations will allow us to describe a register of possible neoepitopes candidates of HLA class I and II, respectively.

**HLAthena** (http://hlathena.tools/) This open-access portal is based on identification of HLA-I loaded peptides by exhaustive and systematic LC-MS/MS characterization. Here, more than 185,000 eluted peptides were analyzed from HLA A, B, C and G of 95 monoallelic human cell lines. The typical peptide motifs of each HLA allele was determined, as well as the unique and shared binding submotifs across alleles and the ones associated with a different peptide length. In addition, this database is developed by combining datasets with transcript abundance and the knowledge of peptide processing, which provides some prediction models of specific allele length for endogenous peptide presentation. These models predict HLA class I peptides compared with existing ligands tools and identify more than 75% of HLA I loaded peptides studied in 11 tumoral patients' cells (melanoma, glioblastoma and clear cell renal cell carcinoma) with high accuracy. In summary, HLAthena database allows to systematically decipher the rules of presentation of endogenous antigens in tumoral cells (Abelin et al., 2017; Sarkizova et al., 2020). Other studies have used this database to predict and reduce the number of peptides to be studied in the face of the urgent need to develop a SARS-CoV-2 vaccine because the stimulation of an adequate immune response leading to protection is highly dependent on the presentation of epitopes to circulating T cells *via* the HLA complex. In this study 174 SARS-CoV-2 epitopes with high binding prediction scores were identified and validated to stably bind to 11 HLA allotypes (Prachar et al., 2020).

**NetMHC 4.1** (http://www.cbs.dtu.dk/services/NetMHC/) is a method for predicting peptides bound to MHC class I using gap sequence alignment. This method is based on artificial neural network to align the amino acid sequence of peptides assembled in the HLA complexes, which allows insertion and deletion in the alignment. Alignment-based prediction methods including deletions and insertions show higher throughput than strategies trained on single-length peptides (Buus et al., 2003; Nielsen et al., 2003; Andreatta and Nielsen, 2016). Similarly, they exemplify how the position of the deletion can help explain the peptide-MHC binding pattern, such as when a long peptide protrudes from the HLA groove or protrudes at either end. And they also demonstrated that this method can predict the length distribution of different HLA molecules, and used this prediction algorithm to quantify the reduction in the experimental workload required to identify potential epitopes. There are several studies that use this database to carry out studies of very diverse pathologies (Conley et al., 2018; Khanna and Rana, 2019). As an example, one study uses this database to predict T-cell epitopes of *Mycobacterium tuberculosis*. As the identification of T-cell or B-cell epitopes on the target antigen is the main goal in epitope-based vaccine design, immunological diagnostic tests and antibody development, it is essential to provide a robust and reproducible system that can assist in the diagnosis of *M. tuberculosis* (Khanna and Rana, 2019).

**SYFPEITHI** (http://www.syfpeithi.de/) This approach contains a collection of MHC class I and II ligands and peptide motifs from humans as well as additional species (such as apes, cows, chickens, and mice), which is constantly being updated. You can search for HLA alleles and motifs, and you can find natural ligands, T cell epitopes, source of proteins and organisms and their references. It includes links to The European Molecular Biology Laboratory (EMBL) and PubMed databases. In addition, ligand prediction can be used for various HLA allele products. The content of the database is limited to the availability of published datasets; however, it is highly useful to correlate the prediction of T cell epitopes and HLA-loaded peptides (Rammensee et al., 1999).

The prediction based on published motifs (such as natural ligands and cluster sequencing) considers the amino acids at anchor positions. Calculation of a reliable score is for anchoring is done by giving some amino acids of determined peptides specific values depending on whether they are anchors, auxiliary anchors or preferred residues. Ideal anchoring is 10 points, unconventional anchoring is 6 to 8 points, auxiliary anchoring is 4 to 6, and residues are preferably 1 to 4 points. Also, some amino acids which have a molecular weight that is considered to have a negative outcome on binding capacity are between −1 and −3 (Rammensee et al., 1999). This novel database has already been used in the characterization (previously discussed) of HLA ligandome in chronic lymphocytic leukemia, among others (Kowalewski et al., 2015).

**IPD-IMGT/HLA** (https://www.ebi.ac.uk/ipd/imgt/hla/) includes 25,000 allelic sequences of more than 40 genes, which are encoded by the MHC of the human genome. The IPD-IMGT/HLA is a stable, highly accessible and easy-to-use database which provides access to many alternative sequences of this genetic system to the medical and scientific communities, essential for, for example, successful transplant results. The challenge for this database is to continue providing a highly selected sequence variation database while keeping an increased amount of submissions and the complexity of the sequence (Robinson et al., 2020).

This database has multiple tools, either its own or that have been incorporated from data libraries to the existing tools available from the European Bioinformatics Institute (https://www.ebi.ac.uk/) (EMBL-EBI) (Valentin et al., 2010). Among them, you can find: i.-Sequence alignment, ii.-Allele query, iii.-sequence search tool (FASTA and BLAST) and iv.-cell query (original materials) (Labarga et al., 2007; Valentin et al., 2010; Robinson et al., 2014; Albrecht et al., 2017; Madeira et al., 2019).

**The Immune Epitope Database (IEDB)** (https://www.iedb.org/home_v3.php) is an open-access database supported by the National Institute of Allergy and Infectious Diseases (NIAID). It catalogues, in the context of infectious disease, allergy, autoimmunity and transplantation, different experimental studies on antibody and T cell epitopes examined in humans as well as non-human primates and several animal species (Vita et al., 2019). The IEDB also hosts tools to help in the prediction and analysis of epitopes. In one recent study, they use the IEDB database to design a model for vaccine design by prediction of B-

epitopes in perturbations in the sequence of a multitude of peptides, in various source or host organisms (González-Díaz et al., 2014).

### Analysis of LC-MS/MS Data Sets of MHC-I and MHC-II Loaded Peptides

In addition to the information repositories about the peptides assembled in HLA molecules, tools for analyzing data extracted from LC-MS/MS characterization (including the different MS/MS instrumentation) and databases are increasingly common, including:

**DeepRescore** (https://github.com/bzhanglab/DeepRescore) is an immunopeptidomics data analysis device that supports deep learning-derived peptide characteristics to arrange peptide-spectrum matches (PSMs). It could take MS/MS raw data (in MGF format, for example) and recognize results from search engines as input. The last version supports four well-establish search engines like MS-GF+, Comet, X! Tandem and MaxQuant (Li et al., 2020). DeepRescore includes peptide characteristics derived from deep learning predictions (among which stand accurate retention time and MS/MS spectrum prediction) with previously used functions to reproduce peptide spectrum matching. Using two public immunopeptideomics data sets, it is demonstrated that compared with existing methods, the scoring performed by DeepRescore increases the accuracy and sensitivity of the recognition of MHC binding peptides and neoantigens. It also shows that, to a large extent, performance improvements are driven by features derived from deep learning. Thus, this post-processing tool is freely available to the scientific community and can be used to identify sensitive and reproducible HLA binding peptides and neoantigens from immunopeptidomic datasets.

**PEAKS X PRO** (https://www.bioinfor.com/ Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) is a commercially available software platform with several tools for the detection of peptide characteristics. Here, the three main characteristics are: DeepNovo is an extensive neural network model for *de novo* peptide sequencing. The DeepNovo design includes the latest developments in convolutional neural networks (CNN) and recurrent neural network (RNN) to learn the characteristics of peptides, as well as fragment ions and sequence patterns of tandem mass spectra. These systems are also integrated with local dynamic programming to solve the complex improvement tasks of *de novo* sequencing. DeepNovo has an accuracy growth at the amino acid level by 7.7% to 22.9% and an accuracy improvement at the peptide level by 38.1% to 64.0%. DeepNovo was used to automatically construct again the entire sequence of the mouse antibody light and heavy chain, without the need for others auxiliary databases to achieve 97.5% to 100% coverage and 97.2% to 99.5% accuracy. In addition, it can be retrained to modify any data source and supplies a whole end-to-end training and prediction solution for *de novo* sequencing problems (Tran et al., 2017). The second one is DeepIso, which combines the latest developments in CNN and RNN to estimate the peptide intensity and detect their features of different charge states. DeepIso consists of two different deep learning-based components, which can learn multiple levels of the high-dimensional data itself represented by multiple layers of neurons and can be adapted to new situations. The peptide characteristics list investigated with this model matches with 97.43% of high quality MS/MS identifications in a standard dataset (Zohora et al., 2019). The third one is DeepNovo-DIA, *de novo* peptide sequencing Independent data acquisition (DIA) method of mass spectrometry data. It also uses a neural network to capture cross m/z, retention time and intensity. Besides, DIA combined with peptide sequence pattern solves height problems multiple spectra, allowing us to identify novel peptides in human antigens and antibodies (Tran et al., 2019).

**MaxQuant** (https://www.maxquant.org/) is a quantitative proteomics software package developed for analyzing large mass-spectrometric data sets. It is the most used software with this type of raw data due to its versatility for handling and the interesting options with quantitative results. It is composed by a set of algorithms that extract information from raw MS data with high efficiency and sturdiness, capable of count elevated peptide identification rates, as well as high precision protein quantification for thousand proteins in complex proteomes (Cox and Mann, 2008). In recent years, the demand of MaxQuant has increased due to the great development of methodological strategies to identify peptides of interest in different pathologies (Schaab et al., 2012; Tyanova et al., 2015; Tyanova et al., 2016).

## DISCUSSION

### Relevance of Immunopeptidomics Characterization in Oncology and Infections

For personalized medicine, a huge effort has been made to explore endogenous and exogenous processed ligands by diverse HLA heterodimers; which is an important from multiple points of view, such as: knowing the specific binding preferences of HLA could help predict secure and preferably immunogenic epitopes for independent patients with different pathologies. Technological and methodological advances (LC-MS/MS instrumentation, data search algorithms, acquisition methods, …) has increased the number and size of MS data repositories and their correlation with other multi-omics info (Vita et al., 2015; Fleri et al., 2017; Creech et al., 2018).

In addition, another advance has been the creation of international consortiums dedicated to establishing standardization strategies related to peptides isolated from HLA molecules. Hence, a few years ago, it was launched The Human Immuno-Peptidome Project (HIPP) (https://www.hupo.org/Human-Immuno-Peptidome-Project), was launched under the umbrella of the Human Proteome Project Organization (HUPO), whose main goal is to use mass spectrometry technology to map the entire spectrum of peptides presented by HLA molecules and allow any immunologist, clinicians and other biomedical scientist to perform reliable analysis (Admon and Bassani-Sternberg, 2011; Caron et al., 2017). Within this framework, the immunogenicity of the predicted antigen will be confirmed through *in vitro*

validation studies to evaluate the accuracy as well as the efficiency of present prediction algorithms investigated by numerous industrial and academic laboratories.

### Limitations of Immunopeptidomics Characterization

Bearing in mind all the methodological processes that must be carried out for the identification of immunogenic peptides assembled on HLA molecules, there are several limitations to be taken into account for their discovery.

The first limitation observed is the need to use an optimal lysis buffer, which allows us to obtain the maximum amount of membrane proteins, where the HLA molecules are subcellular located, and consequently the peptides assembled on them, to achieve a huge increment of relative protein abundance. If other specific lysis buffers are used for other subcellular compartments or for total protein extraction, maybe the relative abundance is not modified to enrich on HLA molecules with the subsequent decrease of peptides identification. In a similar manner, the elution buffer is also quite critical because it also might affect the yield on peptide isolation and identification (Chong et al., 2018).

Depending on the main goal of each immunopeptidome characterization, the immunoprecipitation strategy to be used must be taken into consideration; because another limitation observed is directly related to the pan-antibodies; because these pan-antibodies are optimal for maximizing the coverage, however, it also might lead to false HLA-restriction attribution to the eluted peptides (Bassani-Sternberg et al., 2010; Chong et al., 2018).

And finally, it must be taken into account that there are peptides with a weak and/or poor immunogenicity and involved in a possible immunosuppression, so that, only by using mass spectrometry, the identification of all the peptides bound to an HLA molecule may be limited (Purcell et al., 2019).

## FUTURE PERSPECTIVES AND CHALLENGES

Despite the great advances in the knowledge and prediction of HLA-bound ligands, challenges remain because the antigens of interest currently represent a very small fraction of HLA-ligandome. Then, the integration of this knowledge with other multi-omics technologies is required, such as antigen identification based on whole exome sequencing and the subsequent predictive power of the algorithms that predict HLA ligand binding. However, these investigations emphasize the clinical possibilities of naturally processed HLA ligands based on MS sequencing, especially when merged with other "omics" strategies such as RNA-seq or next-generation sequencing (NGS) (Dutoit et al., 2012; Nelde et al., 2019).

Another challenge which needs to be overcome is the current need for plenty patient materials and the time frame required for patient-specific groups of HLA ligands and antigen analysis, which hinder and delay the implementation in the clinical environment and routines (Ott et al., 2017; Sahin et al., 2017; Creech et al., 2018). For example, tumor biopsy, HLA typing or whole exome sequencing and mutation detection may take about two weeks. At the same time, once the new antigen target is determined, HLA ligand enrichment, MS data collection and analysis may take several days to several weeks, while the design and manufacture of personalized vaccines may take several weeks. The total time limit for these procedures could be several months, which at this moment is incredibly lengthy for routine use in the clinic (Creech et al., 2018).

In addition, it is important to take into consideration that the response to personalized therapies will depend on the state of the immune system of each patient (Strønen et al., 2016). In order to understand how to optimize and adapt adaptive immune responses, and to improve the prediction and prioritization of antigenic determinants, further research is needed on the expected antigen T cell responses with TCR sequencing and epitope antigen prediction from TCR segments. Bearing this in mind, more studies which combine HLA-ligandome and TCR sequencing for a perfect recognition match between peptide loaded HLA and epitope mapping are required.

Predictions for potential antigens could provide candidates, in many recent studies predominantly for HLA class I epitopes due to the high accessibility of experimental data for class I prediction algorithms in contrast to class II. When CD4 + T cell responses have been studied in preclinical and clinical vaccination investigations (Dutoit et al., 2012; Kreiter et al., 2015; Sahin et al., 2017), it was shown that the processing and presentation of the HLA class II epitope may also play a critical role in the treatment of many diseases. However, although there are prediction algorithms for both classes, those of class II are less accurate because the peptide-binding groove allows longer peptides to bind, increasing the heterogeneity and complexity of epitope presentation (Depontieu et al., 2009; Mommen et al., 2016; Khodadoust et al., 2017). Therefore, more extensive analysis is required to better understand the characteristics of HLA class II bound peptides and the cellular procedures required for processing and presentation of class II epitopes (Kim et al., 2017).

Overall, improving prediction algorithms and combining MS HLA ligand profiles with other "omics" approaches is essential to create opportunities for customized peptide vaccines against various pathologies targeting antigens (self- and non-self) of interest and enable personalized immunotherapies (in oncology or infectious diseases) with large-scale clinical applications (Creech et al., 2018). For this reason, LC-MS/MS technology should continue to promote the improvement of epitope prediction and our knowledge of epitope processing and presentation for personalized immunotherapies which would transform the way patients with infectious, autoimmune diseases or even cancer are treated today.

## AUTHOR CONTRIBUTIONS

Conceptualization: PJ-V, AL-V, VA-F, EM, RG, MF; Resources: PJ-V, APH, MLG-V, CA-H; Writing-original draft preparation: PJ-V, CA-H, HB, MF; Supervision: HB, EM, RG, MF; Funding acquisition: MF. All authors contributed to the article and approved the submitted version.

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# Studies of the Parasite-Midgut Interaction Reveal *Plasmodium* Proteins Important for Malaria Transmission to Mosquitoes

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Malaria transmission relies on parasite-mosquito midgut interaction. The interactive proteins are hypothesized to be ideal targets to block malaria transmission to mosquitoes. We chose 76 genes that contain signal peptide-coding regions and are upregulated and highly abundant at sexual stages. Forty-six of these candidate genes (60%) were cloned and expressed using the baculovirus expression system in insect cells. Six of them, e.g., PF3D7_0303900, PF3D7_0406200 (Pfs16), PF3D7_1204400 (Pfs37), PF3D7_1214800, PF3D7_1239400, and PF3D7_1472800 were discovered to interact with blood-fed mosquito midgut lysate. Previous works showed that among these interactive proteins, knockout the orthologs of Pfs37 or Pfs16 in *P. berghei* reduced oocysts in mosquitoes. Here we further found that anti-Pfs16 polyclonal antibody significantly inhibited *P. falciparum* transmission to *Anopheles gambiae*. Investigating these candidate proteins will improve our understanding of malaria transmission and discover new targets to break malaria transmission.

**Keywords: *Plasmodium*, malaria transmission, sexual stage, parasite-mosquito interaction, Pfs16, mosquito midgut invasion**

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## INTRODUCTION

Malaria is a deadly infectious disease spread by mosquitoes. It affects half of the world's population and claims nearly a half-million lives annually. Although the global malaria cases have decreased since 2010, the ten most burdened countries in Africa still had 3.5 million more cases than the previous year (Who, 2018). The fast spread of insecticide-resistant mosquitoes partially causes this. Mosquitoes become resistant to the five major chemical pesticides, making traditional vector control methods by pesticides often fail to control malaria epidemics (Alout et al., 2017). Concerns also come from the artemisinin-resistant *Plasmodium falciparum* throughout Cambodia and parts of Thailand. The spread of drug-resistant parasites may cause devastating consequences in the future (Chookajorn, 2018; Conrad and Rosenthal, 2019). Malaria vaccines that protect humans from infecting would be ideal for controlling malaria epidemics. However, no malaria vaccines have been developed. The most promising malaria vaccine candidate is RTS,S. It is mainly composed of circumsporozoite protein fragments presented on lipoprotein particles (Duffy and Gorres, 2020).

However, the efficacy of RTS,S in Phase III trial was less than 36% (Rts, 2014). These issues have prompted researchers to find new strategies to stop the spread of malaria.

Since *Plasmodium*-infected mosquitoes spread malaria, a vaccine or medicine that prevents parasites from infecting mosquitoes is an alternative for malaria control. The targets of transmission-blocking reagents may participate in parasite developmental processes such as differentiation of sexual forms of microgametes and macrogametes, zygote formation, ookinete invasion of mosquito midgut, and sporozoite infection (Acquah et al., 2019). Several antigens from parasites have been examined as targets to block malaria transmission. Antibodies against Pfs25 or members of s48/45 6-Cysteine family (Pfs230, Pfs48/45, and Pfs47) on the surface of gametocytes (MacDonald et al., 2016) and zygotes (Young et al., 2005) have been shown to inhibit malaria transmission. Some proteins targeted by TBV studies were summarized in a recent review (Bennink et al., 2016).

The interaction between parasites and the mosquito midgut is a crucial determinant of successful infections in a mosquito. Quite some studies have shown mosquito proteins are essential for the ookinete formation in the mosquito midgut and their penetration of the peritrophic matrix (PM) and midgut epithelial cells. Mosquito proteins AnAPN1 and FREP1 have been identified to play roles in malaria transmission. AnAPN1 localizes on the apical surfaces of *Anopheles gambiae* midguts (Blagborough and Sinden, 2009), and studies have shown that anti-AnAPN1 antibodies significantly inhibited malaria infection in mosquitoes (Dinglasan et al., 2007; Pritsch et al., 2016). FREP1 was identified by a direct association study of clinically circulating *P. falciparum* infection in wild *An. gambiae* mosquitoes (Li et al., 2013). FREP1, located at PM, interacts with parasites, thereby facilitating malaria transmission (Zhang et al., 2015). Antibodies targeting the FREP1 FBG domain inhibited transmission of *P. falciparum* and *P. vivax* to *An. gambiae* and *An. dirus*, respectively (Niu et al., 2017). CRISPR/Cas9-mediated gene knockout of FREP1 further elucidated the function of FREP1 as a host factor in mosquitoes (Dong et al., 2018).

Parasitic proteins involved in gametogenesis, fertilization of macro- and micro-gametes, zygote-to-ookinete transformation, and penetration of the midgut endothelium are also critical for malaria transmission (Bennink et al., 2016). Recently, protein Pfs47 (PF3D7_1346800) was reported to interact with AgP47Rec in the mosquito midguts to evade mosquito immunity (Molina-Cruz et al., 2020). Also, some soluble proteins secreted by the sexual stage parasite or membrane-associated proteins such as chitinase and von Willebrand factor A-domain-related protein (WARP) are also important to complete parasite transmission to mosquitoes (Li et al., 2004). More research is required to study parasitic proteins that interact with mosquito midguts to elucidate molecular mechanisms of malaria transmission and provide more targets for malaria control.

Besides targeted by transmission-blocking vaccines, these interactive proteins can also be targeted by small molecules to block malaria transmission. The fungal secondary metabolite *P*-orlandin from *Aspergillus niger* (Niu et al., 2015), asperaculane B from *Aspergillus aculeatus* (Niu et al., 2020b), and pulixin (Niu et al., 2021) that prevent FREP1 in mosquito midgut from binding to sexual stage *Plasmodium falciparum* inhibit malaria transmission to mosquitoes. A synthetic polysulfonated polymer limits malaria transmission by inhibiting the interaction between midgut chondroitin sulfate glycosaminoglycans and ookinetes (Mathias et al., 2013).

Therefore, this study focused on sexual stage parasitic proteins that interact with mosquito midguts. These proteins should be at the cytoplasmic membrane or secreted from the parasites. The identification of these proteins will help us to understand the molecular mechanisms of malaria transmission and provide targets for vaccines and drugs to control malaria transmission.

# MATERIALS AND METHODS

## Rear Mosquitoes

*An. gambiae* (G3 strain) obtained from BEI Resources was maintained in an insectary set at 28°C, 80% relative humidity, 12 hours (h) day/night cycle. Larvae were fed with grounded fish food, and adult mosquitoes were maintained with 8% sucrose solution. The commercial human (AB+, Oklahoma Blood Institute, Oklahoma City, OK) was washed with the same volume of RPMI-1640 three times by centrifugation (500xg for 5 minutes (m)). The human serum (O+, Interstate Blood Bank, Memphis, TN) was heat-inactivated at 56°C for 30 m. The red blood cells and serum were mixed at the ratio of 1:1 (v/v) to feed mosquitoes through a glass feeding device (Chemglass, Vineland, NJ) to lay eggs.

## *P. falciparum* Culture

*P. falciparum* (NF54 strain) was obtained from BEI. The parasites were cultured with the RPMI-1640 (Gibco) complete medium that contains 4% fresh $O^+$ human red blood cells (Oklahoma Blood Institute, Oklahoma City, OK), 12.5 μg/mL hypoxanthine, and 10% human AB+ serum (Interstate Blood Bank, Memphis, TN) in a candle jar at 37°C. The culture was initiated at 0.25-0.5% parasitemia, and the medium was replaced daily. The day 15-17 *P. falciparum* centrifugation at 500×g for 3 m) and resuspended in human AB+ serum/packed $O^+$ red blood cells (1:1 by volume) and used for standard membrane feeding assays (SMFA) (Zhang et al., 2015).

## Prediction of Transmembrane Regions in *P. falciparum* Proteins

The sequences of *P. falciparum* (3D7) proteins were downloaded from PlasmoDB. The protein sequences in fasta format were uploaded into TMHMM Server (v2.0) online (http://www.cbs.dtu.dk/services/TMHMM/). The output format parameter was "Extensive, no graphics". The number of transmembrane regions in each protein was predicted with the TMHMM algorithm (Krogh et al., 2001).

## Selection of Candidate *P. falciparum* Proteins

The information of the signal peptide for each protein was obtained from PlasmoDB. The data of the gene expression of the published RNA-Seq data (Lopez-Barragan et al., 2011) at the different developmental stages of the parasites was downloaded from PlasmoDB. It is inside mosquito midguts, and the gametocytes become gametes that further form ookinetes. The interactions between midguts and gametocytes as well as between midguts and ookinetes are important. These genes are expected to be upregulated at the sexual stage, and their products are cytoplasmic membrane proteins or secretory proteins. Therefore, the candidate genes were selected according to the following three criteria: 1) the coding proteins containing signal peptides; 2) gene expression at sexual stages (median values of stage II gametocytes, stage V gametocytes, and ookinetes) were >5-fold higher than that at any asexual stages (maximum schizont); and 3) genes are abundantly expressed at the sexual stage with >60 RNA-Seq reads at any sexual stages (minimum value). The protein annotations, subcellular locations, and other features were obtained from the UniProt and PlasmoDB. We did not use the mass spectrometry proteomics data because it is challenging to separate cells in different stages (Swearingen and Lindner, 2018).

## Cloning and Expression of Candidate Genes With a Baculovirus Expression System in Insect Cells

Total RNA was extracted from the *P. falciparum* (NF54) infected RBCs using RNAzol (Sigma-Aldrich, MO). The cDNA was synthesized using SuperScript First-Strand Synthesis System (Invitrogen, CA). The genes were amplified by PCR using DNA Engine Dyad Thermal Cycler (Bio-Rad, CA) with gene-specific primers (**Table S1**). The DNA fragments were purified by GeneJet PCR Purification Kit (Thermo Scientific) and cloned into the modified plasmid pFastBac1, which contains a 6xHis tag at the C-terminus of the multiple cloning site. The recombinant plasmid was transformed into DH5α competent cells. PCR-positive recombinant plasmids were confirmed by sequencing and then transformed into DH10Bac competent cells to obtain recombinant bacmids. The white colonies on culture plates were picked and confirmed by PCR. About 1 μg of recombinant bacmid in 100 μL of Grace's Insect Medium, unsupplemented (Invitrogen) was mixed with 100 μL of Grace's Insect Medium, unsupplemented (Gibco) containing 5 μL of cellfectin II, and incubated at room temperature (RT) for 15 m. The mixture was then added to 1.5 mL of Grace's Insect Medium, unsupplemented containing $1\times10^6$ sf9 cells in a 6-well culture plate, and incubated for 4 h at 27°C. The supernatant was replaced with 2 mL of Grace's Insect Medium (Gibco) with 10% FBS (Invitrogen) and cultured at 27°C for 3 days to produce recombinant baculoviruses. After amplification for 2-3 generations, 100 μL of the culture supernatant containing the recombinant virion was added to 2 mL of Express Five serum-free medium (Gibco) supplemented with 20 mM L-glutamine and 1 million High Five cells in the wells of a 6-well culture plate. After incubation at 27°C for three days, both supernatant and cells were harvested by centrifugation at 500×g for 5 m at 4°C. The cells were suspended in native cell lysis buffer (Invitrogen) and sonicated (pause for 30 s every 10 s) for 5 m on ice. Cell debris was removed by centrifugation (10,000 g for 5 m). The recombinant protein concentrations in culture medium and cell lysate were measured with anti-His monoclonal antibodies comparing to a standard protein concentration curve.

## Analysis of the Interaction Between a Recombinant *P. falciparum* Protein and Mosquito Midgut Lysate Using ELISA

The 3-5-day old adult female mosquitoes were fed with fresh blood using a membrane feeding device. Starting 16 h post blood meal and lasting more than 4 h, ~100 midguts were dissected from blood-engorged mosquitoes. The blood was carefully removed from the midguts. The midguts were then placed in a 1.5 mL plastic tube containing lysis buffer (50 mM Tris-HCl, 0.15 mM NaCl, 0.2% Tween-20, pH7.8) and grounded with a micro pestle (Sigma-Aldrich). Subsequently, the tissues were lysed in native cell lysis buffer (Clontech) with ultrasonication for 10 m on ice with 30 s pulse and 10 s sonication. The debris was removed by centrifugation (10,000 g for 5 m) at 4°C. The protein concentration in the supernatant was measured by the Bradford method.

About 50 μL of 1 mg/mL midgut proteins were used to coat each well on a 96 well plate at 4°C overnight. The plate was then blocked with 100 μL of 2% BSA in PBS for 2 h at RT. About 100 ng of recombinant candidate proteins in 50 μl PBS were added to each well. Each corresponding recombinant protein was heat-inactivated for 15 m at 65°C as a control. The plates were incubated for 1 h at RT. Then, 50 μl anti-His mouse monoclonal antibody (Sigma, 1:1, 000 dilutions in blocking buffer) was added and incubated at RT for 1 h, followed by incubation with 50 μl of goat anti-mouse IgG-Alkaline Phosphatase conjugate (Sigma, 1:10, 000 dilutions in blocking buffer) for 1 h at RT. Finally, 50 μL of *p*-nitrophenyl phosphate (pNPP) (Sigma-Aldrich, MO) was added to develop the plate. The plate was then washed three times with PBST (1xPBS containing 0.2% Tween 20) between two incubations. The signal was detected with an Epoch Microplate Spectrophotometer at $A_{405}$ nm (BioTek, Winooski, VT). Because one set of data from the same sample (midgut lysate) was used multiple times (46 proteins), statistical analysis was conducted by multiple t-test using the two-stage linear step-up procedure (Benjamini et al., 2006) with 1% of False Discovery Rate (Q) implemented in GraphPad Prism 8 (GraphPad Software, USA).

## Verification of the Interaction Between a Recombinant *P. falciparum* Protein and Unhomogenized Mosquito Midguts

These six recombinant proteins were further verified by an alternative ELISA. About 10 unhomogenized midguts (collected 18 h after bloodmeal) in a 1.5 mL plastic tube were suspended in 50 μl PBS containing 100 ng of a recombinant candidate protein and incubated for 1 h at RT. The PBS buffer with chloramphenicol acetyltransferase (CAT), which was

expressed with the baculovirus system in the High Five cells, was used as a blank control. Then, the midguts were collected through centrifugation (500 g for 5 m), washed with 100 µl PBS three times, and homogenized in 100 µl lysis buffer (50 mM Tris-HCl, 0.15 mM NaCl, 0.2% Tween-20, pH7.8) by a micro pestle (Sigma-Aldrich) and ultrasonication as described above. After removing the debris by centrifugation (10,000 g for 5 m) at 4°C, 50 µl of the supernatant was used to coat ELISA, followed by incubation with anti-His mouse monoclonal antibody, goat anti-mouse IgG-Alkaline Phosphatase conjugate, and pNPP as described above. The plate was washed three times with PBST (1xPBS containing 0.2% Tween 20) between two incubations. The signal was detected with an Epoch Microplate Spectrophotometer at $A_{405}$ nm (BioTek, Winooski, VT). Statistical analysis was conducted by multiple t-test using the Two-stage linear step-up procedure (Benjamini et al., 2006) with 1% of False Discovery Rate (Q) implemented in GraphPad Prism 8 (GraphPad Software, USA).

## Antibody Transmission-Blocking Assays of *P. falciparum* Infection in *An. gambiae* Mosquitoes

*E. coli*-expression of the full-length mature Pfs16 protein and generation of the antibody was conducted by a company (Bosterbio, Pleasanton, CA). The polyclonal anti-Pfs16 antibody was generated in rabbits and purified with protein A/G immunogen affinity. The titer of the antibody was measured by coating 0.1 µg antigen to the wells in a 96-well plate and calculated by testing a series dilution of the antibody. To conduct antibody transmission-blocking assays, we suspended day 15-17 cultured *P. falciparum* with the fresh O+ type human blood to get the 0.2% final concentration of stage V gametocytes. Approximately 15 µL PBS containing 45 µg purified anti-Pfs16 rabbit polyclonal antibody was mixed with 285 µL infectious blood (final antibody concentration is 0.15 mg/ml), and an SMFA (Niu et al., 2020a) was conducted using 3-5 days old female naïve mosquitoes. After feeding for 30 m, the engorged mosquitoes were separated and maintained with 8% sugar in a BSL2 insectary (27°C, 12-h light/dark cycle, 80% humidity). Seven days after infection, midguts were dissected, stained with 0.1% mercurochrome, and examined using light microscopy to count oocysts. Equivalent amounts of irrelevant antibody or BSA were used as controls. Since the distribution of oocyst numbers does not follow the normal distribution, the results were analyzed with the nonparametric test, Wilcox-Mann-Whitney test, and the experiments were repeated three times. Also, 15 µL PBS containing 45 µg, 15 µg, 3 µg, 1 µg or 0 µg purified anti-Pfs16 rabbit polyclonal antibody were mixed with 210 µL infectious blood to conduct SMFA. Due to infection rates and mosquito mortality, >50 mosquitoes were used for infection.

## Binding Assays of Pfs16 to Mosquito Midguts

Mosquito midguts were isolated from 3-5-day-old naïve mosquitoes. The mosquito midguts were also isolated from mosquitoes 18 hr post bloodmeal. The midguts were cut in half, and the content was rinsed with 200 µL 1xPBS three times. Midguts were incubated sequentially with the recombinant Pfs16 protein (experimental group) or CAT (control group) expressed in High Five cells culture supernatant (10 µg/mL) for 1 h at RT, the rabbit antibodies against Pfs16 protein in PBS (1µg/mL) for 1 h at RT, Alexa Fluor 594-conjugated goat against rabbit antibodies (ThermoFisher, 1:500 dilutions in PBS) for 1 h in the dark at RT. The sample midguts were washed with 1XPBS 5 min for 3 times between each incubation. Finally, the treated midguts were examined under a fluorescence microscope. All midguts were exposed with the same activated light intensity, and images were taken with the same exposure time. The fluorescence pixel in images was measured with Adobe Photoshop 2020.

# RESULTS

## Selection of *P. falciparum* Proteins That Potentially Affect Parasite Invasion in Midguts

We selected *P. falciparum* proteins with signal peptides, and their gene expression was upregulated and abundantly present at the sexual stage as described in methods. Based on protein sequences from PlasmoDB, 1,079 *P. falciparum* proteins contain signal peptides. The RNA-Seq data of seven developmental stages of these genes (Lopez-Barragan et al., 2011) were downloaded from PlasmoDB. Differential gene expression analysis of RNA-seq data was performed using the R package, and a heatmap was generated. The result showed stage-specific gene expression patterns, with about half of them being upregulated at the sexual stage (**Figures 1A**, **S1**). Among them, 670 gene-encoded proteins contained one or more transmembrane regions (TM), and 413 contained signal peptides without TM. We selected the genes that were >5-fold upregulated at sexual stages and abundantly expressed (>60 reads per gene) at the sexual stages (gametocyte II, V, and ookinete). These criteria were selected because only sexual stage proteins are critical for parasites to infect mosquitoes, and abundantly expressed genes have fewer measurement errors and are relatively easier to study their mechanisms. Based on the selection criteria, 76 candidate proteins were obtained for further study (**Figure 1B**). Many important genes, such as CelTOS, GEST, and SOAP, which have been proved to be necessary for parasite invasion, were included in this list (**Table 1**). The expression data from another study (Pelle et al., 2015) were used to verify the expression of candidate genes. Except for gene PF3D7_1115100, the microarray expression profiles of 75 candidate genes were obtained. Results showed that >90% of candidate genes had expression signals greater than 4, confirming their abundance at the sexual stage (**Figure S2A**). Also, the microarray data confirmed that they were upregulated at the sexual stage comparing to the asexual stage (**Figure S2B**).

Because not all membrane proteins have signal peptides, we also investigated the parasitic proteins that had TM while lacking signal peptides. About 949 *P. falciparum* proteins have TM and

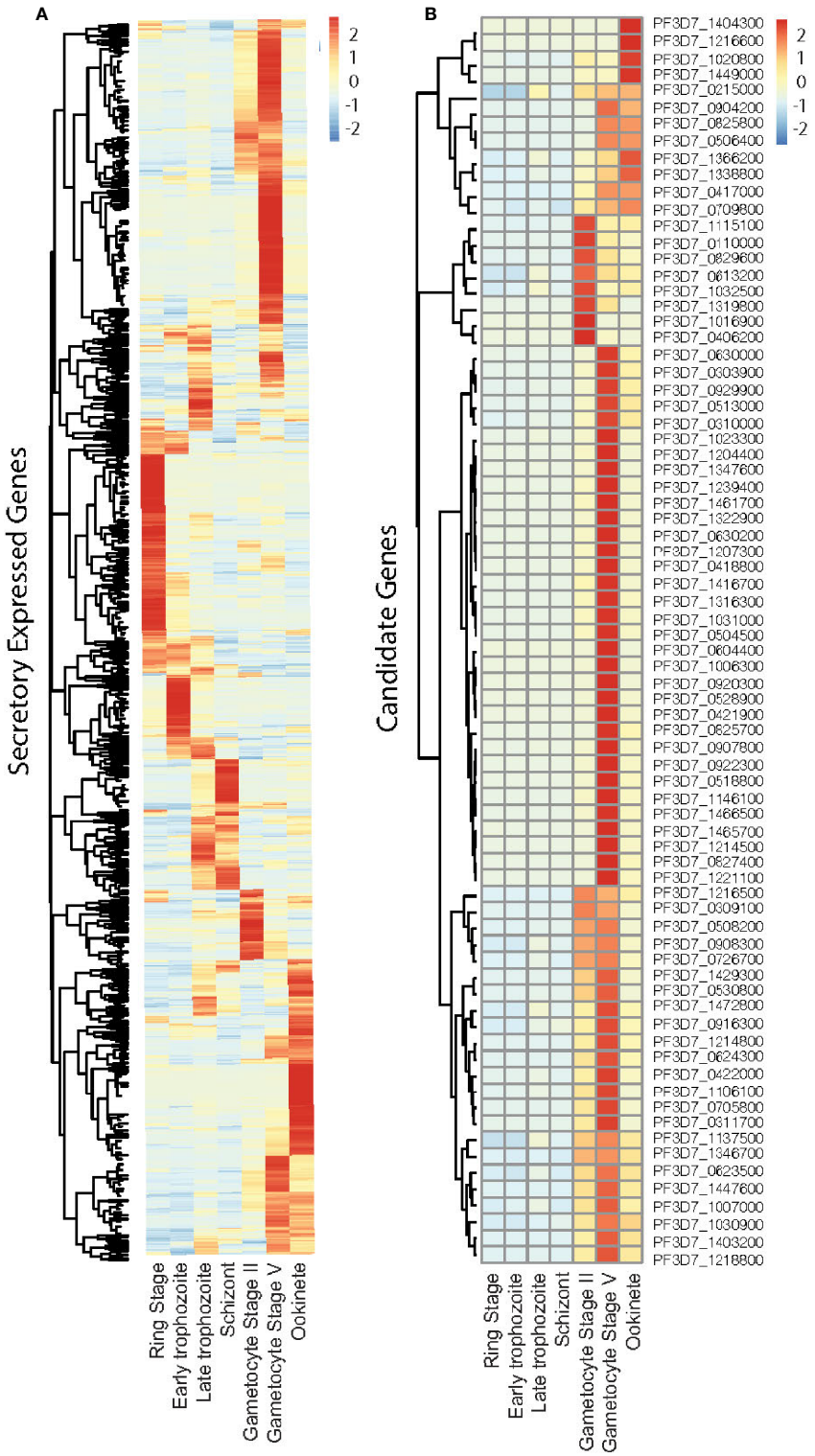


**FIGURE 1** | Expression profile of the *Plasmodium falciparum* genes containing signal peptide at different developmental stages. **(A)** The expression profiles of 1,079 parasitic proteins containing signal peptides using RNA-seq data (Lopez-Barragan et al., 2011). **(B)** The expression profiles of candidate genes.

**TABLE 1 |** The features of selected candidate proteins.

| *Gene ID (PF3D7)* | Expression based on RNA-seq data | | | | | | | *Product Description* | *TMM* | *Length (aa)* | *Location* | *C&E* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ring | ET | LT | ST | GII | GV | Ookinete | | | | | |
| *0310000* | 1.14 | 3.09 | 29.91 | 12.51 | 117.95 | 475.94 | 190.3 | 50S ribosomal protein L9, apicoplast, putative | yes | 197 | apicoplast | yes |
| *0508200* | 5.25 | 2.87 | 40.18 | 34.21 | 604.92 | 704.38 | 144.83 | longevity-assurance (LAG1) protein, putative | yes | 355 | apicoplast | yes |
| *0613200* | 6.5 | 1.67 | 66.31 | 30.45 | 269.35 | 163.88 | 121.91 | conserved Plasmodium protein, unknown function | yes | 60 | apicoplast | |
| *0623500* | 15.91 | 7.27 | 41.39 | 17.78 | 165.71 | 260.26 | 150.4 | superoxide dismutase [Fe] (SOD2) | no | 266 | apicoplast | yes |
| *0726700* | 4.97 | 6.87 | 41.46 | 6.04 | 325.31 | 367.53 | 121.77 | conserved Plasmodium protein, unknown function | no | 170 | apicoplast | |
| *0829600* | 2.32 | 0 | 14.88 | 2.41 | 401.49 | 193.37 | 98.58 | early transcribed membrane protein 8 (ETRAMP8) | yes | 170 | apicoplast | yes |
| *0907800* | 32.74 | 7.98 | 73.41 | 35.48 | 238.38 | 1960.34 | 307.28 | ribosomal protein L35, apicoplast, putative | no | 191 | apicoplast | |
| *0908300* | 6.48 | 2.34 | 83.13 | 39.8 | 424.56 | 486.84 | 150.68 | conserved protein, unknown function | yes | 305 | apicoplast | |
| *0929900* | 2.36 | 0.53 | 13.9 | 9.68 | 60.51 | 359.01 | 126.53 | conserved Plasmodium protein, unknown function | yes | 191 | apicoplast | yes |
| *1020800* | 20.15 | 5.42 | 8.46 | 2.58 | 113.51 | 78.63 | 321.75 | dihydrolipoamide acyltransferase component E2 | no | 640 | apicoplast | |
| *1023300* | 8.5 | 1.91 | 53.69 | 7.74 | 120.29 | 822.3 | 117.75 | conserved protein, unknown function | yes | 79 | apicoplast | yes |
| *1106100* | 6.08 | 11.91 | 35.52 | 13 | 175.17 | 379.06 | 66.1 | apicoplast ribosomal protein S15 precursor, putative | yes | 269 | apicoplast | yes |
| *1137500* | 4.58 | 2.07 | 78.58 | 31.02 | 213.01 | 276.4 | 163.35 | apicoplast ribosomal protein S14p/ S29e precursor, | no | 172 | apicoplast | yes |
| *1472800* | 4.51 | 1.68 | 71.14 | 23.82 | 145.41 | 355.88 | 130.75 | HSP20-like chaperone, putative | yes | 363 | apicoplast | yes |
| *0604400* | 3.8 | 0.86 | 0.7 | 1.15 | 178.99 | 4730.33 | 85.86 | conserved protein, unknown function (Pfs37) | yes | 178 | cell surface | yes |
| *0904200* | 5.41 | 1.74 | 1.28 | 0 | 269.16 | 3109.6 | 2284.3 | PH domain-containing protein, putative | yes | 292 | cell surface | yes |
| *1204400* | 3.24 | 1.02 | 1.55 | 0 | 260.54 | 2094.45 | 148.73 | sexual stage-specific protein G37, putative | yes | 349 | cell surface | yes |
| *1338800* | 5.14 | 7.23 | 32.17 | 13.49 | 78.81 | 178.26 | 280.73 | CPW-WPC family protein | no | 550 | cell surface | |
| *0418800* | 5.84 | 0.78 | 0.95 | 0 | 803.15 | 5476.06 | 624.45 | MOLO1 domain-containing protein, putative | yes | 261 | crystalloid | yes |
| *0825700* | 3.89 | 0.5 | 0.27 | 0 | 108.54 | 2480.37 | 580.03 | crystalloid-specific PH domain-containing protein | no | 305 | crystalloid | yes |
| *1207300* | 4.12 | 0.46 | 0.76 | 1.88 | 264.59 | 1771.22 | 202.82 | LIMP protein, putative | no | 109 | crystalloid | yes |
| *0309100* | 9.18 | 3.14 | 13.98 | 6.92 | 668.75 | 586.6 | 155.1 | conserved Plasmodium protein, unknown function | no | 178 | cytoplasm | |
| *0630200* | 2.08 | 1.13 | 3.07 | 6.07 | 931.97 | 6706.56 | 740.02 | secreted ookinete protein, putative (PSOP6) | no | 135 | cytoplasm | yes |
| *0920300* | 2.41 | 0.81 | 7.32 | 1.1 | 305.88 | 5919.52 | 334.91 | conserved Plasmodium protein, unknown function | no | 187 | cytoplasm | yes |
| *0922300* | 4.66 | 1.29 | 7.26 | 5.23 | 194.98 | 1926.68 | 276.13 | conserved protein, unknown function | no | 315 | cytoplasm | yes |
| *1239400* | 0.94 | 0.85 | 0.7 | 0 | 170.17 | 1061.44 | 107.42 | conserved protein, unknown function | no | 179 | cytoplasm | yes |
| *1366200* | 12.38 | 14.2 | 48.88 | 19.14 | 62.95 | 129.63 | 216.7 | conserved protein, unknown function | yes | 150 | cytoplasm | |
| *1416700* | 2.56 | 1.15 | 5.96 | 0 | 1072.11 | 4267.03 | 499.5 | conserved protein, unknown function | yes | 132 | cytoplasm | yes |
| *1461700* | 4.46 | 3.62 | 14.12 | 1.63 | 1388.9 | 8222.2 | 808.08 | conserved Plasmodium protein, unknown function | no | 126 | cytoplasm | yes |
| *1465700* | 2.2 | 0.4 | 0.97 | 0.53 | 63.24 | 1233.72 | 154.13 | plasmepsin VIII, putative | no | 385 | cytoplasm | yes |
| *1032500* | 11.58 | 29.21 | 142.95 | 45.35 | 566.79 | 191.19 | 238.5 | DER1-like protein, putative (Derlin) | yes | 263 | endoplasmic reticulum | yes |
| *0422000* | 6.86 | 8.4 | 20.98 | 1.92 | 116.99 | 318.5 | 70.83 | steroid dehydrogenase, putative | yes | 321 | membrane | |
| *0406200* | 216.87 | 522.9 | 1153.39 | 744.61 | 431324.27 | 52190.48 | 16181.79 | sexual stage-specific protein precursor (Pfs16) | yes | 157 | membrane | yes |
| *0504500* | 3.08 | 0.56 | 4.23 | 5.23 | 849.57 | 4697.28 | 741.87 | MOLO1 domain-containing protein, putative | yes | 275 | membrane | |
| *0624300* | 5.48 | 3.29 | 23.54 | 9.99 | 1668.26 | 3629.97 | 1111.5 | CPW-WPC family protein | yes | 185 | membrane | yes |
| *0827400* | 11.9 | 5.13 | 6.47 | 9.42 | 150.05 | 2537.45 | 477.67 | conserved Plasmodium protein, unknown function | yes | 218 | membrane | |
| *0916300* | 3.09 | 0.7 | 22.49 | 27.47 | 86.12 | 209.96 | 69.37 | conserved Plasmodium protein, unknown function | yes | 292 | membrane | |
| *1007000* | 10.63 | 18.78 | 40.9 | 11.86 | 133.55 | 243.47 | 108.27 | transmembrane protein 147, putative | yes | 260 | membrane | yes |

*(Continued)*

**TABLE 1** | Continued

| *Gene ID (PF3D7)* | Expression based on RNA-seq data | | | | | | | *Product Description* | *TMM* | *Length (aa)* | *Location* | *C&E* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ring | ET | LT | ST | GII | GV | Ookinete | | | | | |
| *1016900* | 21.82 | 50.13 | 807.63 | 136.34 | 89715.85 | 2928.45 | 3224.83 | early transcribed membrane protein 10.3 (ETRAMP10.3) | yes | 108 | membrane | |
| *1030900* | 18.62 | 4.66 | 44.37 | 89.53 | 576.08 | 1043.52 | 694.99 | ookinete surface protein P28 (Pfs28) | no | 218 | membrane | |
| *1031000* | 9.35 | 1.64 | 8.8 | 8.52 | 4217.91 | 23349.99 | 3477.26 | ookinete surface protein P25 (Pfs25) | yes | 217 | membrane | |
| *1403200* | 3.3 | 0.52 | 1.71 | 0 | 282.14 | 685.68 | 356.55 | conserved Plasmodium protein, unknown function | no | 291 | membrane | yes |
| *1216600* | 4.95 | 1.67 | 3.19 | 6.77 | 90.42 | 64.18 | 1725.74 | cell traversal protein for ookinetes and sporozoites (CelTOS) | no | 182 | microneme | yes |
| *1404300* | 7.25 | 1.26 | 54.23 | 146.41 | 94.81 | 198.7 | 10377.13 | secreted ookinete adhesive protein, putative (SOAP) | no | 202 | microneme | |
| *0518800* | 4.44 | 0.5 | 0.41 | 0 | 1039.54 | 10083.58 | 1662.31 | secreted ookinete protein, putative (PSOP13) | no | 203 | nucleus | |
| *0311700* | 4.58 | 0.94 | 10.11 | 2.86 | 1190.93 | 3185.75 | 369.83 | plasmepsin VI | yes | 432 | osmiophilic body | |
| *1214800* | 5.66 | 0.46 | 5.31 | 1.88 | 1108.51 | 2803.96 | 869.86 | conserved Plasmodium protein, unknown function | yes | 109 | osmiophilic body | yes |
| *1216500* | 3.83 | 1.15 | 65.56 | 4.65 | 6183 | 5110.23 | 2927.77 | male development gene 1 (MDV1) | no | 221 | osmiophilic body | yes |
| *1449000* | 3.64 | 0.21 | 6.7 | 3.32 | 189 | 203.55 | 1188.67 | gamete egress and sporozoite traversal protein (GEST) | no | 248 | osmiophilic body | yes |
| *0110000* | 14.94 | 13.25 | 79.5 | 43.91 | 4159.99 | 1545.12 | 780.59 | conserved Plasmodium protein, unknown function | yes | 234 | unknown | |
| *0215000* | 6.07 | 6.28 | 66.04 | 28.19 | 86.63 | 103.68 | 107.44 | acyl-CoA synthetase (ACS9) | no | 885 | unknown | |
| *0303900* | 5.43 | 0 | 11.58 | 4.17 | 555.38 | 3358.77 | 1091.71 | phosphatidylethanolamine-binding protein, putative | no | 197 | unknown | Yes |
| *0417000* | 4.72 | 3.7 | 2.87 | 0.75 | 110.98 | 263.42 | 251.48 | conserved Plasmodium protein, unknown function | yes | 275 | unknown | Yes |
| *0421900* | 2.36 | 0.71 | 1.16 | 0 | 218.42 | 3456.57 | 358.06 | conserved Plasmodium protein, unknown function | no | 71 | unknown | yes |
| *0506400* | 3.39 | 0 | 3.92 | 1.76 | 113.35 | 1323.48 | 1237.32 | conserved Plasmodium protein, unknown function | yes | 118 | unknown | yes |
| *0513000* | 7.76 | 10.4 | 21.47 | 6.12 | 104.32 | 538.55 | 227.69 | conserved protein, unknown function | no | 269 | unknown | |
| *0528900* | 23.74 | 4.24 | 2.55 | 0.9 | 202.94 | 2764.89 | 251.14 | conserved protein, unknown function | no | 228 | unknown | |
| *0530800* | 12.22 | 6.61 | 57.56 | 33.19 | 1237.56 | 2048.45 | 289.69 | CPW-WPC family protein | no | 254 | unknown | yes |
| *0630000* | 4.03 | 0.55 | 0.74 | 0 | 157.87 | 1484 | 442.85 | CPW-WPC family protein | yes | 289 | unknown | yes |
| *0705800* | 4.38 | 2.11 | 4.08 | 0 | 682.37 | 1631.31 | 286.22 | cysteine-rich secretory protein, putative | yes | 193 | unknown | yes |
| *0709800* | 13.07 | 4.71 | 14.76 | 3.18 | 61.39 | 89.27 | 106.78 | conserved protein, unknown function | yes | 64 | unknown | |
| *0825800* | 5.58 | 1.03 | 0.93 | 0 | 105.54 | 2523.89 | 2355.77 | conserved protein, unknown function | no | 446 | unknown | |
| *1006300* | 7.47 | 2.13 | 1.45 | 0 | 301.55 | 4881.44 | 354.62 | conserved Plasmodium protein, unknown function | no | 143 | unknown | |
| *1115100* | 23.35 | 8.62 | 26.54 | 34.18 | 1085.19 | 398.79 | 415.29 | conserved protein, unknown function | no | 307 | unknown | yes |
| *1146100* | 3.86 | 0 | 4.26 | 0 | 995.55 | 7107.6 | 1081.7 | conserved protein, unknown function | no | 175 | unknown | |
| *1214500* | 9.99 | 9.56 | 4.46 | 1.1 | 258.57 | 5263.68 | 705.22 | conserved Plasmodium protein, unknown function | yes | 186 | unknown | yes |
| *1218800* | 3.72 | 2.04 | 7.27 | 1.18 | 108.64 | 259.86 | 120.4 | secreted ookinete protein, putative (PSOP17) | no | 349 | unknown | |
| *1221100* | 1.74 | 0.98 | 6.23 | 2.37 | 150.63 | 3971.38 | 628.74 | conserved Plasmodium protein, unknown function | no | 260 | unknown | |
| *1316300* | 7.01 | 0.97 | 0.79 | 0 | 675.09 | 3205.13 | 533.55 | conserved Plasmodium protein, unknown function | no | 104 | unknown | yes |
| *1319800* | 5.04 | 1.05 | 7.14 | 1.41 | 5387.24 | 2344.32 | 112.06 | conserved Plasmodium protein, unknown function | yes | 145 | unknown | yes |
| *1322900* | 49.77 | 16.63 | 27.17 | 14.07 | 336.07 | 2473.53 | 292.96 | conserved protein, unknown function | yes | 263 | unknown | |
| *1346700* | 3.91 | 0.45 | 3.9 | 0.92 | 109.52 | 128.45 | 80.61 | 6-cysteine protein (P48/45) | yes | 448 | unknown | |
| *1347600* | 4.48 | 0.92 | 10.95 | 4.45 | 181.53 | 1539.74 | 108.79 | conserved protein, unknown function | yes | 277 | unknown | yes |
| *1429300* | 5.94 | 7.41 | 37.22 | 6.1 | 1131 | 1842.23 | 378.5 | CPW-WPC family protein | no | 371 | unknown | yes |
| *1447600* | 5.57 | 1.26 | 2.73 | 0 | 373.24 | 687.62 | 312.91 | conserved protein, unknown function | yes | 121 | unknown | yes |
| *1466500* | 6.74 | 0 | 1.99 | 0 | 501.1 | 3660.75 | 692.51 | conserved Plasmodium protein, unknown function | no | 126 | unknown | yes |

*ET, early trophozoite; LT, late trophozoite; ST, Schizont; GII, stage II gametocyte; GV, stage V gametocyte; C&E, successfully cloned and expressed.*

lack signal peptides (**Table S2**). However, none of them satisfied the expression criteria, e.g., >5-fold upregulated at sexual stages and abundantly expressed at the sexual stages (>60 reads per gene).

About 39 of the 76 candidate proteins contained TM. These 76 candidate proteins were localized in different subcellular compartments, although subcellular locations of 35% of these proteins (n=27) are still unknown (**Figure 2**). The rest were distributed in 9 different locations, including rhoptry neck, apicoplast, cell surface, crystalloid, cytoplasm, endoplasmic membrane, microneme, nucleus, and osmiophilic body. Among them, 18% of these proteins (n=14) are located on the apicoplast. The apicoplast is a specific organelle important for *Plasmodium* development. Also, a decent number of candidate proteins are located on the membrane (15%), which include parasitophorous vacuole membrane and cytomembrane.

## Determination of Candidate *P. falciparum* Proteins That Interact With the Mosquito Midgut

Candidate genes were PCR-cloned and the recombinant proteins were expressed in High Five insect cells using the baculovirus expression system in the serum-free medium. A monoclonal antibody against the 6xHis tag at the C-terminus of recombinant proteins was used to quantify the recombinant protein concentration. We have finally cloned and expressed 46 genes successfully at ELISA detectable level for further analysis (**Figure 3A**).

To determine if *P. falciparum* candidate proteins interacted with *An. gambiae* midgut lysate, we performed ELISA. Mosquitoes were fed with blood, and their midguts, including peritrophic matrix, were isolated 15-21 h post blood meal. A 96-well 100 ng of recombinant protein was added into wells to determine the interaction between a candidate protein and the midgut lysate. The heat-inactivated proteins were used as the corresponding negative controls. Both the experimental group and control were done in triplicates per treatment. CAT, which was expressed with the baculovirus system in the High Five cells, was used as a blank control. The $A_{405}$ of experimental and control wells were measured (**Figure 3B**). Since the same samples were used in 46 genes, the $A_{405}$ values were analyzed with multiple t-test using the two-stage linear step-up procedure (Benjamini et al., 2006) with 1% of False Discovery Rate (Q). The result showed that six *P. falciparum* proteins (PF3D7_0303900, PF3D7_0406200 (Pfs16), PF3D7_1204400 (Pfs37), PF3D7_1214800, PF3D7_1239400, PF3D7_1472800) were retained by midgut lysates at a level significantly higher than the corresponding controls (**Figure 3B**, False Discovery Rate (Q) < 0.01).

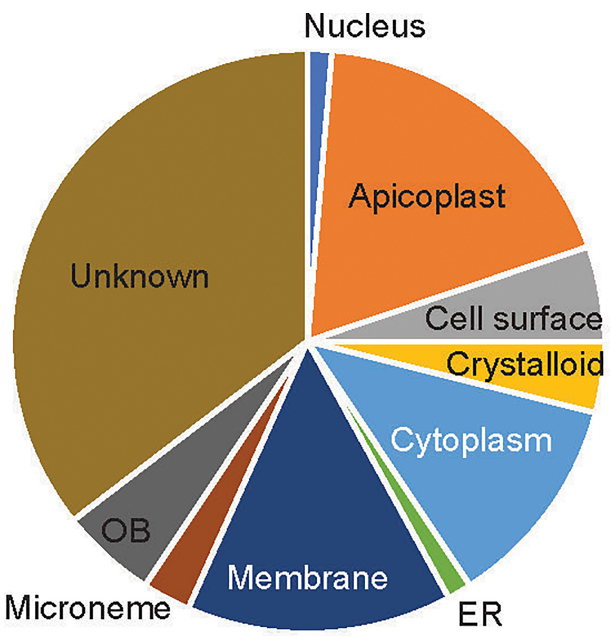


**FIGURE 2** | The subcellular location of the candidate proteins. ER, endoplasmic reticulum; OB, osmiophilic body.

These six recombinant proteins were further verified by an alternative ELISA using unhomogenized midguts to pull down the recombinant proteins following by homogenizing and detection. The results (**Figure 4**) showed the difference between each recombinant protein and the blank control was significant (Q < 0.01), which confirmed the interaction between the candidate proteins and midguts.

## Functional Analysis of *P. falciparum* Proteins That Bound to Mosquito Midguts

To understand the function of candidate proteins, we searched their orthologs in *P. berghei* and analyzed the *P. berghei* infectious phenotypes for their knockout in public databases RMgmDB (Khan et al., 2013; Gomes et al., 2015), PlasmoGEM (Bushell et al., 2017), and other works (Zhang et al., 2018). Results (**Table 2**) showed that three *P. berghei* genes were knocked out successfully, and two *P. falciparum* genes could be mutated by transposon insertions (Zhang et al., 2018). Disruption of orthologs of PF3D7_12148, PF3D7_1204400 (Pfs37) or PF3D7_0406200 (Pfs16) did not change *P. berghei* infection at asexual stage. Knockout of PBANKA_1430600, the ortholog of PF3D7_1214800, did not affect asexual stage parasites, and the effect on sexual stage parasites and transmission has not been reported yet. Knocking out the orthologs of Pfs37 or Pfs16 rendered *Plasmodium* parasites to generate fewer oocysts in mosquitoes. Pbg37, the ortholog of Pfs37, is a protein of 37 kDa with a signal peptide and multiple transmembrane domains and only expressed at sexual stages of parasites such as gametocytes, zygotes, and ookinetes. Knockout of Pbg37 led to reduced gametocyte production and significantly affected the further development of ookinetes from gametocytes. Notably, the anti-Pbg37 antiserum from the immunized mice significantly reduced the number of oocysts in the midgut, which indicates that it could be a target against malaria infection (Liu et al., 2018).

Next, we selected Pfs16 to study the antibody effects on malaria transmission. Pfs16 was found in the *P. falciparum*

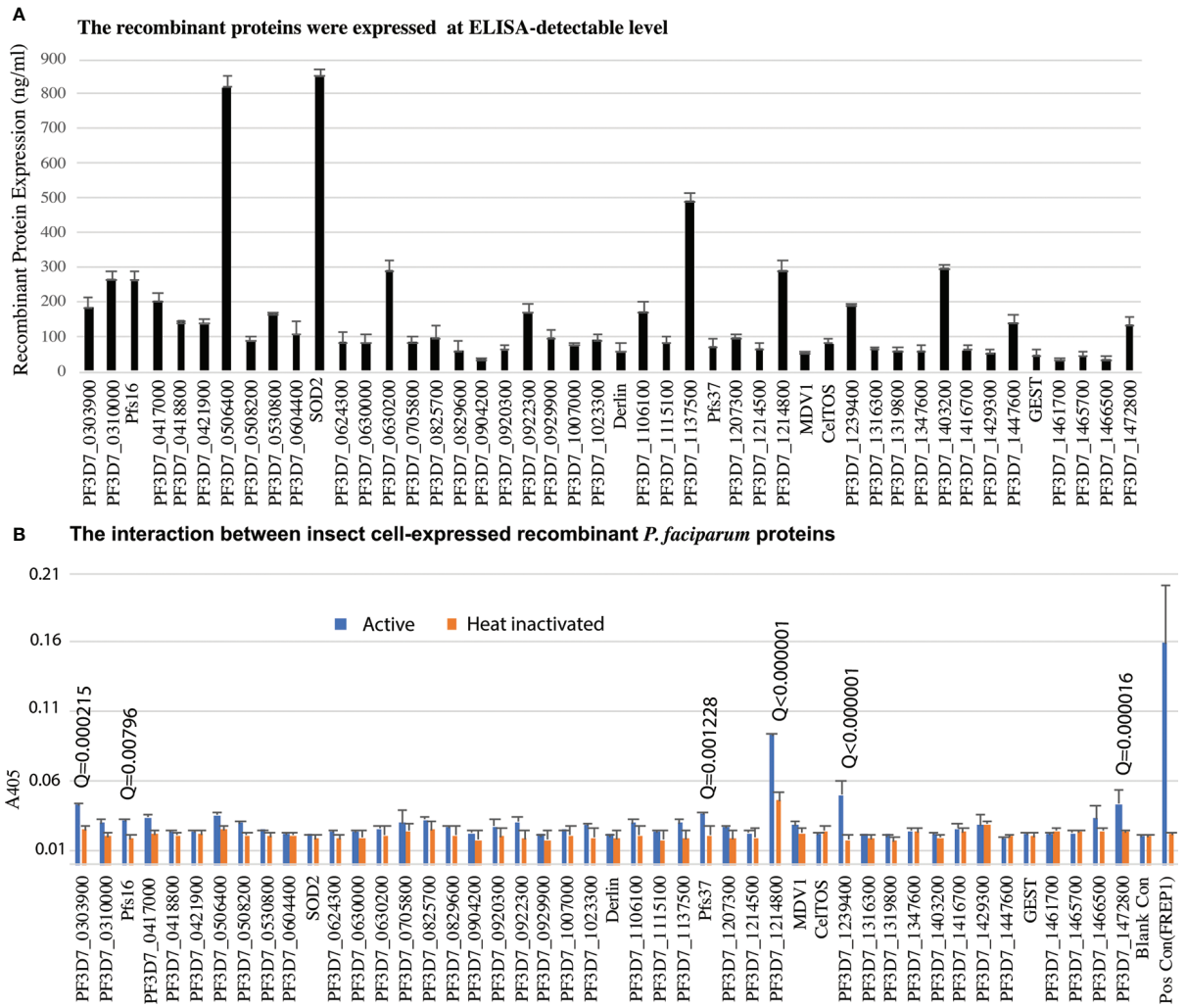


**FIGURE 3** | The insect-cell expressed recombinant parasitic proteins and their interaction with midguts. **(A)** The expression of candidate proteins in high five cells. Totally, forty-six genes were expressed in high five cells with a baculovirus expression system at a detectable level. Each sample was measured with triplicates. The expression experiments were conducted three times. **(B)** Interaction between recombinant proteins and midgut lysate. The concentration of recombinant proteins was normalized. Naïve recombinant proteins and heat-inactivated proteins were used as experimental groups and control groups with triplets. The false discovery rates (Q) were calculated by multiple t-test using the Two-stage linear step-up procedure (Benjamini et al., 2006). Those proteins with Q <1% were labeled with the Q values. The same experiments were repeated three times, and the results were similar.

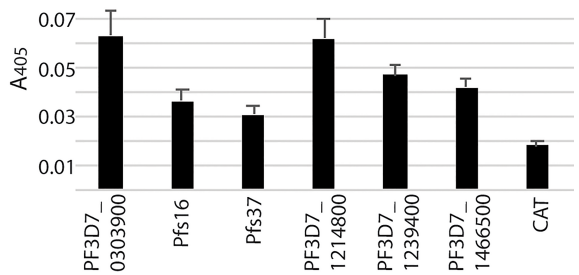


**FIGURE 4** | The interaction between the candidate proteins and unhomogenized mosquito midguts.

gametocyte parasitophorous vacuole membrane. Knockout of PBANKA_1003900 (Pbg16), the ortholog of Pfs16 in *P. berghei*, did not change *Plasmodium* asexual stage (Kongkasuriyachai et al., 2004) or formation of ookinetes (Deligianni et al., 2018). Disruption of Pbg16 reduced the number of oocysts in mosquitoes (**Table 2**) (Kongkasuriyachai et al., 2004). However, the amino acid sequences of Pfs16 and Pbg16 are largely different. Thus, we further examined the function of Pfs16 on parasite transmission using transmission-blocking assays.

The anti-Pfs16 polyclonal antibody was generated in rabbits immunized with *E. coli*-expressed Pfs16 as the antigen and further purified using protein A/G immune affinity column. The specificity of the anti-Pfs16 antibody was verified with Western blot analysis using the recombinant protein expressed in insect cells. The result (**Figure 5A**) showed two bands with the size of about 19kD and 16kD, which matched the precursor (with signal peptide) and mature forms of recombinant protein containing 6xHis and V5 tags. Therefore, a rabbit polyclonal antibody against Pfs16 could specifically recognize recombinant Pfs16. Next, we determined the inhibition of anti-Pfs16 polyclonal antibody against *P. falciparum* transmission to mosquitoes. The *P. falciparum* culture containing 0.2% stage V gametocytes and 0.15 mg/ml anti-Pfs16 antibody (titer = $10^3$/mg) was used to infect *An. gambiae* (G3) using SMFA. The equal amounts of unrelated polyclonal antibody (anti-V5) or BSA were used to substitute anti-Pfs16 antibody as the control. The results showed that compared with the anti-V5 or BSA controls,

**TABLE 2** | *P. berghei* phenotypes of candidate gene knockouts.

| Gene ID (*PF3D7*) | Pb ID (*PBANKA*) | Coverage % | Identity % | The function of gene products | # of TM | location | knock out | Phenotype | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | AS | GAM | OK | OC | SP |
| *1239400* | *1453900* | 96 | 45 | unknown | 0 | cytoplasm | fail/mutable | | | | | |
| *1214800* | *1430600* | 97 | 35 | unknown | 1 | osmiophilic body | succeed | ND | | | | |
| *1204400* | *0603300* | 97 | 64 | sexual stage-specific protein G37 (Pfs37) | 7 | cell surface | succeed | ND | ATTN | ATTN | ATTN | |
| *0406200* | *1003900* | 35 | 26 | sexual stage-specific protein (Pfs16) | 2 | membrane | succeed | ND | ND | ND | ATTN | ND |
| *0303900* | *0402500* | 96 | 53 | phosphatidylethanolamine-binding protein | 0 | unknown | fail/mutable | | | | | |
| *1472800* | *1336000* | 72 | 60 | HSP20-like chaperone | 1 | cytoplasm | fail/ nonmutable | | | | | |

*AS, asexual stage; GAM, gametocyte; OK, ookinete; OC, oocyst; SP, sporozoite.*
*ND, no difference; ATTN, the number of the knocked parasite was attenuated compared with wild type strain. "Fail/succeed" was based on individual gene knockout. Mutable/nonmutable was based on transposon mutagenesis (Zhang et al., 2018).*

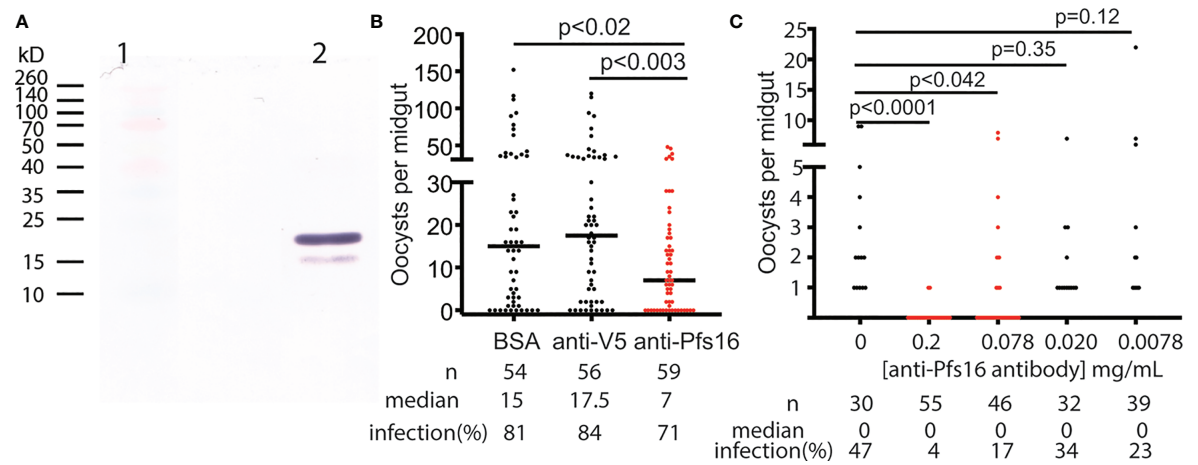


**FIGURE 5** | The functional analysis of Pfs16 on *Plasmodium* transmission to mosquitoes. **(A)** Western blot assay showed that anti-Pfs16 antibody specifically recognized the insect cell-expressed recombinant Pfs16 protein in lane 2. Two bands in lane 2 are the precursor and mature forms of Pfs16 with His- and V5-tag Lane 1 displayed standard protein markers with some color proteins. **(B)** Rabbit anti-Pfs16 polyclonal antibody inhibited *P. falciparum* transmission to *An. gambiae* by SMFA. The same concentration of BSA and an unrelated antibody (anti-V5) were used as the controls. The p-values between the experimental and the control groups were calculated using Wilcoxon-Mann-Whitney tests. The assays were repeated, and the results were similar. Horizontal bars are the median numbers of oocysts per mosquito midguts. **(C)** The antibody inhibition on malaria transmission was dose-dependent. This experiment was repeated twice, and the results were similar.

the anti-Pfs16 Ab significantly reduced the number of oocysts by 50% and 57%, respectively (**Figure 5B**), supporting that Pfs16 is a potential target for malaria control. This experiment was repeated three times and obtained similar results. We also examined the different concentrations (0.2 mg/mL, 77 μg/mL, 20 μg/mL, 7.7 μg/mL and 0 μg/mL) of anti-Pfs16 on *P. falciparum* transmission to *An. gambiae* using SMFA. The results showed that the antibody inhibition rate decreased with the reduction of antibody concentration (**Figure 5C**). When antibody concentration was greater than 0.078 mg/mL, the inhibition against malaria transmission to mosquito was significant ($p < 0.05$).

## Recombinant Pfs16 Protein Bound to Blood-Fed Mosquito Midguts

Finally, we examined the binding tissues of Pfs16. The naïve mosquito midgut contains endothelia and the basal lamina, while

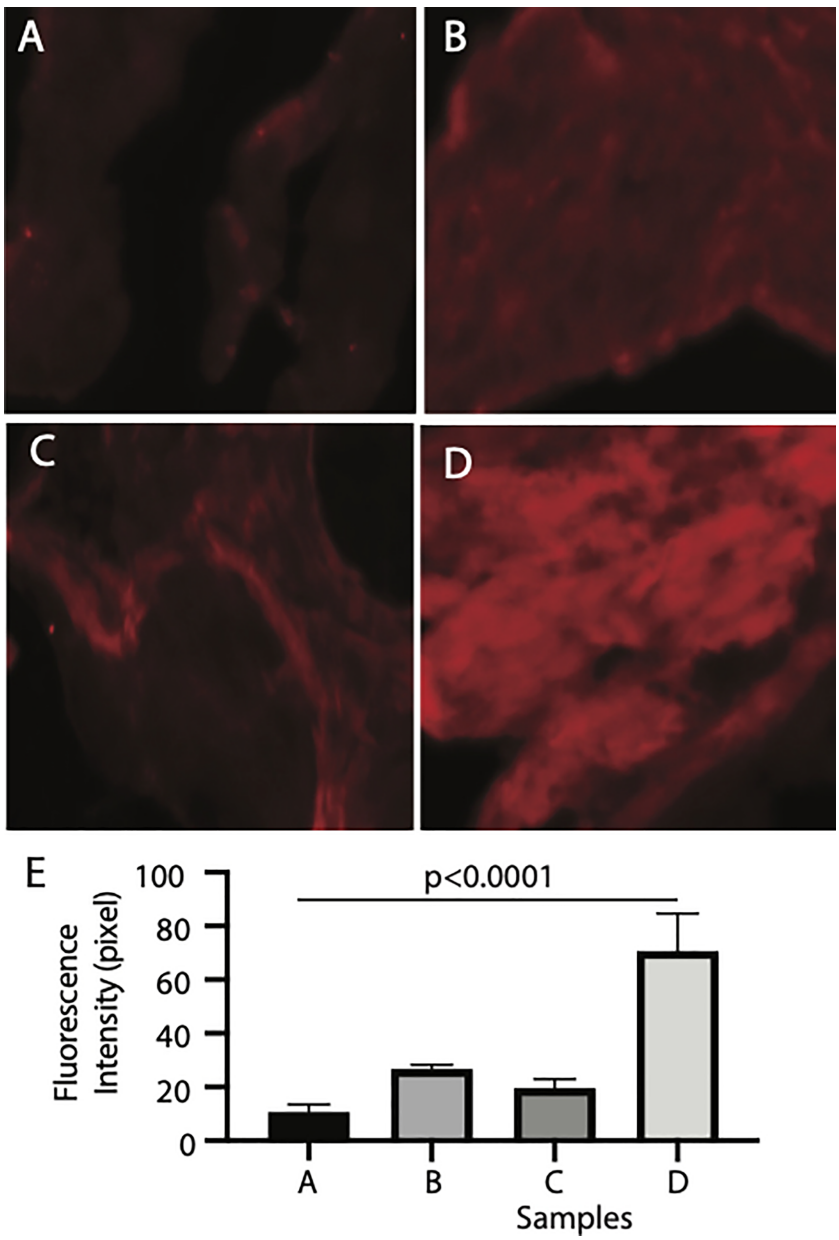


**FIGURE 6** | Recombinant Pfs16 protein bound to blood-fed mosquito midguts. **(A)** The naïve mosquito midguts were incubated with unrelated protein (CAT). **(B)** The blood-fed mosquito midguts incubated with un-related protein (CAT). **(C)** The naïve mosquito midguts incubated with Pfs16. **(D)** The blood-fed mosquito midguts incubated with Pfs16 protein. **(E)** The median fluorescence intensity of differently treated midguts. P. Calculated with one-way ANOVA.

a peritrophic matrix was formed after a blood meal. The mosquito midguts were isolated from the naïve and blood-fed mosquitoes and incubated with insect cell-expressed recombinant Pfs16 protein or the non-specific insect cell-expressed protein (CAT) as the control. The rabbit polyclonal antibody against Pfs16 was used to detect the bound Pfs16. Results showed that the fluorescence from naïve mosquitoes incubated with CAT was the weakest (**Figure 6A**). Substitution of the CAT with Pfs16 protein resulted in a slighter increase of fluorescence intensity (**Figure 6C**); however, it was similar to the blood-fed mosquito midguts incubated with CAT and anti-Pfs16 (**Figure 6B**). The fluorescence was the highest for the blood-fed mosquito midguts incubation with Pfs16 and anti-Pfs16 (**Figure 6D**). The medians of fluorescence intensity of four treatments were shown in **Figure 6E**. Collective, these data suggest that Pfs16 protein interacted with the midgut peritrophic matrix.

## DISCUSSION

Mosquito midgut is the first place for *Plasmodium* development into ookinetes and the subsequent invasion. The interaction between *Plasmodium* parasites and mosquito midgut plays a key role in malaria transmission (Zhang et al., 2015). However, the parasite-midgut interactome is not well-understood. This project investigated parasitic secreted proteins that interact with mosquito midguts.

About 76 parasitic proteins with signal peptides and abundant at the sexual stage of parasites were selected for investigation. Many of these candidate proteins reportedly play essential roles in malaria transmission. For instance, the CelTOS is essential for ookinetes to rupture the midgut epithelial cell membrane (Kariu et al., 2006), and GEST is the gamete egress and sporozoite traversal protein vital for gametes exit hosts in mosquito midguts (Stone et al., 2018). Our data showed that both CelTOS and GEST did not directly bind to the mosquito midguts, consistent with the reported mechanisms that CelTOS was secreted directly into endothelial cytoplasm (Kariu et al., 2006) and PEST ruptured gametocyte parasitophorous vacuole membrane (Talman et al., 2011).

The 76 candidate proteins locate at different subcellular locations, such as apicoplast, osmiophilic body, and microneme. Previous studies have shown that the abundant proteins in apicoplast have been often the targets to develop malaria drugs (Biddau and Sheiner, 2019). Micronemes play a critical role during the invasion of parasites in mosquitoes (Li et al., 2004). Three ookinete-secreted proteins, PgCHT1 chitinase, WARP, and CTRP from micronemes, can break mosquito midgut to facilitate malaria transmission. Antibodies against these proteins interfere with parasite invasion (Li et al., 2004). There are several membrane proteins e.g., Pfs25 (PF3D7_1031000), P28 (PF3D7_1030900), and P48/45 (PF3D7_1346700) that under investigation as TBV by communities.

In the meantime, our bioinformatics analysis might miss some genes if they lack signal peptides or their expression data were incomplete. For instance, mosquito AgP47Rec was recently reported to interact with parasitic protein PF3D7_0134800 (P47) (Molina-Cruz et al., 2020). It was missed in our candidates because the RNA-seq expression data lacks its expression at schizonts (Lopez-Barragan et al., 2011).

Out of the 76 candidate genes, 30 were not cloned into the plasmid due to incorrect annotation or were cloned into plasmids with low expression in insect cells. This is because some proteins such as SOAP rapturing insect cells and some membrane proteins are hard to clone and express in insect cells. Notably, forty-six genes (60.5%) were successfully cloned and highly expressed with a baculovirus expression system in High Five cells. By screening these proteins *via* ELISA analysis, we identified six *P. falciparum* proteins that interacted with the midgut of mosquitoes. The orthologs of these six *P. falciparum* genes were found in *P. berghei*. Knockout of Pbg37 and Pbg16, orthologs of Pfs37 and Pfs16, respectively, from *P. berghei* reduced *Plasmodium* oocysts in mosquitoes (Kongkasuriyachai et al., 2004; Liu et al., 2018). Disruption of the Pbg37 gene negatively impacted malaria transmission, which might be caused by attenuated gametocytes. Importantly, the anti-Pbg37 antiserum inhibited *P. berghei* transmission to mosquitoes (Liu et al., 2018), supporting Pfs37 is an excellent target to break malaria transmission.

Knockout of the Pbg16 gene did not change parasites at the sexual stage, and the formation of ookinetes was normal. The amino acid sequences between Pfs16 and Pbg16 are strikingly different, with only 35% Pfs16 was covered by Pbg16, and covered regions shared 26% identical amino acids. Therefore, we generated polyclonal antibodies to examine Pfs16 function in malaria transmission. Our data show that the ingested antibodies against Pfs16 significantly inhibited parasite transmission to mosquitoes. This result supports that Pfs16 is accessible to extracellular proteins such as antibodies. Pfs16 was observed as a gene at the onset of *P. falciparum* gametocytogenesis and often used as a marker for the production of the sexual stage of parasites (Bruce et al., 1994; Dechering et al., 1997; Lanfrancotti et al., 2007). Pfs16 seems not essential in the development of gametocytes and is not involved in the formation of zygote and ookinetes. However, it may play some role in the optimal production of sexual parasites. During the entire gametocyte maturation from stages I–V Pfs16 (Lobo et al., 1994; Dechering et al., 1997; Berry et al., 2009), it was detected to be localized on the parasitophorous vacuole membrane (Moelans et al., 1991; Baker et al., 1994; Eksi and Williamson, 2011). One publication reported that anti-Pfs16 antisera did not show any transmission-blocking activities. However, no data were presented in that paper (Moelans et al., 1995). Differently, our results showed that the purified anti-Pfs16 polyclonal antibody (0.15 mg/mL) significantly inhibited parasite transmission to mosquitoes.

Successful parasite invasion of mosquitoes begins with parasites' interaction with mosquito midguts. Through bioinformatics analysis followed by protein interaction assays, we identified six midgut-binding parasitic proteins. Moreover, we showed that

antibodies against Pfs16 inhibited malaria transmission. Further investigation of these proteins will improve our understanding of the molecular mechanisms of malaria transmission and provide targets to break malaria transmission.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

GN and YC conducted experiments, interpreted the data, and wrote the manuscript draft. XW conducted bioinformatics and statistical analysis. YK expressed Pfs16 proteins in insect cells. JL conceived concepts, designed the project, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.654216/full#supplementary-material

**Supplementary Table 1 |** Specific primers for candidate genes.

**Supplementary Table 2 |** The expression data of genes that contain transmembrane domains and lack signal peptides.

**Supplementary Figure 1 |** The heatmap of 1,079 parasitic proteins containing signal peptides.

**Supplementary Figure 2 |** The expression of parasitic proteins based on microarray showing the abundance of candidate genes at sexual stages.

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