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Pathogenicity and virulence of *Entamoeba histolytica*, the agent of amoebiasis

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ABSTRACT

The amoeba parasite *Entamoeba histolytica* is the causative agent of human amebiasis, an enteropathic disease affecting millions of people worldwide. This ancient protozoan is an elementary example of how parasites evolve with humans, e.g. taking advantage of multiple mechanisms to evade immune responses, interacting with microbiota for nutritional and protective needs, utilizing host resources for growth, division, and encystation. These skills of *E. histolytica* perpetuate the species and incidence of infection. However, in 10% of infected cases, the parasite turns into a pathogen; the host-parasite equilibrium is then disorganized, and the simple lifecycle based on two cell forms, trophozoites and cysts, becomes unbalanced. Trophozoites acquire a virulent phenotype which, when non-controlled, leads to intestinal invasion with the onset of amoebiasis symptoms. Virulent *E. histolytica* must cross mucus, epithelium, connective tissue and possibly blood. This highly mobile parasite faces various stresses and a powerful host immune response, with oxidative stress being a challenge for its survival. New emerging research avenues and omics technologies target gene regulation to determine human or parasitic factors activated upon infection, their role in virulence activation, and in pathogenesis; this research bears in mind that *E. histolytica* is a resident of the complex intestinal ecosystem. The goal is to eradicate amoebiasis from the planet, but the parasitic life of *E. histolytica* is ancient and complex and will likely continue to evolve with humans. Advances in these topics are summarized here.

Introduction

The persistence of a parasite species is the trade-off between the rapid reproduction of the parasite and, in doing so, the measured exploitation of the host's resources; disruption of this paradigm can result in host damage and hence loss of fitness for the parasite. Virulence is the term used to designate the mechanisms leading to the breakdown of homoeostasis in the parasitized host, whereas the extent of damage, due to the appearance of virulence, is the measure of pathogenicity. Important mechanisms increase parasite survival during pathogenicity, including the presence of different genotypes within a single parasite species leading to rapid growth, the efficient establishment of stress response systems, and the induction of resistant cell forms. These parasitic skills leading to its adaptation, reduce the likelihood that the hosts will kill them; or at least, allow parasites to persist within tolerance limits. A successful parasite, surviving throughout the life of the host without damaging it, should be considered a commensal-like organism, but emerging notions point out that in complex ecosystems, commensals can disrupt homoeostasis. This is the case of the human intestinal ecosystem because the presence of non-virulent parasites can modify the composition of the microbiota [1,2] and consequently, they impact the nutritional and immune status of the host, without any sign of intestinal damage [3,4]. This complexity has led to the idea of naming eukaryotic intestinal microbes as symbionts, thus encompassing mutualists, commensals, and parasites [3] and, therefore, enriching perspectives for the study of host-microbial eukaryote interactions. Under certain circumstances, parasites can become pathogenic, for example when their numbers increase indiscriminately in the absence of an effective host immune response.

Ancient parasites have evolved with humans as in the case of the *Entamoeba* species, which are endobiotic amoebae colonizing animal species [5,6]. The persistence of at least seven species of *Entamoeba* depends on their ability to infect humans primarily in the intestinal tract, where they divide and encyst. Most *Entamoeba* infections in humans appear to be caused by non-pathogenic species (e.g. *Entamoeba dispar* and *Entamoeba coli*), the only amoebic species that could become virulent and harm the host is

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Entamoeba histolytica, the causative agent of amoebiasis, responsible for dysentery and liver abscesses [7]. Asymptomatic infections are most common, while symptoms of invasive amoebiasis develop in about 10% of infected people [8]. Clinical features of symptomatic amoebic infection may include blood and mucus in diarrhoeal stools, which are dysentery phenotypes; in some cases, the parasite invades the liver and forms abscesses that may occur for months or years after travel or residence in a geographic region where amoebiasis is endemic. The exact burden of amoebiasis is difficult to quantify due to limited diagnostic and surveillance capacities in endemic regions, including developing countries in Central and South America, Africa, and Asia. This infectious disease is also present in industrialized countries mainly due to returning travellers or immigrants from endemic countries [9]. Although poorly quantified overall, the impact of amoebiasis remains significant as prevalence estimates are as high as 40% in some populations (e.g. Mexico [10], China [11], India [12], Turkey [13]). No vaccine against amoebiasis currently exists; the drug of choice for therapy is metronidazole, which may cause severe side effects in humans [14] and on anaerobic members of the microbiota [15]. Therefore, understanding what determines the outcome of amoebic infection and the nature of amoebic virulence is a key challenge for *Entamoeba* research to advance therapeutic and vaccine development.

Here is reviewed our current knowledge of the lifecycle and mechanisms of pathogenic conversion of *E. histolytica* considering several characteristics by which this parasite survives in the human intestine. Due to the abundant literature in the field, it is important to note several compilations of various aspects of pathogenesis already published [7,16–18].

The lifecycle of *Entamoeba histolytica*

The occurrence of amoebiasis is largely based on the simple two-stage lifecycle of *E. histolytica*, with an environmental infectious cyst and a dividing trophozoite residing in the human intestine. Although other non-pathogenic amoeba species share this same lifestyle, *E. histolytica* has been able to take advantage of gene regulation and activated pathways to adapt to the host responses when the balance between the parasite and the intestinal ecosystem is disturbed. The cyst, which is the contaminating form, ensures the survival of the species because it resists to environmental changes and is easily transmitted. Due to their high resilience, cysts overcome the eradication of the pathogen by the immune system or by antibiotic treatment. Mature cysts are excreted in the host's stool and are transferred to another human via a faecal-oral route through contaminated food or water or person-to-person contact; thus, amoebic infection occurs when water quality, sanitation, and hygiene practices are inadequate. This finding has been largely confirmed during the COVID-19 pandemic, as good hand hygiene compliance in endemic regions significantly correlated with a reduced rate of *E. histolytica* and other intestinal parasitic infections [19]. After ingestion by a new host, in the small intestine, the cyst emerges into four disease-causing trophozoite forms that migrates and colonizes the large intestine, where they feed on bacteria and divide or encyst themselves (Figure 1). While most infected people are asymptomatic carriers, they shed millions of cysts daily and can perpetuate amoebic infection.

Formation of cysts in *Entamoeba*

Encystation in *Entamoeba* occurs after a slowdown in cellular metabolism following food deprivation or
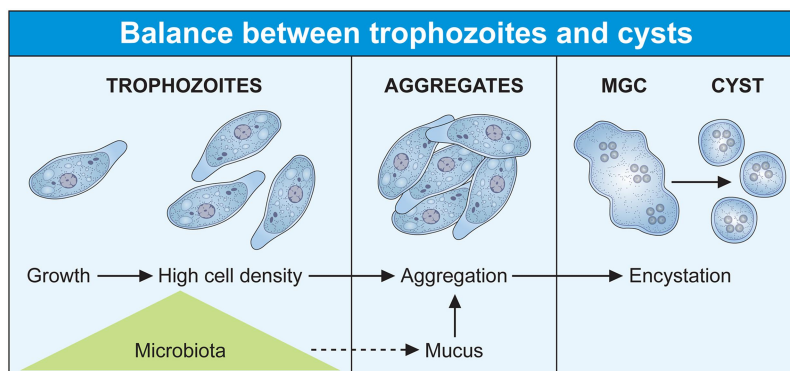

Figure 1. The lifecycle of *Entamoeba histolytica* is influenced by the microbiota. (Two cellular forms make up the lifecycle of *Entamoeba*, the contaminating cyst and the trophozoite. Infection occurs directly upon ingestion of the cyst by a new host, without intermediate hosts as a vector. The trophozoite colonizes the large intestine as a commensal-like, where it feeds on bacteria and divides. Trophozoite populations can reach high densities and aggregates due to amoebic surface molecules recognizing galactose ligands (e.g. lectins) that interact with mucus. High trophozoite density is a key parameter of encystation, and aggregation should trigger the transition from exponential growth to encystation following the formation of multicellular giant cells (MGC) where the cyst forms. The MGC phenotype depends on the transcription factor ERM-BP which has no role in the aggregation process.

environmental conditions unfavourable to the growth and division of the parasite. The common model to study amoebic encystation in laboratory conditions is *Entamoeba invadens* (a reptile pathogen), which has a lifecycle similar to *E. histolytica*. Trophozoites retract their cytoplasmic projections such as pseudopodia, round off, detach from the substrate, and aggregate into multicellular clumps [20,21]. At early stages of encystation, a tetra nucleated intermediate cell (early cyst) forms due to two successive cycles of chromosomal replication without cell division [22,23]. Gradual dehydration induces the decrease in cytoplasmic volume by approximately 80% leading to retraction of the cytoplasm from the cyst wall, glycogen accumulation, and maturation of cysts by addition of the wall elements [22] such as chitin-rich microfibrils that are associated with the lectins Jacob and Jessie. The main biochemical and molecular features of *Entamoeba* encystation have been reviewed [18,24]. Clearly, changes in the environment such as starvation of the carbon source or osmotic shock induces encystation.

Signalling molecules involved in cyst formation include galactose-terminated ligands, sphingolipids (especially long-chain fatty acids), small-molecule intermediates in signalling pathways as cyclic adenosine monophosphate, calcium, phospholipase D, cholesteryl sulphate, and the metabolic cofactor nicotinamide adenine dinucleotide (NAD+). Encystation is due to the activation of specific amoebic surface receptors including a β1-adrenergic receptor sensitive to catecholamines [25] and lectin receptors sensitive to galactosidase-terminated ligands [26]. Profiling gene transcription in *Entamoeba* species (both *E. histolytica* and *E. invadens*) determined the role of at least three transcription factors involved in encystation: Myb [27], ERM-BP [28], and NF-YC [29,30], these factors have temporary sequential activities according to the cyst stage. Epigenetic modifications also control encystation since treatment of trophozoites with histone acetylation modifiers lead to the downregulation of expression for genes implicated in the synthesis of chitin, cyst wall proteins, polyamines, or gamma-aminobutyric acid pathways [31]. The data suggest that the genetic regulations and entire signalling pathways activating gene transcription necessary for *Entamoeba* encystation are far from completely identified.

The trophozoite as the vegetative cellular form of *Entamoeba histolytica*

E. histolytica trophozoites (20 to 40 μm in size) reside in the intestinal lumen, feed on bacteria and divide by binary fusion. The genome of *E. histolytica* is aneuploid; sometimes larger than tetraploid and contains one or more extra copies of shorter truncated chromosomes [32]. Cytokinesis in *E. histolytica* is not coupled with nuclear division, therefore, cell division can be asymmetrical [33], delinked from S phase and mitosis [34], leading to daughter cells with zero, one, or several nuclei [33]. Nevertheless, genome analysis determines that *E. histolytica* has orthologues of all cytokinesis-related proteins, compared to budding yeast, but the fact that amoebae can accumulate polyploid cells suggests that checkpoints regulating the synchrony between genome duplication and cell division are absent [35,36].

The current AT-rich genome assembly of *E. histolytica* is sized at 20 Mbp with 8,201 predicted genes of which the vast majority (~76%) do not contain introns and encode short open reading frames (on average ~389 amino acids), compared with other unicellular eukaryotes. There are distinctive genomic features during the evolution of *Entamoeba* species such as the remarkable loss of genes related to adaptation to low oxygen niches (e.g. genes involved in the tricarboxylic acid cycle and oxidative phosphorylation), a fact correlated with the absence of classic mitochondria [37] and as a result, glycolysis is the primary source of energy for cellular functions [35,38], and ethanol is the main end-product of glucose catabolism [39].

Entamoeba species retain a mitochondrion-derived organelle called mitosome devoid of an organellar genome [40,41], it is the seat for the synthesis of sulfolipids [42]. The mitochondrial iron – sulphur assembly system of eukaryotes is replaced in *E. histolytica* by an unrelated nitrogen fixation system whose coding genes were acquired by lateral gene transfer (LGT) from ε-proteobacteria [43,44]. Recently, another organelle, the peroxisome, has been identified in *E. histolytica* [45]; this finding breaks the paradigm of peroxisome absence in anaerobic parasites. Amoebic peroxisomes contain myo-IDH, an enzyme that catalyses the conversion of myo-inositol, which is a substrate for the synthesis of phosphatidylinositol and phosphoinositide derivatives. It seems that mitosomes and peroxisomes are involved in the mechanism of lipid synthesis and transfer, but the interplay between components of mitosomes, peroxisomes, and the cytosolic membrane system is still under investigation [46]. In addition to gene loss, *Entamoeba* genomes also show expansion in some gene families such as those encoding proteins containing leucine-rich repeats (LRRs) homologous to bacterial adhesins (BspA from *Bacteroides forsythus*) [47], surface antigen Ariel1 and AIG1-like GTPases [6].

An interesting aspect of the biology of *E. histolytica* is the absence of well-defined morphological structures corresponding to the rough endoplasmic reticulum (rER), Golgi bodies [36] or microtubular spindle [48]. However, there are protein orthologs of established eukaryotic endomembrane systems, and functional ER and Golgi can be predicted [49,50]; in addition, α, β, and γ tubulin have been identified but they differ significantly from other eukaryotic tubulins, sharing only 50%, 58%, and 46% identity, respectively [48]. The actin-rich cytoskeleton plays a crucial role in all life processes of *E. histolytica*: division, encystation, motility, endocytosis, and in other processes supporting pathogenesis, such as phagocytosis of human cells and parasite resistance to host immune responses. However, *E. histolytica* has a single actin protein, which differs significantly in structure from its human counterparts [51,52], but actin performs all the various functions of the actin-rich cytoskeleton through many actin-binding proteins, 390 of them have been identified by bioinformatics [53], but their specific functions are only partially determined

Encystation of trophozoites in the presence of the microbiota

The four primary hallmarks of *E. histolytica* encystation (e.g. acquisition of a spherical shape, resistance to detergents, chitin accumulation at the cell wall, and tetranucleation); these steps are accomplished in the complex environment of the human colon where proliferation of trophozoites occurs simultaneously. Extensive research under laboratory conditions, using *E. invadens* as an amoebic encystation model, has provided rich information on encystation pathways; nevertheless, the results may not fully reflect the reality of this phenomenon in *E. histolytica* due to genetic differences between the two species and the need of distinct hosts for their proliferation [54]. Moreover, the intestinal environment complexity is different from the culture medium due to the fact that the human intestinal lumen harbours the microbiota which plays important roles in tissue homoeostasis including protection against pathogens [55], maintenance of mucosal barrier integrity [56], host nutritional elements sustain [57], and factoring the efficiency of the immune response [56]. For instance, some bacteria from the microbiota can degrade complex non-digestible carbohydrates (fibbers) to produce short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate [58], which are energy providers for colon epithelial cells and parasite growth and SCFAs can inhibit encystation [59]. Levels of glucose in the human colon does not reflect the

carbon source deprivation, which is important for *in vitro* encystation, and SCFAs also stimulate the production of mucin 2 [60], which contains oligosaccharides that might be used as a carbon source. Moreover, the potential impact of eukaryote symbionts on intestinal homoeostasis is not often considered; for example, microbiota composition is modified due to intestinal colonization by *Blastocystis* spp [61], which can be associated with other protozoa such as non-pathogenic *Entamoeba* species [62] and these changes are associated with amoebiasis [63,64]. Likewise, more than 140 fungal genera have been discovered as permanent or transient biota in the intestinal tract; many are either beneficial or commensal (e.g. *Saccharomyces*); as well as viruses and bacteriophages [65].

Altogether, these diverse microbes and their derivative metabolic products, suggest their potential specific roles in the control of intestinal homoeostasis and therefore in amoebic encystation. Thus, the major question that arises is how *E. histolytica* maintains the balance between vegetative growth and the onset of encystation? The answer to this question is largely unknown due to a significant gap in the development of experimental models mimicking the human colon, which are needed to study the amoebic lifecycle and amoebiasis [66,67]. Significant efforts have been made to this end; for example, 30% of cyst-like structures were obtained after treatment of trophozoites with hydrogen peroxide and metals, but these structures are not tetranucleate and may reflect a transient trophozoite survival response when subjected to a stressful environmental state [68,69]. An encouraging result was obtained using media conditioned by *Escherichia coli* and *Enterococcus faecalis* and an alkaline pH, the cyst-like cells obtained were multinucleated, but were not viable for excystation [70]. Recently, a promising model for encystation studies was obtained using two key parameters: high trophozoite density and glucose limitation in the culture medium [71]. The cysts are viable and exhibit all four characteristics of the cyst phenotype: round shape, chitinous cell wall, four nuclei, and detergent resistance. These cysts are viable for excystation generating the growing trophozoite form. A remarkable difference compared with *E. invadens* is that encystation commitment of *E. histolytica* was enhanced by SCFAs in this model, with acetate having the strongest effect [59]. Moreover, treatment with SCFAs induced minimal transcriptional changes in *E. histolytica* [72], all data support the recently proposed working hypothesis that epigenetic regulations leading to encystation of *E. histolytica* could occur under the influence of microbiota metabolites, such as SCFAs [73] and reinforce the idea that the encystation

signals of *E. histolytica* only partially overlap those of *E. invadens*.

High trophozoite density in glucose-free medium allows cyst formation in *E. histolytica* in the above cited model. This characteristic is already known for *E. invadens*, the cells aggregate and merge to give multi-nuclear giant cells (MGC) where cysts form [26,74]. The signal molecules and pathways inducing trophozoite aggregation are not known, but the addition of galactose (Gal) inhibits aggregation and hence encystation [75], a fact indicating that surface molecules sensitive to Gal are involved in amoebae interactions necessary for aggregation. Gal-inhibitable cell surface lectins have been proposed as an important Gal-responsive component bridging trophozoites [26] or allowing their interaction with the extracellular medium rich in Gal-terminated ligands including mucin [26,76]. Several lectins from *E. histolytica* are potential candidates for trophozoite aggregation, including a protein recognizing oligosaccharides found in oval-bumin [77], β trefoil lectin [78], and galactose/N-acetyl galactosamine lectin (Gal/GalNAc lectin) which recognizes Gal and N-acetyl galactosamine [79,80]. The molecular mechanism and signalling pathways implicating these lectins in encystation are far from determined; in particular, the Gal/GalNAc lectin is a versatile receptor activating multiple overlapping signalling pathways depending on the cellular function to be performed [18]. Nevertheless, encystation signalling pathways may occur through changes in the actin-rich cytoskeleton including reduction in actin levels that lead to rounded quiescent cells [81] or disruption of actin polymerization dynamics [82]; moreover, the carboxyl terminal domain of the Gal/GalNAc lectin heavy chain interacts with the cytoskeleton [18] triggering the activation of the above-described transcription factors involved in encystation. Recent data reinforce this hypothesis; aggregation occurs as an independent step leading eventually to encystation since the ERM-BP transcription factor participates in MGC formation in *E. invadens*, but silencing ERM-BP has no effect on the aggregation process [29]; indicating that trophozoite aggregation follows specific molecular regulation independent of MGC formation. Furthermore, ERM-BP is an important factor for MGC induction in *E. histolytica* following heat stress under conditions preceding cell death [29], these observations correlate stress responses and encystation. The MGC phenotype depends on the ERM-BP transcription factor which uses NAD+ as a cofactor, the level of NAD+ increases during both phenomena [29] and genes involved in cyst wall formation (e.g. chitinase and Jacob) are upregulated in both conditions [83]. These observations are reinforced

by a detailed view of *E. histolytica* transcriptome, which confirm that the gene encoding the cyst wall-specific glycoprotein Jacob 1 (EHI_028930) is upregulated during Oxidative Stress (OS) [84] and HS [85]; the gene encoding chitinase 1 is upregulated during HS, and ERM-BP is upregulated during HS (8 times) but downregulated in OS. However, orthologous genes, including cyst wall – specific glycoprotein Jacob 2, chitinase Jessie 1–2, −3, and −4 are not significantly altered during HS, indicating the existence of selective regulation for encystation-related genes.

Trophozoites, oxidative stress, and the microbiota

The epithelium provides a physical separation between the immune cells and the microbiota present in the intestinal lumen and the smooth mucus. In tissue homoeostasis, epithelial cells participate in immune defence by secreting mucus and releasing reactive oxygen species (ROS), antimicrobial peptides, chemokines, and cytokines. ROS derive from the reduction of oxygen to form: (i) superoxide (O_2-) a reaction involving NADPH oxidase and dual oxidase; (ii) hydrogen peroxide (H_2O_2) generated by dismutation of two molecules of O_2- by the enzyme superoxide dismutase (SOD), (iii) hydroxyl radical (HO) produced by either oxidation of water or hydroxide ions. Catalase or glutathione (GSH) reduce H_2O_2 [86]. Some bacteria from the microbiota contribute to ROS levels in the intestinal lumen [87], bacteria as well as other intestinal microorganisms accommodate to live with ROS levels related to homoeostasis. During the onset and establishment of intestinal diseases ROS level may increase triggering oxidative stress (OS), which damage lipids, proteins, and DNA and lead to cell death.

E. histolytica as a single-celled microaerophilic organism lives in low oxygen tension conditions, this parasite faces ROS during their growth and undergoes OS upon contact with bacteria and upon activation of immune responses from the host. *E. histolytica* lacks known important components of antioxidant defence mechanisms (e.g. catalase, peroxidase, glutathione, and the glutathione recycling enzymes glutathione peroxidase and glutathione reductase) and uses L-cysteine, instead of glutathione, as the main thiol. Nevertheless, this parasite has several anti-oxidative enzymes, including peroxiredoxins, SOD, dismutase, flavoprotein A, ferredoxin, thioredoxin, and thioredoxin reductase. Additionally, other scavenging proteins have been identified, such as rubrerythrin and hybrid-cluster protein. ROS cause oxidation of amoebic proteins and trigger OS responses [88], including transcription changes for

genes whose protein products are involved in mRNA translation, signalling regulatory cascades, DNA repair processes, energy metabolism, stress response, and solute transport [84,89,90]. A stress-responsive transcription factor (HRM-BP) in *E. histolytica* has been identified [91].

Commensal or pathogenic enterobacteria [84] and their derived metabolites (e.g. oxaloacetate [92], queuine [93]) protect trophozoites against OS. The molecular mechanisms of such bacterial protection on trophozoites are being studied, and it has already been determined that: (i) *Enterobacteriaceae* modify the transcription of numerous amoebic genes (approximately 31% of the coding sequences), including genes involved in protein synthesis and homoeostasis (ribosomal proteins and translation-related factors), nutrition (hydrolases, peptidases and trafficking), cell survival, encystation factors, as well as genes for several types of antioxidant enzymes (e.g. peroxidases and thioredoxins) [84]; (ii) oxaloacetate, as a non-enzymatic antioxidant H_2O_2 scavenger, reduces the amount of oxidized proteins [92], and (iii) queuine, as a hypermodified nucleobase, leads to hypermethylation of certain tRNAs affecting the protein synthesis [93] and, by unknown mechanism induce the upregulation of genes encoding anti-stress proteins, including heat shock protein 70, antioxidant enzymes and enzymes involved in DNA repair [93]. The protective effect of *Enterobacteriaceae* on *E. histolytica* is not universal since the production of H_2O_2 by *Lactobacillus acidophilus* (a probiotic) modifies the amoebic transcriptome differently compared to other *Enterobacteriaceae* [84]. *L. acidophilus* causes oxidation of vital proteins of *E. histolytica* (e.g. Gal/GalNAc lectin heavy chain, thioredoxin, cysteine protease A5, oxidoreductases, and signalling molecules), thus triggering parasite death [94].

In summary, the success of the parasitic lifestyle, due to the balance of the two cell forms of *Entamoeba* (trophozoite and cyst), is strongly influenced by the microbiota which, on the one hand, provides the bacteria and its derived nutrients promoting the growth of trophozoites and, on the other hand, acts as a motor for the survival of the parasite in stressful situations. Thus, the microbiota appears as the natural and adapted fuel to increase the population density of trophozoites. Their interaction with the extracellular environment, and their number, allow trophozoites aggregation; then, initiating the process of encystation after resources limitation. The cyst spreads in nature with the human stools, infects a new host and perpetuates the parasitic species (Figure 1). In some intestinal conditions (explained later), the lifecycle of *E. histolytica* can be disrupted switching trophozoites to a virulent phenotype.

Virulence of *E. histolytica* strains isolated from asymptomatic or symptomatic patients, genetic diversity

The outcomes of *E. histolytica* infection are mostly asymptomatic, only a fraction of those infected develop dysentery and, in rare cases, liver abscesses. In areas where amoebic infections are endemic, the incidence rate of *E. histolytica* has been found to be higher than that of *E. dispar*, while the population does not suffer from amoebiasis [95]. Molecular diagnosis among asymptomatic carriers determined a significant number of cases of mixed infections with *Entamoeba* [95,96] or in association with another protozoan [2]. However, a possible synergy in the mixed species population favouring the pathogenicity of *E. histolytica* has not yet been considered; mainly because it is not possible to conduct field experiments with these parasites strictly infecting humans. Nevertheless, this hypothesis is plausible because, as mentioned above, the presence of different protozoa in the same individual leads to changes in the microbiota [2] and potentially, interactions between amoebic species could modify the parameters of the lifecycle; for example, by inducing efficient aggregation of trophozoites because many molecules on the amoebic surface are common to various species [80]. Relevant knowledge on amoebic virulence has been acquired by studying strains of *E. histolytica* isolated in different geographical areas: while HM1: IMSS strain, isolated in Mexico from a patient suffering from amoebiasis is virulent; the Rahman strain, isolated in England from an asymptomatic carrier, is naturally attenuated and shows reduced virulence in experimental models [97,98]. Isolates of *E. histolytica* with various virulence patterns have also been reported from other countries [99]. To decipher the mechanisms supporting these varying levels of virulence among strains, parasite genotypes have been compared to determine changes in genetic polymorphism (e.g. using polymorphic markers linked to tRNA or interspersed nuclear retrotransposable elements) [100–103]; data suggest a high genetic diversity in strains of *E. histolytica* isolated worldwide [104]; but their link to the outcome of infection is still elusive.

Genome sequencing of trophozoites isolated worldwide has established [105] single nucleotide polymorphisms (SNPs) in these strains. Although their divergence from the reference genome is small (between 0.312 and 0.857 SNPs per kilobase), the

SNPs indicate genomic plasticity and suggest genetic recombination in *Entamoeba* [105]. To test a potential association between SNPs and disease outcome, samples of human stool and liver abscess aspirates were collected from areas endemic for amebiasis. After short-term xenic cultures of parasites, extensive DNA sequencing and bioinformatics analysis identified SNPs from 21 loci [106]. Two genes (EHI_065250 and EHI_080100) have SNPs with a significant link to parasites isolated from liver abscesses. Recently, SNPs at non-repetitive loci, which should be genetically stable, were identified in 49 isolates of *E. histolytica* from human cases with different disease outcomes (22 were from diarrhoeal, 7 from asymptomatic, and 20 with liver abscess) [107]. A total of 26 SNPs were present in three coding DNA sequences (lysine and glutamic acid – rich protein gene, *kerp1*, EHI_098210; glutamic acid and lysine – rich protein 2 gene, *kerp2*, EHI5A_120210; and amoebapore C gene, *apc*, X76903), and in two non-coding sequences (intron AY956435, intergenic region AY956439). Analysis of the genetic distribution within the *kerp2* gene shows that 10 SNPs are associated with disease outcome clusters (7 asymptomatic, 2 liver abscess). These SNP-disease correlations, in addition to potential recombination events and significant population distinction between pathogenic and non-pathogenic strains of *E. histolytica*, indicate that SNPs might be under constant selection pressure from the host and may be a potential determinant of disease outcome regarding amoebiasis [107]. SNPs of the *kerp2* gene are the first to be suggested as potential prognostic markers linked to a high risk of developing liver abscesses in people with persistent asymptomatic *E. histolytica* infection. Overall, these encouraging data, implying an impact of genetic diversity of field isolates of *E. histolytica* on the outcome of the disease, call for more studies, with a large sampling, in different geographical areas where amoebiasis is endemic, to obtain genetic markers relevant for detection of *E. histolytica* strains capable of invading the intestinal parenchyma.

Entamoeba histolytica and humans: from asymptomatic to symptomatic phenotype

The complex balance between "commensal-like" and pathogenic phenotypes for *E. histolytica* has allowed this parasite to become one of the most common human pathogens causing life-threatening infections. As a pathogenic parasite, *E. histolytica* leads to intestinal invasion in the 10% of infected people. For reasons still poorly understood, trophozoites begin to destroy the muco-epithelial barrier, thereby inducing the overproduction of mucus, killing host cells, and causing inflammation and dysentery. Trophozoites may disseminate through the portal vein system and invade the liver where it forms abscesses [108]. These stages of amoebiasis, illustrating amoebic virulence, depend on the capacity of the parasite to adapt rapidly to the new environments it encounters (mucus, epithelium, blood). An effective immune response to the parasiticinvasion leads to trophozoites death since in nature they cannot encyst outside the intestinal lumen. Invasive trophozoites trigger pathogenicity that is associated with several parasitic factors, including markers for: adhesion to mammalian cells, motility, extracellular matrix (ECM) degradation, cytotoxicity and engulfment of human cells, induction of host cell death and inflammation. Due to extensive literature and complexity of amoebic infection; in this topic we will focus on some newly identified features correlating to the very early virulent stages of *E. histolytica*, the reader is referred to excellent overviews elsewhere on the pathogenic process [7,17,109].

Entamoeba histolytica virulent trophozoites and the heat-shock proteins

Amoebic virulence is generally attributed to the ability of the parasite to produce liver abscesses in susceptible laboratory animals (e.g. gerbils and hamsters); however, long-term culture of *E. histolytica* HM-1: IMSS strain drifts towards an irreversible loss of the virulent phenotype [110,111]. The reasons for the attenuation of trophozoite virulence should be multiple [112]. To date, several studies have highlighted the correlation between attenuation and the lack of amoebae ability to maintain an intracellular hypoxic environment due to reduced performance of their OS responses [113,114]. Moreover, glycolytic flux is irreversibly decreased in attenuated trophozoites exposed to O_2 [115] reinforcing the idea of a link between virulence and survival mechanisms under host immune attack.

Gene expression analysis of virulent trophozoites cultured under OS indicates massive upregulation of genes encoding heat shock proteins (e.g. ClpB, HSP70, HSP90, and HSP101); in contrast, this response is absent in cultured attenuated trophozoites [113]. Careful differential expression analysis of transcriptome data obtained by RNASeq reinforces and clarifies these observations [116]: in highly virulent amoebae, 31 *hsp* genes are exclusively upregulated (e.g. *hsp70*), 14 *hsp* genes are upregulated in both strains (e.g. *hsp101*), and only 1 *hsp* gene is exclusively upregulated in attenuated trophozoites; moreover, 42 *hsp* genes are expressed

under OS at comparable levels in the two types of trophozoites (Figure 2).

Hsps are molecular chaperones that allow cells to survive during stress by maintaining proper protein folding limiting their denaturation and aggregation, some Hsps also have a role in maintaining genomic stability [117,118]. These proteins are classified according to their molecular mass: Hsp100(−101, ClpB), Hsp90, Hsp70, Hsp60, Hsp40, and Hsp20. In *E. histolytica*, Hsp70-A (EHI_113410), belonging to the Hsp70 family (13 members), is cytosolic [119], the coding gene is upregulated following O_2, H_2O_2, or NO treatments and during the formation of hepatic abscesses [89,113,116,120]; therefore, inhibition of Hsp70-A blocks virulence [113]. Hsp101 is highly over-expressed when trophozoites are treated with NO [120], and the only gene exclusively overexpressed in attenuated parasites is EHI_087630, which encodes an isoform of the Hsp transcription factor, the other 5 isoforms are expressed at the same level in all trophozoites. Overall, the data support the conclusion that the attenuation of virulence in *E. histolytica* involves the inhibition of the stress response through Hsps. To date, efforts to reverse the virulence-attenuated parasite phenotype have been unsuccessful and, consistent with these recent data, this objective call for experiments devoted to reverse the Hsp gene expression in attenuated parasites.

Virulent *E. histolytica* invade and destroy the intestinal mucus, the role of glycosidases

Human intestinal mucus is composed of highly glycosylated mucins, mainly mucin-2 (MUC2) [66,121]. O-glycans make up about 80% of the MUC2 mucin mass, these have a sialic acid on the GalNAc component attached to the protein core [121]. Mucus also contains several other molecules, such as IgGFc binding protein and calcium-activated chloride channel 1 [122]; these components are produced and secreted by goblet cells [66]. Some members of the microbiota, pathogenic bacteria, and parasites are mucus degraders [123–125], these are known to harbour glycosyl hydrolases that cleave specific glycan bonds in mucin. Degradation of the intestinal mucus layer by *E. histolytica* is crucial for colonic invasion and this activity induces rapid hypersecretion of new mucus from goblet cells [126]. Thanks to the human colon explant model of amoebiasis [127], the early pathophysiological pathways of intestinal invasion by virulent *E. histolytica* HM-1: IMSS show mucus degradation in 2 hours [127]. Mucus breakdown by *E. histolytica* was recently visualized using the in vitro 3D scaffold model of the human colon containing goblet cells [67]. Immunofluorescence with specific anti-MUC2 antibodies and two-photon microscopy locate mucin fragments either in the
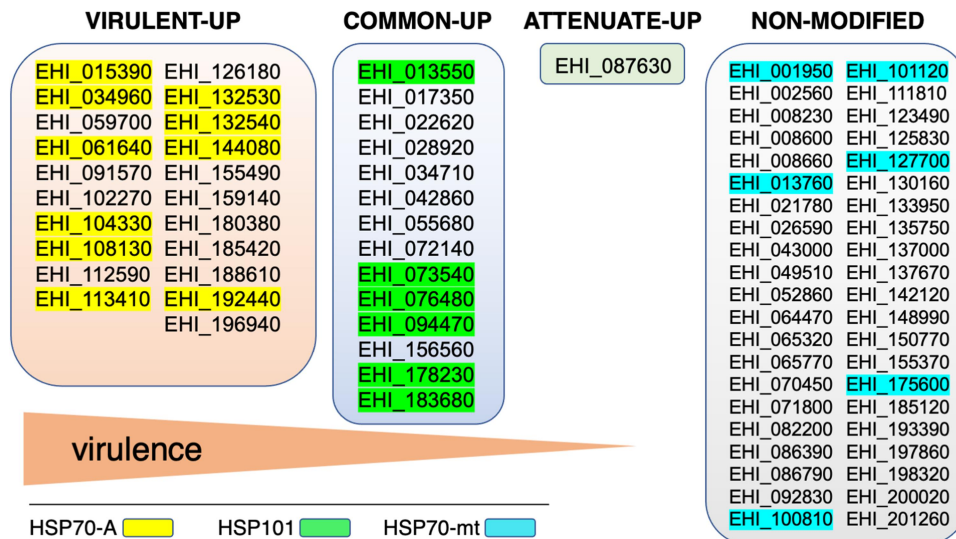

Figure 2. The stress response of virulent trophozoites involves heat shock proteins of the Hsp70 family. In the genome of *E. histolytica* HM1:IMSS strain, 86 genes encoding HSP proteins have been identified; of these, 78 genes have a transcriptional response in trophozoites undergoing OS. Gene expression analysis of stressed trophozoites indicates that the gene family encoding Hsp70 is preferentially upregulated (fold changes >3) in highly virulent amoebae; the Hsp101 encoding genes appear preferentially upregulated in virulent and attenuated amoebae, whereas only a gene encoding one HSP transcription factors is upregulated in attenuated trophozoites. In addition, 42 genes encoding HSPs are expressed at comparable mRNA levels in both types of trophozoites subjected to OS. The EHI_ prefix followed by numbers matches the gene identity in AmoebaDB database (https://amoebadb.Org).

mucosal environment or inside trophozoites. The amoebic signalling pathways activated by ingestion of MUC2 remain to be determined.

E.histolytica poses various glycosidases, such as N-acetylgalactosamidase, N-acetyl-glucosaminidase, β-galactosidase, β-N-acetyl-hexosaminidase, and sialidases [128]; some of these enzymes can cleave polysaccharides present in MUC2, as they can be secreted [125] or be a component of cytoplasmic granules released by exocytosis after amoeba interaction with collagen [129]. *E. histolytica* meeting the colon rapidly changes its transcriptomic profile [116]. The increased expression of genes related to carbohydrate and glycosylated residue metabolisms of virulent amoebae compared with attenuated amoebae correlates with gene expression patterns obtained by comparing virulent HM-1:IMSS and non-virulent Rhaman strains, in the same colon explant model [130]. The glycosidases encoding genes highly upregulated include: β-galactosidase which catalyzes the hydrolysis of β-galactosides into monosaccharides, as well as α- and β-amylases acting on starch, glycogen, and related polysaccharides and oligosaccharides [131]. Knock down of genes encoding β-amylases leads to trophozoites that are unable to degrade the mucus layer and therefore, with limitations to invade the human colon [130].

In agreement with the pathophysiological analysis and the transcriptomic profile of virulent trophozoites, it can be hypothesized that the degradation of saccharides within the mucus layer is an important source of energy promoting the viability of amoebas during mucosal invasion. Mucus-derived components can compensate for the deprivation of carbon sources due to the decrease in the bacterial population characteristic of the deep layer of mucus. Moreover, this expected nutritional need could be accompanied by the stress response of amoebae imposed by the passage from the lumen to the mucosal environment. This hypothesis correlates the upregulation in *E. histolytica* of genes encoding β-amylases during intestine invasion, heat shock, and OS [85]; in contrast, β-amylase genes are downregulated in trophozoites subjected to OS in the presence of bacteria [84], a fact that reinforces the microbiota role in parasite survival. Links between nutritional requirements and stress responses for β-amylase genes have been observed in plants where they respond positively to growth, development, and abiotic stress [132,133]. The importance of these β-amylase gene behaviours during amoebic pathogenesis is still under investigation; however, humans lack β-amylase and the fact that blocking the amoebic enzyme suppresses pathogenesis makes this enzyme a highly relevant candidate target for new anti-amoebiasis drugs.

Virulent *E. histolytica* invade and destroy the intestinal mucosa, the role of cysteine protease A5

Cysteine proteases (CP) play a key role in the pathogenesis of *E. histolytica* [134]; among them, cysteine protease A5 (CP-A5, EHI_168240) is present only in *E. histolytica* [135]; it is an important amoebic factor for mucus depletion because it targets peptides in glycosylated poor regions of MUC2 [135,136]. This enzyme localizes to the surface of the trophozoite [137], contains an Arg-Gly-Asp (RGD) integrin-binding motif that has also been described in higher eukaryotic cathepsin X [138]. CP-A5 binds via its RGD motif to the $\alpha_V\beta_3$ integrin on enterocyte-like cells and stimulates inflammatory responses [139]. The interaction of amoebic CP-A5 with integrin $\alpha_V\beta_3$ triggers in goblet cells the outside-inside signalling that result in mucin secretion with activation of several kinases (e.g. kinases of the SRC family, the focal adhesion kinase FAK, PI3K, PKCδ, and MARCKS) [140]. An *E. histolytica* cyclooxygenase-like regulates CPA5 activity through a protein–protein interaction that stabilizes CP-A5 and increases virulence [141]. Recent data indicate the role of CP-A5 in the degradation of bacterial biofilms [142].

To enter the tissues, trophozoites disorganize the loose collagen scaffold to escape into the mucosa [127]. Epigenetically silenced trophozoites for CP-A5, which are negative for its proteinase activity, fail to trigger the tissue inflammatory response [127] or to induce collagen remodelling necessary for lamina propria invasion [143]. In the explant colon model, enzymatic activities of human matrix metalloproteinases (MMP-1 and −3) have been determined to be responsible for the alteration of collagen fibrillar structures during intestinal invasion by *E. histolytica* [144]. Clearly, CP-A5 activity convert pro-MMP-3 into active MMP-3 enzyme, which in turn activates pro-MMP-1, leading to collagen degradation [144]. In addition to the role in intestinal amoebiasis, CP-A5 activity is also required to induce the formation of liver lesions in hamsters [145] and, is implicated in the loss of the actin cytoskeleton and the organization of focal adhesion structure triggering endothelial cell death [146].

Overall, we can conclude that CP-A5 is a multifunctional enzyme that plays a critical role in the pathogenesis of *E. histolytica* in the human colon: by degrading mucus and bacterial biofilms, binding to αvβ3 integrin receptors on goblet cells to induce pro-inflammatory responses, by activating the remodelling of the intestinal fibrillar collagen structure and in addition, by playing a role in the formation of liver abscess.

Interplay of virulent *E. histolytica* and human intestine at early stages of amoebiasis

To be successful during intestinal invasion, *E. histolytica* must be motile and the actin-rich cytoskeleton is central to the complex invasive process [147]. In the early stages of intestinal invasion [148], once the protective mucus layer has been destroyed, virulent trophozoites penetrate the intestinal parenchyma, adhere and destroy the extracellular matrix (ECM) through the action of amoebic surface receptors recognizing ECM components and proteinase activities [149]. The trophozoite evolves in the colon epithelium by attaching to the mammalian cell by means of surface glycosylated components like the Gal-GalNAc lectin and the glycocalyx-bound lipopeptidophosphoglycan (LPPG) [18]; or by direct amoeba-cell contact involving proteins like KERP1, KERP2 [150]; serine, threonine-, and isoleucine-rich proteins STIRPs [151] and the adhesin ADH112 [152]. Cell-cell junctions of epithelial cells are disrupted by proteinases [153], mammalian cell killing, and phagocytosis occurs; alternatively, after attaching to host cells, amoebae "chew off" and ingest host cell fragments by trogocytosis [154,155]. Onset of host immune responses begins with neutrophil infiltration to sites of parasitic invasion, followed by activation of mast cells, macrophages, natural killer T cells, and subsequent secretion of pro-inflammatory cytokines [148]. By studying the pro-inflammatory response and mucus secretion due to *E. histolytica*, two recent data indicate that: (i) pro-inflammatory responses correlate with the downregulation of the cell differentiation-related transcription factor Math1 (homologous mouse atonal 1) [156], the inhibition of which leads, during tissue development, to a reduction in the population of goblet cells [157]; (ii) microbiota restore normal responses for mucus secretion and pro-inflammatory cytokine activation, in a mouse model of antibiotic-induced dysbiosis infected with *E. histolytica* [158]. These results suggest firstly a direct effect of trophozoites on the regulation of goblet cells persistence and secondly, a role of the microbiota in the control of pro-inflammatory responses, which acts in addition to the nutritional and protective role of amoebae mentioned above. Research on the nature, activity, and importance (in terms of pathogenicity) of amoebic "virulence factors" and signalling supporting the intestinal invasive process has been intensive and are the subject of detailed reviews [7,159]. Several aspects will be summarized below aimed at further enriching these publications.

Disruption of epithelial cells junctions by *E. histolytica*

The epithelium facing the intestinal lumen is composed of a single layer of polarized cells (enterocytes, goblet, enteroendocrine, Paneth, microfold, cup and chemosensory) constituting the physicochemical and immunological barrier against luminal antigens and enteric microbiota while allowing the absorption of nutrients and water [66]. Sealing cells together needs of tight junctions (TJs), adherens junctions (AJs), and desmosomes (DSMs) [160]; particularly, TJs regulate paracellular traffic of macromolecules and ions across the epithelium. TJ-specific families of integral membrane proteins include occludin, claudins, and junctional adhesion molecules (JAMs) that are responsible for maintaining the ion barrier of the paracellular space; claudins interact with scaffolding proteins like zona occludens (ZO-1 and ZO-2), which link them to other cytoplasmic proteins and actin microfilaments [161]. Occludins regulate the flux of macromolecules, while claudins provide control of ionic flux and limit the diffusion of proteins and lipids acting as a membrane fence [162] and, in combination with JAMs, regulate epithelial polarity [163].

The cysteine proteinase CP112 from *E. histolytica* is involved in the degradation of the cell-cell junction [164]. CP112 has been described with a canonical catalytic domain and an RGD motif, this enzyme degrades type I collagen, fibronectin, and haemoglobin. The proteinase CP112, together with the adhesin ADH, forms the virulence complex EhCPADH [165]. Trophozoites overexpressing CP112 or the purified enzyme cause a drop in transepithelial electrical resistance of cells and disrupt the fence function of TJs in enterocyte-like cells (Caco-2 cells); in contrast, CP112 does not affect the paracellular permeability of macromolecules [164]. By means of high-resolution confocal microscopy and immunoprecipitation essay, the authors demonstrated that purified CP112 affects claudin-1 and claudin-2 integrity; no effect was found on claudin-4, occludin, ZO-1, or ZO-2; treatment of mice with CP112 protease confirmed these data. Additional findings suggest that trophozoites could also affect AJs and DSMs and indicate a role for CP112 in the multifactorial virulence event of *E. histolytica* [166]. CP112 is not the only factor disrupting TJs; for example, trophozoites secretes the inflammatory molecule prostaglandin E_2 (PGE_2), which, in mammalian cells, activates secretion of chloride and breaks TJs ion barrier acting on claudins with the increased Na+ permeability; PGE_2 from *E. histolytica* dissociates claudin-4 from mammalian cell membrane affecting TJs ion barrier [167].

Other additional proteases participate in alterations of TJ proteins: CP-A5 (tested by amoebic infection in Muc2−/− mice) [168] and serine proteases participating in transepithelial electrical resistance dropping of epithelial cells [169].

Due to the primary function of cell junctions as regulators of ion transport in the intestine, it is tempting to correlate the activities described above, concerning TJ disruption by amoebic proteases, with two important sets of data regarding the early stages of amoebic infection. Based on the cytotoxic properties of *E. histolytica* leading to mammalian cells death, the authors screened a library of human interfering RNAs (RNAi) to select enterocyte-like cells resistant to die in the presence of virulent trophozoite [170]. The identification of genes significantly enriched, whose silencing by RNAi increase cell survival upon contact with *E. histolytica*, highlight ion transporters channels for Ca2±dependent K+ (5 genes); Cl− (4 genes), Na+ (3 genes) and Ca2+ (2 genes). Further drug inhibition of K+ efflux prevented the death of intestinal epithelial cells and macrophages, indicating direct activation of K + efflux as an early marker of tissue responses following intestinal invasion by *E. histolytica* [170]. The second dataset corresponds to the transcriptome and proteome analysis of human factors significantly modulated at the early stages of the interaction between virulent *E. histolytica* and the 3D scaffold model of the human colon [67]. Upregulated genes for receptors acting as ion channels have been identified: the epsilon subunit of the gamma-aminobutyric acid receptor; the CL channel involved in ulcerative colitis [171], and the epsilon subunit of the acetylcholine receptor that can open ion channels [172]. The long non-coding SLC9A3 antisense RNA 1 [173] targeting the SLC9A3 gene that encodes the Na+/H+ exchanger NHE3 associated with congenital diarrhoea [174] was also upregulated. At the protein level, abundantly secreted are components of desmosomes and factors linking them to the cytoskeleton (desmoplakin, plakophilin-3, and plectin-1); these are important for the maintenance of tissue integrity [175].

Taken together, the data suggest that disruption of structures responsible for cell-cell junctions combined with disruption of ion channels, are major lesions occurring at the early stages of contact between *E. histolytica* and the intestinal epithelium, they both lead to the collapse of tissue architecture. Therefore, mucus secretagogue activity, tissue leakage, and epithelial collapse, are responsible for the early symptoms of amoebic dysentery. They are the premises for the expected death of host cells, although how epithelial cells die because of interaction with *E. histolytica* remains an open question.

Mammalian cell death upon contact with *E. histolytica*

Following the entry of *E. histolytica* in the intestinal mucosa, the cells die. The data demonstrates that the destruction of mammalian cells requires contact with trophozoite that trigger calcium influx. Adhesion of trophozoites to enterocytes occurs via various factors on the amoebic surface, including LPPG, Gal-GalNAc lectin, KERP1, STIRP, and the CPAHD112 complex [18]; the contact led to the death of human cells in large proportions; approximately 40% of cultured enterocyte-like cells are killed within 30 minutes of interaction [150,176]. Dead cells can be ingested by phagocytosis, which is an important process for clearance of pathogens, necrotic or apoptotic cells [177]. In *E. histolytica*, phagocytosis is a way to acquire nutrients as this parasite is a professional phagocytic cell ingesting bacteria [178] and diverse mammalian cell types [179]. However, the interaction of living cells (e.g. epithelial, erythrocytes or lymphocytes) and *E. histolytica* involves complex steps. Amoebae that encounter mammalian cells rapidly induce trogocytosis, a phenomenon where amoebae bite and ingest distinct host cell fragments, contributing to cell killing [180]. Uptake and display of mammalian membrane proteins on the surface of the amoeba protect the parasite from lysis by inhibiting the complement cascade, indicating that trogocytosis contributes to amoebic defence strategies for immune evasion [181,182]. Trogocytosis and phagocytosis occur by different mechanisms, although many of the early steps and molecules involved may be common [183,184].

Contact-dependent cell killing by *E. histolytica* triggers apoptosis of T cells and neutrophils through activation of host cell caspase 3 [185], but caspase-independent death mechanisms in T cells have also been described; these include protein tyrosine dephosphorylation and ROS generation and are associated with the amoebic kinases, PI3K and PKC [186]. Human enterocytic cell lines can undergo apoptosis (associated with caspases-3 and −9 activation) upon interaction with *E. histolytica* [187]. In search of the molecular mechanisms inducing the apoptotic process of enterocytes, the authors investigated non-coding RNAs, such as microRNAs (miRNAs), which are negative regulators of gene expression. They revealed a set of six miRNAs with complex miRNA-mRNA co-regulatory networks in which the miRNAs target the

anti-apoptotic genes XIAP, API5, BCL2, and AKT1 [187]. One of them, miR-643 exerts post-transcriptional downregulation of XIAP by targeting its mRNA 3'-UTR. The use of antagonists of miR-643 leads to the restoration of XIAP levels and the suppression of apoptosis and activation of caspases 3 and 9. The authors conclude that there is a functional role for the miR-643/XIAP axis in the apoptosis of enterocytes activated by *E. histolytica* [187]. The role of the other miRNAs discovered in this work remains ongoing experimentation; as well as the role of upregulated long non-coding RNAs discovered in the 3D scaffold model of the human colon [67].

LncRNAs are involved in many cellular processes and can interact with miRNAs to modulate their roles, including in epigenetic modifications regulating gene expression [188]. This area of research is *terra incognita* with respect to amoebic infection and progression of amoebiasis; however, interesting advances have been made in the study of intestinal host-bacteria interactions. Induction of miRNA has been correlated with the relative abundance of several microbiota taxa [189]; suggesting that the microbiota modulates miRNA induction mainly through metabolites [190]. Conversely, miRNAs secreted by intestinal epithelial cells modify the composition of the microbiota [190]. Certainly, new avenues of research concerning the control by human cells of the amoebic cytotoxic effect call for studies determining the involvement of non-coding human RNAs in the regulation of enterocyte death; in return, it is also important to understand the role of specific amoebic natural antisense RNAs [85] appearing during the invasion of the human colon in the control of virulence factors.

Human host defences at the epithelium and cell-mediated innate immunity responses against *E. histolytica*

Antimicrobial peptides followed by cell-mediated innate immunity corresponds to the second level of intestine defence against invasion by *E. histolytica*; these include defensins, regenerating islet-derived proteins REGs and alarmins. Defensins are small peptides that integrate into the membranes and form pores devoted to kill the invading microbe. *E. histolytica* induce secretion by enterocytic-like cells of α and β defensins [191], which, when purified, can kill trophozoites in vitro [192]; but at the same time, the genes encoding β defensins are downregulated in the 3D-scaffold model of infection [67] and in the human xenograft model [193], probably as an amoebic defence mechanism that requires more investigations.

The antimicrobial peptide REG1A (anti-apoptotic) and REG1B mRNA increase during acute amoebic infection in humans [194], and REG4 (anti-inflammatory) is secreted by cells in the 3D-intestinal scaffold model [67]. Alarmins are endogenous molecules released to signal cell or tissue stress or damage. The ubiquitous nuclear DNA-binding protein call high-mobility group box 1 protein (HMGB1) is an alarmin activating innate immunity, it is released by necrotic cells or secreted by macrophages, natural killer cells, and dendritic cells. HMGB1 is activated and released from macrophages in response to *E. histolytica* invasion [195]. HMGB1 secretion depends on the microbiota and this molecule shapes the magnitude of the pro-inflammatory response during amoebiasis.

A very broad view of intestinal immune responses emerges when considering the activities of the microbiota (for example, the production of metabolites) and the diversity of responses of the immune system due to the presence of enteric pathogens [196]. A brief summary of these responses highlights several factors. The Toll-like receptors (TLR) on enterocytes surface, which recognizes microbial-derived compounds and trigger signalling cascades ending with nuclear translocation of NF-κB transcription factor and secretion of cytokines and chemokines, (e.g. TNF, IL-6, IL-8, IL-18, and CCL20). These molecules activate immune cells underlying the enterocytes layer, which migrate to the infection site (e.g. dendritic cells, monocytes, neutrophils, and T lymphocytes). Antimicrobial factors, including β-defensins and the inducible nitric oxide synthase generating nitric oxide, are also induced following TLR activation. The pathogen and the inflammation damage the enterocytes, these can physically expel themselves from the intestinal epithelium by a mechanism that involves the activation of inflammatory caspases (caspase-1, −4, and −11) as along with caspase-8. Activated caspases induce the macromolecular signalling complex called "inflammasome," triggering the processing and secretion of the pro-inflammatory cytokine IL-18, as well as the induction of a specialized form of inflammatory cell death called pyroptosis [197]. The last step of pyroptosis requires the cleavage of the pore-forming protein gasdermin D by caspase-1; the N-terminus end of gasdermin D forms a transmembrane pore that disturbs ion and water regulation, and the dying cells release cytokines such as IL-1β, IL-18 and the prostaglandin PGE_2, resulting in subsequent interferon-γ (IFN-γ) production and neutrophil recruitment [198].

Many of these components marking immune responses are evoked during invasion of the colonic

mucosa by *E. histolytica*, which triggers a complex inflammatory process with production of interleukins (IL-1β, IL-6, IL-8), IFN-γ, and Tumour Necrosis Factor (TNF) [17]. These factors activate immune cells, including neutrophils, lymphocytes, and macrophages, which are further expected to kill trophozoites through the action of ROS and NO.

Regarding macrophages, the LPPG of *E. histolytica* is recognized by TLR 2 and TLR4, with activation of NF-kB and release of IL-8, IL-10, IL-12p40, and TNF [199]. Caspase-1 (processed by CP-A5) activates the NLRP3-like inflammasome [200] and cleaves pro-IL-1β into the active form IL-1β, the secretion of which also depends on the activity of caspase-4 and gasdermin D [201].

Neutrophil infiltration is intense in the human colon due to the density of *E. histolytica* [202], and they kill *E. histolytica* in the presence of TNF and IFN-γ primarily via ROS [203,204]. Decreased microbiota diversity in humans or in the mouse model increases the severity of *E. histolytica* infection, likely due to decreased neutrophil recruitment to the infection site [205]; however, the mechanisms of amoeba–neutrophil interaction, and consequent parasite and cell death, are far from determined. Exposure of human neutrophils to *E. histolytica*, or purified LPPG, induced the release of neutrophil extracellular traps (NET) in a time- and dose-dependent manner [206,207], but it is not clear that these NETs are able to kill *E. histolytica*. Mechanisms of NETosis involve translocation of elastase to the nucleus due to generation of ROS by NADPH oxidase (NOX2) or by activation of peptidyl arginine deiminase 4 (PAD4); both mechanisms result in DNA decondensation. Amoeba-induced NETosis is independent of ROS and PAD4 activity; however, it depends on the presence of extracellular calcium and serine protease activity [207], the signalling pathway leading to NETosis involves the activation of Raf/MEK/ERK and NF-κB [208]. Besides oxidative and nitrosative stresses, some cytokines (e.g. TNF) can act as a chemoattractant for *E. histolytica* by increasing trophozoite motility and directionality [209], the amoebic surface protein CSP has homologies with human TNF receptor, even a blockage of CSP abolishes the chemotaxis towards TNF and blocks the parasitic invasion of explants of the human colon [210].

Intestinal epithelial cells normally produce interleukin-25 (IL-25), but this is decreased in humans with amoebic colitis [211]; this cytokine plays a role in maintaining intestinal barrier function and inhibit TNF production. IL-25-mediated protection is accompanied by a decrease in TNF levels in experimental models of amoebiasis [211]. IL-33 (nuclear alarmin)
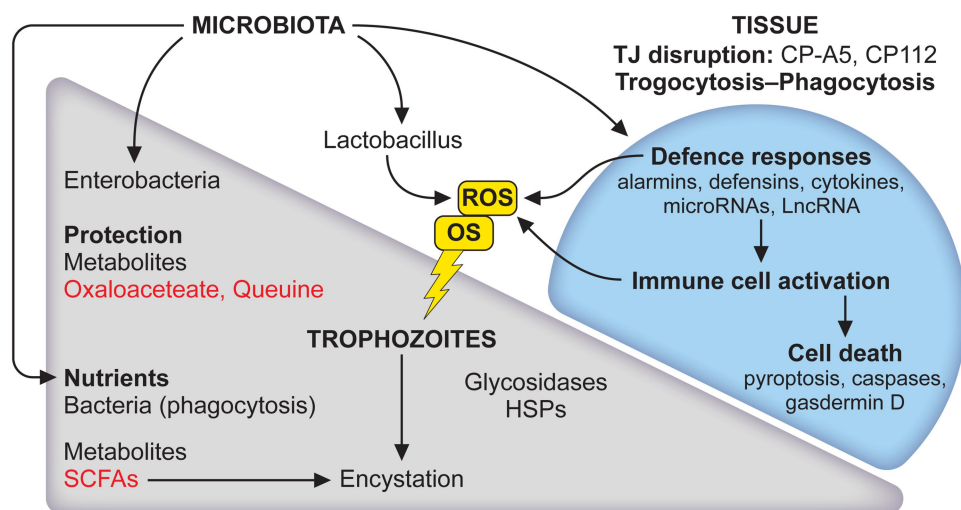

Figure 3. The microbiota has a role to play in the lifecycle of *E. histolytica* and tissue responses. The balance between trophozoite and cyst is strongly influenced by the microbiota. First, amoebae phagocytose bacteria and derived nutrients promote the growth of trophozoites. Secreted bacterial products such as short-chain fatty acids (SCFAs) are expected to provide energy for parasite growth. During carbon source deprivation, SCFAs stimulate the production of mucin, which could be used as a carbon source. Acetate is a SCFA that induces encystation *in vitro* in *E. histolytica*. Second, an important activity of bacteria is to act as a parasite survival aid in stressful situations like oxidative stress (OS); it regulates the expression of amoebic genes, metabolites such as oxalacetate and queuine, derived from *Enterobacteriaceae*, protect *E. histolytica* from OS. This response is not universal since *Lactobacillus acidophilus* (a probiotic) modifies the amoebic transcriptome differently and causes oxidation of vital *E. histolytica* proteins leading to the death of trophozoites. Major lesions occurring at the early stages of contact between *E. histolytica* and the intestinal epithelium led to disruption of tight junctions combined with disruption of ion channels tissue architecture collapse. Mammalian cells die by trogocytosis, apoptosis and phagocytosis. The inflammatory response occurs leading to cell death, the microbiota influence alarmins activity. For references see the main text.

activates innate lymphoid cell inducers of type 2 immune responses; the IL-33 encoding gene is upregulated in humans with colonic amebiasis, suggesting that IL-33 is involved in the induction of barrier repair mechanisms to protect intestinal tissue from *E. histolytica* [212]. Overall, this rapid overview of the data confirms the complexity of innate immune responses against *E. histolytica* considering the diversity of activated cells, secreted products and their consequences on tissue integrity (Figure 3).

Multiple other factors of interest implicated in the intestinal defence against *E. histolytica*, or having an impact on the development of amoebiasis, include genetic [213,214], social and nutritional aspects [215–217]; they will not be discussed in this review. For the implications of virulence-related factors as potential targets for novel therapies for amoebiasis, the following publications should be considered [218–221].

Conclusion

Research on the pathways of intestinal amoebiasis and its prevention remains a real challenge due to the complex and multifaceted relationship between virulent *E. histolytica* and the host during the different stages of amoebiasis. The confrontation of trophozoites with microbiota, human immunity and their stress-inducing products, activate regulatory pathways necessary for the genetic, transcriptomic, and epigenetic phenomena that maintain fitness of *E. histolytica* and ensure its survival in the human body. Although insufficiently considered, the microbiota plays a major role in the relationship of *E. histolytica* with the intestine; remarkably, it can influence lifecycle, stress response, and pathogenicity. The simplicity of the amoebic lifecycle guarantees the dissemination of the parasite among humans and therefore the persistence of *Entamoeba* species as commensal-like (or symbiotic) microbes. Even in the future, when virulence and pathogenicity will be controlled by new pharmacological treatments or vaccination, *E. histolytica*, as a parasite, will probably continue to evolve with humans like other species of *Entamoeba*.

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