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Article

Insights into the mechanisms of *Lactobacillus acidophilus* activity against *Entamoeba histolytica* by using thiol-redox-proteomics

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Abstract:

Amoebiasis is an intestinal disease transmitted by the protist parasite, *Entamoeba histolytica*, following the ingestion of contaminated food and water. In the colon, *E. histolytica* can phagocytose bacteria that are the main components of the microbial flora. Most infected individuals are asymptomatic but for unknown reasons, the parasite can become virulent and invasive, causes amebic dysentery, and migrates to the liver, where they cause hepatocellular damage. For the last few decades, it has become evident that *E. histolytica* virulence is directly linked to its interaction with the gut microbiota. *Lactobacillus acidophilus* is a common inhabitant of healthy human gut and a probiotic that present antimicrobial activity against many pathogenic bacteria, fungi and parasites. The purpose of this study was to examine the mechanisms behind the innate amebicide activity of *L.acidophilus*. We found that this activity is mediated by hydrogen peroxide (H_2O_2) produced by *L. acidophilus*. Redox proteomics shows that *L. acidophilus* triggers the oxidation of many essential amebic enzymes like pyruvate:ferredoxin oxidoreductase, the lectin Gal/GalNAc and cysteine proteases (CPs). Further, trophozoites of *E.histolytica* cultivated with *L.acidophilus* show reduced binding to mammalian cells. These results support *L.acidophilus* as a prophylactic candidate against amoebiasis.

Keywords: *Entamoeba histolytica*; *Lactobacillus acidophilus*; probiotic; redoxomics; cysteine proteases.

1. Introduction

Amebiasis is an enormous global medical problem because of poor sanitary conditions and unsafe hygiene practices existing in many parts of the world. According to the World Health Organization, 50 million people in India, Southeast Asia, Africa, and Latin America suffer from amebic dysentery and amebiasis causes the death of at least 100,000 individuals each year. The main mode of transmission of amebiasis is the ingestion of food or water that is contaminated with feces containing *E. histolytica* cysts. After the cyst form has been swallowed by the host, excystation occurs in the intestinal lumen, followed by colonization of the large intestine by the trophozoites where they continue to divide and encyst. Eventually, both trophozoites and cysts are excreted in stools. Only 10% of the infected individuals will develop acute intestinal and extra-intestinal diseases. One possible explanation for this observation is the difference in the gut microbiota between individuals that may significantly influence the host's immune response in amebiasis and *E. histolytica*'s virulence [1]. Over the last few decades, it has become evident that *E. histolytica*'s pathogenicity is directly linked to the parasite's interaction with the gut microbiota [2], as the parasites are reported to feed on bacteria and cellular debris found in the large

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intestine [1]. However, such feeding is very selective, where only those bacteria with the appropriate recognition molecules are ingested by the parasite [3]. Amebiasis is characterized by acute inflammation of the intestine with release of pro-inflammatory cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS) from activated cells of the host's immune system. ROS and RNS are the major cytotoxic effectors for killing *E. histolytica* and cause oxidation and nitrosylation of amebic proteins, trigger stress responses, inhibit glycolysis and the activity of some virulence factors [4-7]. Cellular means of subverting the toxicity of oxidative stress (OS) are important for the success of infectious diseases. No vaccine against amebiasis currently exists; the drug of choice for treating amebiasis is metronidazole, which may cause severe side effects such as nausea, vomiting, headaches, a metallic or bitter taste in the mouth, and more serious effects such as anorexia, ataxia and skin rashes/itching [8,9]. Additionally, some clinical strains of *E. histolytica* are less sensitive to metronidazole, suggesting the emergence of metronidazole-resistant strains [10,11].

Probiotics are live organisms which when administered in adequate amounts confer a health benefit to the host [12] [13]. Probiotics and commensal bacteria have been suggested to have some influence on the outcome of protozoan infections [14-16]. As an alternative bio-therapeutic for amoebiasis, there are a number of studies which have been conducted, interestingly most of these studies are aimed at the efficiency of the probiotic at inhibiting adhesion of the protozoa to the intestinal mucosal surface [17,18]. Recently we have shown that *Lactobacillus acidophilus* is detrimental to *E. histolytica* [19]. This detrimental effect is associated with the transcription by the parasite of genes encoding major signaling molecules, such as kinases, regulators of small GTPases and oxidoreductases and genes encoding proteins necessary for ribosome structure. It has been suggested that the probiotic effect of certain bacteria (such as *L. acidophilus*) is mediated by the ability to produce H_2O_2 [20] via an NADH-dependent flavin reductase [21] and to maintain a normal, homeostatic microbiota [21]. In this work, we used redox-proteomics to demonstrate that essential *E. histolytica* proteins like cysteine proteases are oxidized by H_2O_2 produced by *L. acidophilus* leading to the death of the parasite. Moreover, we found that *E.histolytica* trophozoites incubated with *L.acidophilus* had reduced binding than control trophozoites to mammalian cells. Our data support the use of *L. acidophilus* as a probiotic against *E.histolytica*.

2. Materials and Methods

2.1. E. histolytica and L. acidophilus culture

E. histolytica trophozoites, the HM-1:IMSS strain (a kind gift of Prof. Samudrala Gourinath, Jawaharlal Nehru University, New Delhi, India), were grown and harvested according to a previously reported protocol [22].

L. acidophilus ATCC4356 strain was cultivated in De Man, Rogosa and Sharpe (MRS) media (Sigma-Aldrich, Jerusalem, Israel) overnight at 37°C with agitation (200 rpm) on a New Brunswick Innova 4300 Incubator Shaker (Marshall Scientific, New Hampshire, USA). Heat-killed *L. acidophilus* was cultivated in MRS media (Sigma-Aldrich, Jerusalem, Israel) overnight at 37°C with agitation, followed by autoclaving at 121 °C and 1.05 kg/cm2 for 15 min.

2.2 Reagents

Catalase from bovine liver (C9322)) were purchased from Sigma-Aldrich (Jerusalem, Israel).

*2.3 Ferrous oxidation-xylenol orange (*FOX*)* assay

The amount of H_2O_2 produced by *L. acidophilus* was determined by the FOX assay according to a previously reported protocol [23].

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2.4 Viability of E. histolytica trophozoites

Trophozoites (~1 × 10^6/ml) were incubated with *L.acidophilus* (~1 × 10^9/ml) in serum-free Diamond's TYI S-33 medium for 120 minutes at 37°C with agitation (200 rpm) in a thermoshaker (ALS-MS-100, Hangzhou Allsheng Instrument, China). The viability of trophozoites was determined by the eosin dye exclusion method [6].

2.5. Detection of oxidized proteins (OXs) by resin-assisted capture RAC (OX-RAC)

The detection of OXs by OX-RAC was performed using a previously described protocol [6]. A protein was considered to be oxidized when its relative amount in the DTT treated lysates was at least two times greater than that in the untreated lysates ($p < 0.05$ according to the results of an unpaired t-test).

2.6. In-Gel Proteolysis and MS Analysis

In-gel proteolysis, MS, and data analysis were performed according to a previously reported protocol [6,24].

2.*7. Classification of OXs According to Their Protein Class*

The OXs were classified according to their protein class using PANTHER Classification System software (http://www.pantherdb.org/ accessed on 28 July 2021) [25].

2.8 Measurement of cysteine proteases (CPs) activity

CPs activity was assayed by using a previously described protocol [26] except that DTT was not systematically added to the reaction buffer.

2.9 *Adhesion assay.*

The adhesion of *E.histolytica* trophozoites to HeLa cells (a kind gift from T. Kleinberger, Faculty of Medicine, Technion) was measured using a previously described protocol [27]. *E. histolytica* trophozoites (2 × 10^5) were incubated with live *L. acidophilus* (2 × 10^8), with heat-killed *L. acidophilus* (DN) (2 × 10^8), with paraformaldehyde-fixed *L.acidophilus* (PLA) (2 × 10^8) and with/without catalase (50µg/ml) for 1 hour at 37°C and then transferred to paraformaldehyde-fixed HeLa cells monolayers for an additional hour of incubation at 37°C. Trophozoites unattached to HeLa cells monolayers were washed once with Phosphate Buffered Saline (PBS) buffer and the trophozoites attached to the HeLa cells monolayer were eluted with 500 µl of a solution of cold galactose (1%) in PBS and counted.

3. Results

3.1 *L. acidophilus* amebicide activity depends on the formation of H_2O_2

The ability of *L. acidophilus* to produce H_2O_2 was measured by the FOX assay. We found that overnight culture of *L. acidophilus* cultivated in MRS media with agitation produces 0.14 ±0.3 mM H_2O_2. We determined the viability of *E. histolytica* trophozoites incubated with *L. acidophilus* or with heat-killed *L. acidophilus*. The viability of *E. histolytica* trophozoites was not affected when the parasite was incubated with *L. acidophilus* for 60 min (Fig 1). However, the viability of *E. histolytica* trophozoites was significantly decreased by 50% when the parasite was incubated with *L. acidophilus* for 120 min. In contrast, the viability of *E. histolytica* trophozoites incubated with heat-killed *L. acidophilus* for 120 minutes was not impaired (Fig 1). Next, we wanted to establish if the amoebical activity of *L. acidophilus* was dependent on the formation of H_2O_2. We incubated *E. histolytica* and *L. acidophilus* in presence of catalase, an enzyme that catalyzes the decomposition of H_2O_2 to H_2O and O_2 [28]. We observed that the amoebicidal activity of *L. acidophilus* was strongly reduced when catalase was added during the incubation of *L. acidophilus* with the parasite (Fig 1). Based on this finding, it strongly suggests that H_2O_2 produced by *L.acidophilus* is the primary cause of parasite death.

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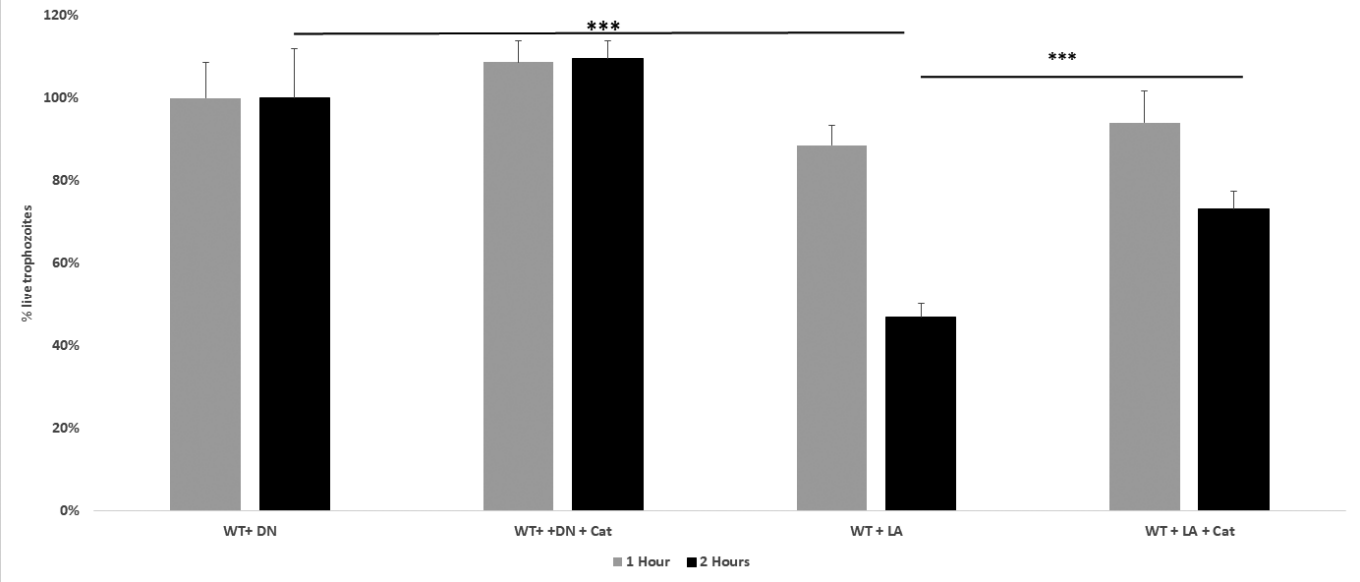

Figure 1: Viability assay of *E. histolytica* trophozoites
E. histolytica trophozoites were incubated with live *L. acidophilus* (LA) or with heat-killed *L. acidophilus* (DN), with/without catalase (50 µg/ml) for 60 and 120 min at 37°C. The data represent two independent experiments performed in triplicate. ***p value< 0.001 by an unpaired Student t test.

3.2 Resin-assisted capture (RAC) of oxidized proteins (OX) coupled to mass spectrometry (OX-RAC) analysis of *E. histolytica* trophozoites exposed to *L. acidophilus*

In order to explore the amebicidal properties of *L. acidophilus*, we used OX-RAC to measure the levels of oxidized proteins (OXs) in *E. histolytica* trophozoites exposed to *L. acidophilus*. In absence of DTT treatment, OXs are not expected to bind to the thiopropyl resin [29]. We observed that the level of OXs in *E. histolytica* trophozoites exposed to heat-killed *L. acidophilus* culture is very low (Fig 2A). These results indicate that heat-killed culture of *L. acidophilus* are not triggering the formation of OXs in *E. histolytica* trophozoites. In contrast, a strong level of OXs was detected in *E. histolytica* trophozoites exposed to live *L. acidophilus* culture (Fig 2A). The addition of catalase during the interaction of *E. histolytica* trophozoites with *L. acidophilus* strongly inhibits completely the formation of OXs in the parasite which confirms that the formation of OXs in the parasite is mediated by H_2O_2 produced by *L. acidophilus* (Fig 2B). These results indicates that the formation of OXs is triggered by H_2O_2 produced by *L. acidophilus*.

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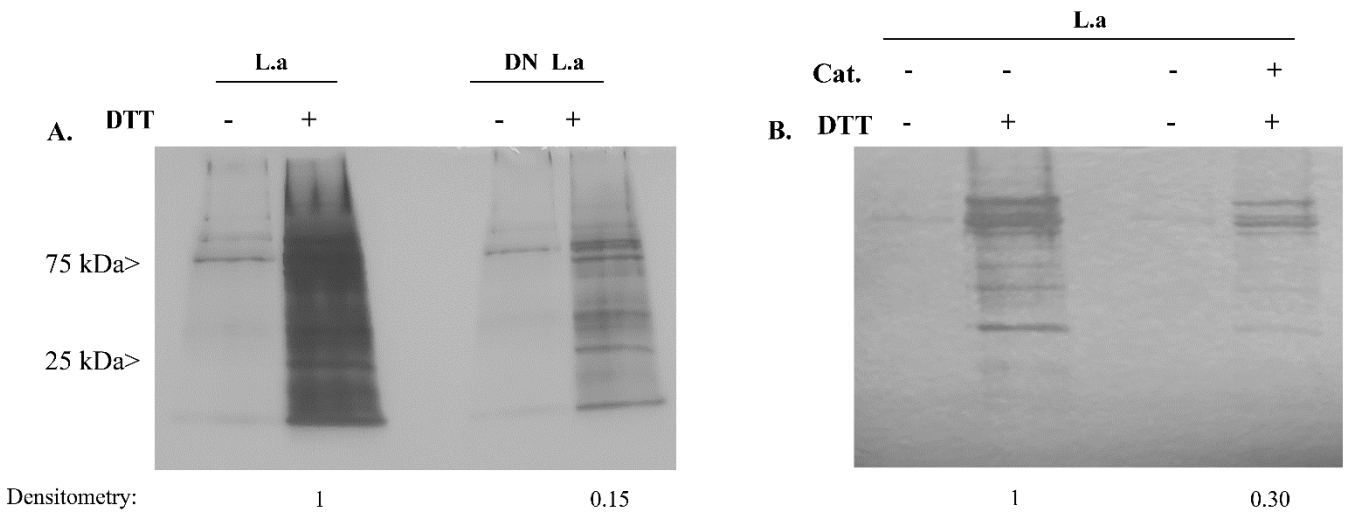

Figure 2: Detection of OXs by resin-assisted capture (OX-RAC) analysis of *E. histolytica*

E. histolytica trophozoites were incubated with live *L. acidophilus* (LA) or with heat-killed *L. acidophilus* (DN) (Fig 2A), with/without catalase (50 μg/ml) (Fig 2B) for 2 hours at 37°C. Total protein lysate was prepared by lysing the trophozoites with 1% Igepal in PBS. The oxidized proteins in the cell lysates were subjected to RAC in the presence of 10mM DTT (+DTT) or the absence of DTT (−DTT). The protein resolved on a 12% SDS-PAGE and stained with silver stain.

The intensity of the protein bands were quantified by densitometry using Image J software [67]. The intensity of the OX-protein bands obtained in presence of DTT in *E.histolytica* trophozoites incubated with live *L.acidophilus* was arbitrary set to 1.

Using MS, we identified 997 OXs in *E. histolytica* trophozoites incubated with *L. acidophilus* (Table S1), which were classified using PANTHER. The most abundant OX families belong to metabolite interconversion enzyme (PC00262), such as thioredoxin (EHI_004490), Protein arginine N-methyltransferase (EHI_158560) or the Galactose-specific adhesin 170kD subunit (EHI_042370), protein modifying enzyme (PC00260) such as cysteine proteinase CP5 (EHI_168240), serine/threonine-protein phosphatase (EHI_031240) or E3 ubiquitin-protein ligase (EHI_050540) and protein-binding activity modulator (PC00095) such as SERPIN domain-containing protein (EHI_119330), AIG1 family protein (EHI_176700) and Rho family GTPase (EHI_070730) (Fig 3A). Of the OXs in *E. histolytica* trophozoites incubated with *L. acidophilus* (Table S2), oxidoreductase (PC00176) and dehydrogenase (PC00092), such as glyceraldehyde-3-phosphate dehydrogenase (EHI_008200), NAD(FAD)-dependent dehydrogenase (EHI_099700), pyruvate:ferredoxin oxidoreductase (EHI_051060), vesicle coat protein (PC00235), such as GOLD domain-containing protein (EHI_023070), beta2-COP (EHI_088220) and coatomer subunit gamma (EHI_040700) and protease (PC00190) such as EhCP-a1 (EHI_074180) and EhCP-a4 (EHI_050570) are significantly enriched according to the PANTHER statistical overrepresentation test (Fig 3B).

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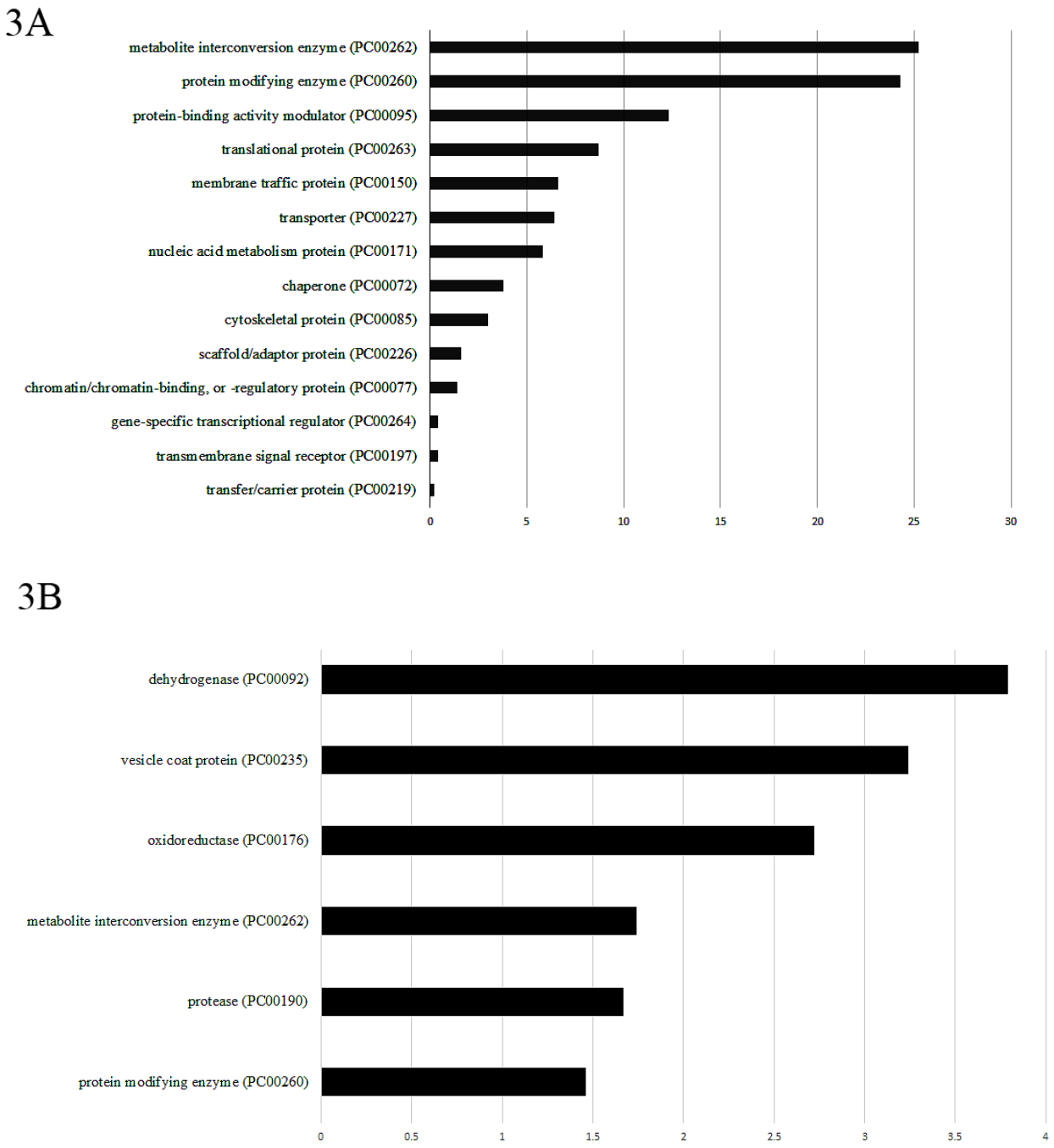

Figure 3. Protein ANalysis THrough Evolutionary Relationships (PANTHER) analysis of OXs in *E.histolytica* incubated with *L.acidophilus*

(A) PANTHER sequence classification of the OXs identified in *E. histolytica* trophozoites co-incubated with *L. acidophilus*.

(B) PANTHER statistical overrepresentation test of the OXs identified in *E. histolytica* trophozoites incubated with *L. acidophilus.*

In order to gain information on the consequence of *L.acidophilus*-mediated-oxidation on the activity of proteins that were identified in the OX-RAC analysis, we decided to focus here on the CPs. When trophozoites are incubated with live *L.acidophilus*, CPs activity is

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strongly inhibited (Fig 4). However, this activity is not inhibited when trophozoites are incubated with *L. acidophilus* in presence of catalase (Fig 4). Addition of DTT in lysates of trophozoites incubated with live *L.acidophilus* partially restored CP activity. Based on these results, it could be assumed that the *L.acidophilus*-mediated-oxidation of CPs' catalytic cysteine residues inhibits CPs, while their reduction by DTT restores the activity. Indeed, the fact that adding catalase to trophozoites incubated with *L. acidophilus* prevents the inhibition of CPs confirms that H_2O_2 produced by *L.acidophilus* inhibits the CPs.

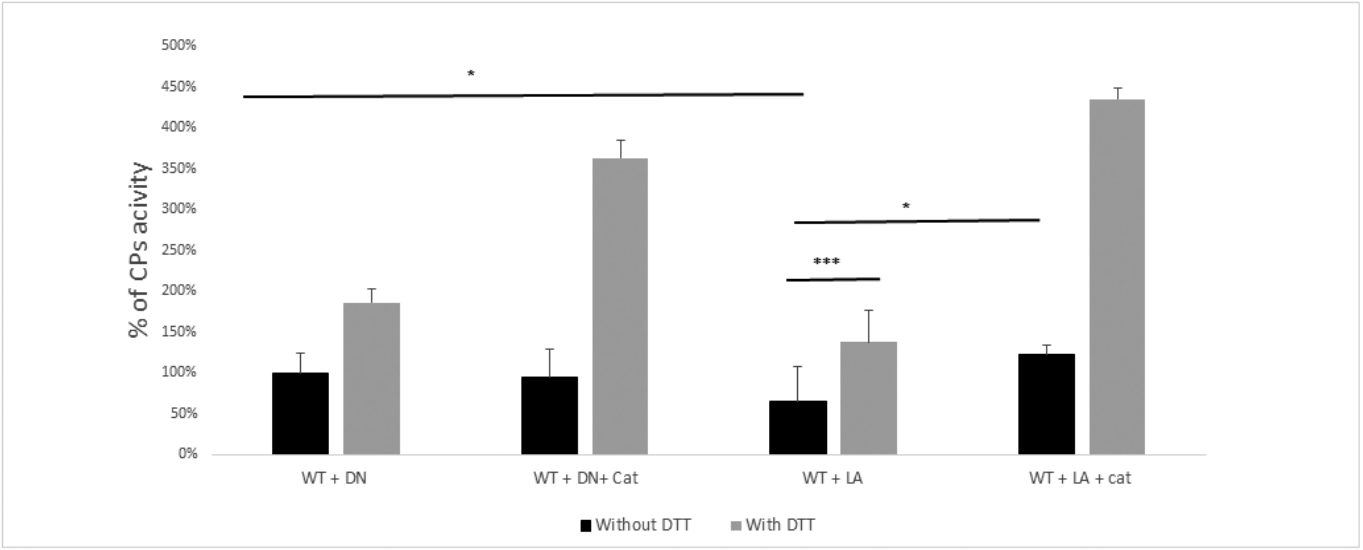

Figure 4: CPs activity of *E. histolytica* trophozoites

E. histolytica trophozoites were incubated with heat-killed *L. acidophilus* (DN) or with live *L. acidophilus* (LA), and with/without catalase (50µg/ml) for 2 hours at 37°C. Total protein was prepared and CPs activity was measured. One unit of CP activity was defined as the number of micromoles of substrate digested per minute per milligram of protein. CP activity of *E.histolytica* trophozoites incubated with heat-killed *L. acidophilus* (WT + DN) was taken as 100% and it corresponds to 0.31 units. The data represent two independent experiment performed in triplicate. *p value<0.05 by an unpaired Student t test. ***p value<0.001 by an unpaired Student t test.

3.3 **Adhesion of *E.histolytica* trophozoites to HeLa cells is impaired by *L.acidophilus***

E.histolytica trophozoites' ability to bind to mammalian cells is the initial step of the amebic infectious process [30]. In our experiment, trophozoites incubated with *L. acidophilus* exhibit, reduced binding to HeLa cells compared to trophozoites incubated with heat-killed *L. acidophilus* or with paraformaldehyde-fixed *L. acidophilus*. However, the binding activity to HeLa cells of trophozoites incubated with *L. acidophilus* in presence of catalase is comparable to the binding activity of heat-killed L. acidophilus or with paraformaldehyde-fixed *L. acidophilus* (Fig 5). These data strongly suggest that the production of H_2O_2 by *L.acidophilus* inhibits *E.histolytica*'s binding to HeLa cells rather than a competition between *L.acidophilus* and HeLa cells.

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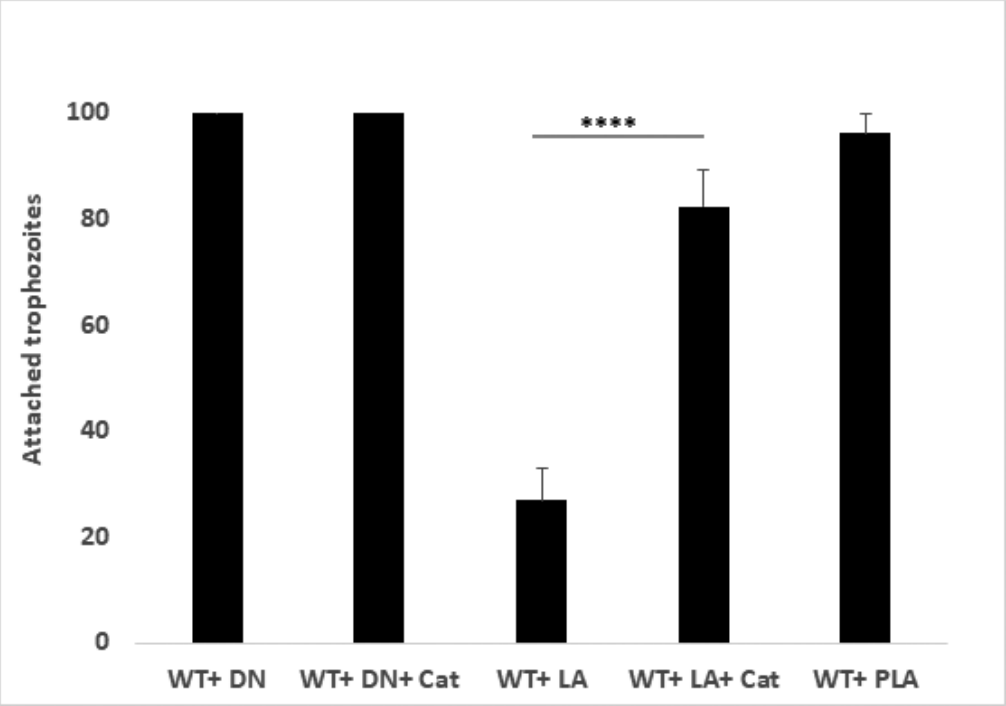

Figure 5. Binding activity assay of *E. histolytica* trophozoites
E. histolytica trophozoites were incubated with live *L. acidophilus* (LA), with heat-killed *L. acidophilus* (DN), with paraformaldehyde-fixed *L.acidophilus* (PLA) and with/without catalase (50µg/ml) for 1 hour at 37°C and then transferred to paraformaldehyde-fixed HeLa cells monolayers. Trophozoites attached to HeLa cells monolayers were counted. The number of trophozoites incubated with heat-killed *L. acidophilus* (WT + DN) that were bound to HeLa cells monolayer (around 75% of the original population) was taken as 100%. The data represent two independent experiments performed in duplicate. ****p value<0.0001 by an unpaired Student t test.

4. Discussion

L. acidophilus is commonly found in the gastrointestinal tract of healthy humans. It is widely used as food preservative and as a probiotic. *L. acidophilus* antimicrobial activity is caused by the production of antimicrobial peptides such as such as lactacins B, organic acids production such as lactic acids and H_2O_2 (recently reviewed in [31]) and immune induction [32]. Whereas the antibacterial and antifungal activity [33,34] of *L. acidophilus* has been well illustrated, the antiparasitic properties of *L. acidophilus* has been less studied. *L. acidophilus* in combination with other probiotics is beneficial in the prevention and treatment of *Giardia lamblia* infection in mice [35], *Toxocara canis* [36], *Trichinella spiralis* [37] and *Cryptosporidium parvum* [38]. In a recent work, we have demonstrated that *L. acidophilus* is detrimental to *E. histolytica* but the amebicide mechanism was unknown [19]. Our work suggests that the formation of H_2O_2 by *L. acidophilus* directly contributes to the amebicide activity of the parasite and the reduction of its cytopathic activity. Numerous OXs identified in this study play an important role in the parasite's biology, supporting this conclusion. Some of these OXs will be discussed here.

Important metabolic enzymes are OXs in the parasite exposed to *L.acidophilus*. Among these proteins is the pyruvate:ferredoxin oxidoreductase (EHI_051060), an Fe-S enzyme which catalyzes the oxidative decarboxylation of pyruvate [39]. This protein has also been identified as an OX in trophozoites exposed to H_2O_2 [6], metronidazole or auranofin [40]. In an oxidatively stressed parasite, pyruvate:ferredoxin oxidoreductase

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becomes strongly inhibited, resulting in an accumulation of pyruvate, which limits ATP production and causes parasite death [41]. Several cysteine residues present within the [4Fe-4S] clusters of close to them are carbamidomethylated suggesting that they are oxidized (Table S2). Destabilization of the Fe-S clusters integrity via oxidation of these cysteine residues in the parasite exposed to *L.acidophilus* will more certainly inactivate the enzyme and consequently contribute to the parasite death.

E. histolytica lacks glutathione, so it relies mainly on thiol for its defense against OS [42]. Thioredoxin (TRX) / Thioredoxin reductase (TRXR) also contributes to redox signaling in *E.histolytica* trophozoites as well as oxidative stress responses [43]). This ubiquitous mechanism of defense is present in many parasites including *Schistosoma mansoni*, *Plasmodium falciparum*, *Giardia lamblia*, and *Trichomonas vaginalis* [43]. TRXs are small redox proteins of around 12kD which act as radical scavengers. In their active site, two cysteine residues are involved in the antioxidant system. The oxidation of these cysteine residues produces disulfide bonds, which will be reduced by TRXR. The presence of TRXs and TRX as OXs in *E.histolytica* exposed to *L.acidophilus* strongly suggests that the parasite is actively responding to H_2O_2 released by the bacteria.

The lectin Gal/GalNAc is essential for parasite attachment to mammalian cells and therefore, for parasite cytopathic activity [44-46]. We previously demonstrated that oxidation of the carbohydrate-recognizing cysteine rich domain (CRD) of Gal/GalNAc lectin renders it inactive [6]. We observed in this study that 170kDa Gal/GalNAc is one of the OXs produced in the parasite exposed to *L. acidophilus*. According to the MS analysis of OXs (Table S2), many cysteine residues are carbamidomethylated in the CRD of Gal/GalNAc lectin, which strongly suggests that they were oxidized leading to an impairment of the parasite's ability to bind mammalian cells (this work and [6]).

CPs are essential for the growth of *E.histolytica* trophozoites and their inhibition by inhibitors of the CPS, such as E64d, causes their death [47]. In this study, we found that many CPs including EhCP-a1 (EHI_074180), EhCP-a4 (EHI_050570), EhCP-a5 (EHI_168240) and EhCP8 (EHI_010850) are oxidized, and that *E.histolytica* CPs activity are inhibited when the parasite is incubated with *L.acidophilus*. Some of these OXs CPs, such as EhCP-A1 and EhCP-A5, are highly expressed in *E.histolytica* [48] and are involved in rosette formation, haemolysis, and erythrocyte digestion [49]. The expression of EHI_010850 (EhCP-8) is upregulated when the parasite is incubated in the presence of hemoglobin, which suggests CP-8 is involved in iron uptake by the parasite. [50]. The mechanisms that lead to oxidants inhibiting CPs have recently been examined [51]. For example, inhibition of papain by H_2O_2 results from the formation of sulfenic acid which reacts with adjacent free thiol to form mixed disulfides. Also, H_2O_2 inhibits cathepsin B by targeting the active site residue (Cys25) to form either sulfenic acid or sulfonic acid around 70% of the time. *E.histolytica* CPs contain four active-site residues, namely Gln, Cys, His, and Asn, the cysteine residue at the active site being present in all *E.histolytica* CPs [52]. According to the MS analysis of OXs (Table S2), this cysteine residue in the active site is carbamidomethylated, which strongly suggests that it was oxidized. By itself, this observation would explain why *E.histolytica*'s CP activity is inhibited by H_2O_2 produced by *L.acidophilus*. As opposed to *E.histolytica*, where H_2O_2 produced by *L.acidophilus* appears to inhibit CPs activity directly, in *Plasmodium* parasites, H_2O_2 mediated inhibition of CPs is dependent on the presence of free hemin, which can be released by quinoline drugs [53].

A functional motility is critical to the survival of *E. histolytica* in order to both dislodge and phagocytose host cells as well as transport virulence factors intracellularly [54]. Rho GTPases play a critical role in the regulation of motility and phagocytic activity of *E.histolytica* [55]. There are several Rho GTPases present in the parasite, and we identified six of them (EHI_126310, EHI_013260, EHI_197840, EHI_029020, EHI_129750, EHI_070730) as OXs. EhRho1 (EHI_029020) regulates phagocytosis by regulating actin polymerization [56]. Numerous studies have shown that ROS regulate Rho GTPases activity [57]. Many Rho family GTPases contain at their N-terminal a cysteine-containing motif (GXXXXGK[S/T]C) which is located directly adjacent to the phosphoryl-binding loop. Oxidation of the cysteine residue in this motif affects the nucleotide binding properties of

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these Rho GTPases [57]. According to the MS analysis of OXs (Table S2), this cysteine residue in the active site is not carbamidomethylated. Instead, we found that cysteine residues located at the C-terminal of these Rho GTPases are carbamidomethylated (Table S2). A ubiquitination region is present in the C-terminal region of many Rho GTPases that may regulate their stability [58]. In light of this information, it is tempting to speculate that the stability of these Rho GTPases is redox-dependent. An example of such regulation occurring in human endothelial cells is described here [59].

Inhibitors of serine proteinases (serpins) control a broad range of biological processes, including pathogen evasion of the host defense system. Cathepsin G, a pro-inflammatory enzyme released by activated neutrophils, is inhibited by serpins [60]. *E.histolytica* expresses a SERPIN that interacts with human neutrophil cathepsin G [61]. In this work, we showed that EhSERPIN is one of the OXs present in *E.histolytica* exposed to *L. acidophilus*. Studies have suggested that SERPINs are redox-regulated by oxidation of cysteine residues in the reactive site loop of these enzymes or its vicinity [62-64]. The presence of carbamidomethylated cysteine residues in the vicinity of the reactive site loop of EhSERPIN (Table S2)[61] suggests that EhSERPIN is also redox-regulated. The effect of oxidation on EhSERPIN activity has yet to be determined.

5. Conclusions

The results of this study show that the production of H_2O_2 by *L. acidophilus* causes oxidation of vital proteins in *E. histolytica* and ultimately results in parasite death. In combination with innate immunity [65,66], these promising properties could lead to the prevention and/or complete elimination of *E.histolytica* infection. However, in-vivo trials are necessary to determine whether these probiotics have health benefits on humans.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1

Table S1: List of all OXs that were enriched by RAC in three independent experiments in *E.histolytica* trophozoites incubated with *L.acidophilus*.

Table S2: List Carbamidomethyl (C)Sites in OXs

Table S3: Description of the parameters that are given in Table S1 and Table S2.

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