Parasitology

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Vitamin supplementation increases the virulence of *Entamoeba histolytica* grown axenically

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Research Article

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Abstract

As a consequence of axenic growth and the elimination of accompanying bacterial flora, *Entamoeba histolytica* virulence decreases rapidly, and pathogenicity is lost. This paper evaluated the impact of vitamin supplementation on the pathogenicity of *E. histolytica*. Growth of *E. histolytica* trophozoites, cultured axenically in PEHPS (a Spanish acronym for the main ingredients – casein peptone, liver, pancreas extract and bovine serum) medium, with or without vitamins, exhibited a similar growth rate. However, the vitamin-enriched PEHPS preparations expressed 2.65 times more haemolytic activity (at 60 min: 98 *vs* 48%, $P < 0.05$), 2.5 times more phospholipase A_2 activity at 150 min of incubation and generated more hepatic abscesses (88 *vs* 60%, $P = 0.05$) than the preparations without vitamins. The haemolytic and phospholipase A_2 activity for the PEHPS – V preparations were restored following vitamin supplementation with A and D. These data highlight, for the first time, that vitamins and specifically vitamin A and D were essential for the recovery of amoebic virulence, lost through axenic growth.

Introduction

Entamoeba histolytica amoebiasis is the third cause of death worldwide, due to a parasitic infection, has a universal distribution affecting 10–20% of the world population, and in some regions, up to 55% (Conde-Bonfil and De la Mora-Zerpa, 1992; Marie and Petri, 2014). Since the introduction of media for cultivation of amoebas in the 70s, research into *E. histolytica* has increased significantly. However, axenization is a long and laborious procedure that involves the gradual adaptation of this parasite to a new way of life (Diamond, 1968; Diamond *et al.*, 1978; Clark and Diamond, 2002).

The metabolism of *E. histolytica* requires many essential nutrients from exogenous sources, including most amino acids, nucleic acids, lipids, vitamins (Marie and Petri, 2014), mammalian blood, tissue extract or serum are common components of mediums used to grow *E. histolytica* axenically. Furthermore, TPS-1, TYIS-33 and PEHPS cultivation medium are the most widely used media (Diamond, 1968; Diamond *et al.*, 1978; Said Fernandez *et al.*, 1988). While Diamond vitamin-Tween 80 mixture© (SAFC Biosciences Inc., Lenexa, KS, USA) was routinely added to the media as a supplement, it has been reported that they are not essential for the growth of *E. histolytica* (Mata-Cardenas and Said-Fernandez, 1990)

The virulence of *E. histolytica* is dependent on multiple factors that determine the capacity of this parasite to damage the host (Marie and Petri, 2014). Furthermore, if the accompanying bacterial flora is eliminated, the virulence of *E. histolytica* decreases rapidly; therefore, some strains that have been grown axenically for many years have lost their pathogenicity (Bos and Van de Griend, 1977; Costa *et al.*, 2006). Moreover, it has been suggested that nutritional modifications may alter the motility, and expression of the virulence factors for this parasite (Marie and Petri, 2014; Gastelum-Martínez *et al.*, 2018). The objective of the current study was to evaluate the impact of vitamin supplementation on the pathogenicity of *E. histolytica* grown axenically.

Material and methods

Protein determination was *via* the Lowry method (Waterborg, 2002); haemolysis, phospholipase A_2 and amoebic hepatic abscess assays were performed on amoebas grown in culture with

or without vitamins. Furthermore, the initial assays were repeated, adding only vitamins A and D to the culture media.

Amoebas

According to the standard protocols described by Said Fernandez *et al.* (1988), axenic cultures of *E. histolytica* strain HMI:IMSS were maintained in PEHPS medium, and used to prepare cultures with vitamins (PEHPS + V) and without (PEHPS − V). PEHPS + V was prepared by adding 250 μL of Diamond vitamin-Tween 80 mixture© (SAFC Biosciences Inc., Lenexa, KS, USA) for each 10 mL of culture media; PEHPS − V was prepared *via* the addition of 250 μL Hank's Balanced Salt Solution (HBSS) that contained 0.7 mM $CaCl_2$, 5.5 mM glucose, 120 mM NaCl, 5.3 mM KCl, 1.7 mM $MgSO_4$, 1 mM Trizma base (Sigma Chemical Co., St. Louis, MO, USA) pH 8.0, and then the media was adjusted to 300 mOsm kg^{-1} with NaCl. Growth curves were built, which allowed us to calculate an equation for the exponential phase, determine the time of duplication, and the growth rate. To prepare culture media only with vitamins A and D, 100 ng of ergocalciferol (Spectrum Chemicals, Gardena, CA, USA), and 100 ng of vitamin A palmitate (Spectrum Chemicals, Gardena, CA, USA) were added to each 5.5 mL of the PEHPS − V. Initial experiments were carried out to evaluate the impact of HBSS on the growth and haemolytic or phospholipase A_2 activities of the amoeba; however, no significant effects were found (data not shown).

Amoeba mass culture and subcellular fraction separation

Spinner flasks (Bellco Glass Inc., Vineland, NJ, USA) containing 600 mL of PEHPS + V or PEHPS − V were inoculated with 5×10^3 trophozoites and incubated with constant agitation, at 37°C, for 3 days. The subcellular fraction was obtained after subsequent steps of centrifugation and homogenization at 4°C; the first amoeba cultures growing in logarithmic phase were chilled and centrifuged at $600 \times \boldsymbol{g}$ for 10 min. The trophozoites were re-suspended in 2 volumes of cold HBSS and disrupted with an Elvehjem-Potter homogenizer (Bellco Glass Inc., Vineceland, NJ, USA). The homogenate was centrifuged again at $135 \times \boldsymbol{g}$ for 15 min, then the supernatant was collected and centrifuged at $30\,000 \times \boldsymbol{g}$ for 30 min, the resultant pellet corresponded to the subcellular fraction (P30), and was separated and resuspended in 2 volumes of HSSB and then stored at −70°C.

Haemolysis assays

The haemolytic activity of *E. histolytica* was assayed as described by Said-Fernández and López-Revilla (1982), briefly, 25 μL of the 2% *Sprague-Dawley* rat erythrocyte suspension (300 mOsm kg^{-1}) was mixed with 25 μL of P30 (150 μg) and incubated at 37°C. The 100% haemolysis control corresponded to erythrocyte suspension mixed only with 25 µl of double-distilled water, and the 0% haemolysis control corresponded to erythrocyte suspension mixed only with 25 µl HBSS. Then chilled to 4°C, and 1 mL of PBS was added to each tube, and then centrifuged at $600 \times \boldsymbol{g}$ for 5 min. The absorbance was determined at 415 nm with a spectrophotometer (PMQ III, model MM3 Zeiss, Germany). The percentage of haemolysis was determined with the following equation:

$$\%\,\mathrm{He} = \frac{\mathrm{ExHR}-\mathrm{SHR}}{\mathrm{MHR}-\mathrm{SHR}} \times 100$$

where '%He' represents the haemolysis percentage, 'ExHR' the experimental haemoglobin release, 'SHR' the spontaneous haemoglobin release in the mixture with HBSS and 'MHR', the maximum amount of haemoglobin released into the mixture with added double-distilled water.

The incubation time effect was assessed measuring haemolysis of P30 (25 μL that contained 150 μg proteins) every 10 min (0–60 min). The dose–response curves contained the assayed P30 doses (having 0–150 μg proteins).

The %He was plotted as a function of the P30 protein concentration and the haemolytic dose of proteins from the P30 – that were required to produce 50% haemolysis (DH_{50}) in the above-described assay mixtures – were identified with these dose–response curves. The haemolysis mixtures were incubated for 1 h at pH 8.0.

Phospholipase A_2 assays

The measurement of phospholipase A_2 was based on the methods described by Vargas-Villarreal *et al.* (1995) with the following modifications. Briefly, 1.5 mL of the borosilicate cone-bottom vials (Bellco Glass Inc., Vineceland, NJ, USA) were mixed with 1.0 mL of 100 mM Tris-HCl (pH 8.0); 1 mM $CaCl_2$; 2% Triton X-100; 0.27 mM phosphorylcholine and 4 μCi of 1,2 dipalmitoyl-[2-palmitoyl-1-^{14}C]-phosphatidylcholine (112 mCi $mmol^{-1}$), (New England Nuclear, Boston, MA, USA), then sonication was applied to the mixture with an Ultratip Labsonic System (Lab-Line Instrument Inc., Melrose Park, IL, USA), at 40 W for 60 s. This substrate preparation was divided into 0.5 mL aliquots and then stored in vials at −70°C until use.

The assays were performed by mixing 10 μL of the above-mentioned substrate preparation with 10 μL of P30 PEHPS + V or PEHPS − V (containing 0–150 μg of total proteins). Following 60 min of incubation at 37°C, the phospholipid hydrolysis was stopped by adding 25 μL of a solution that contained 1 mg mL^{-1} phosphatidylcholine (PC), 1 mg mL^{-1} egg-yolks lysophosphatidylcholine (LPC) and 1 mg mL^{-1} rat-live total free fatty acids (FFA) (Sigma Chemical Co., St. Louis, MO, USA) in 5% trichloroacetic acid-*n*-butanol.

PC, LPC and FFA from the assay mixtures were separated by thin-layer chromatography

Forty-five microliters, of the assay mixture, was applied to 10 × 10 cm silica-gel plates (0.25 mm thickness, 60-mesh; Merck, Darmstadt, Germany). The plates were placed in a thin-layer chromatography (TLC) tank with chloroform, methanol, acetic acid and water (140:40:16:8, by vol.). Lipid spots were developed by exposing the TLC plates to iodine vapours. The identification of the PC, LPC and FFA, spots were compared with their appearance and the respective relative migration coefficients (R_f PC, 0.43; LPC, 0.185 and PA, 0.908) with the corresponding standards (Sigma Chemical Co., St. Louis, MO, USA). The lipid spots were scraped from the TLC silica gel plates and deposited into plastic vials containing 5 mL scintillation liquid (BCS, Biodegradable Counting Scintillation; Amersham International Corporation, Buckinghamshire, England) and the disintegrations per minute (dpm) for each of these was determined with a 1600 Tri-Carb liquid scintillation spectrometer (Packard Instrument Company Inc., Downers Grover, IL, USA) and adjusted to work with the unquenched samples at 96% efficiency.

One unit of PLA activity was defined as 1 ρM of [^{14}C]-palmitic acid hydrolysed (equivalent to the amount of ρM of [^{14}C]-palmitic acid released) during 1 h of incubation. The specific activity was given as the number of units of PLA activity per mg of total protein in the P30 amoeba extracts.

Amoebic hepatic abscess assay

Experiments were performed on 50 male golden hamsters (*Mesacricetus auratus*), 1-month-old, weighing between 50 and

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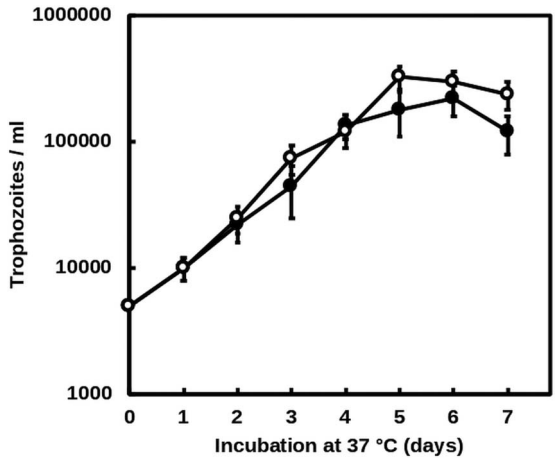

Fig. 1. HMI: IMSS amoebae growth in PEHPS + V and PEHPS − V medium at 37°C for 7 days incubation; each point corresponds to arithmetic average from three determinations, bars represent standard deviation. ○, PEHPS culture media with vitamins. •, PEHPS culture media without vitamins ($P < 0.05$, PEHPS + V *vs* PEHPS − V).

70 g. Animals were randomly allocated into two groups ($n = 25$). One group (PEHPS + V) received amoebas cultivated on PEHPS + V, and the second received amoebas without vitamins (PEHPS − V). The animals were given anaesthesia with sodium pentobarbital proportionated by intraperitoneal injection of 63 mg kg^{-1} (Anestesal, Smith Kline & French, México, México). Then a laparotomy was performed for each hamster under aseptic conditions; the left hepatic lobe was exposed and inoculated with 1×10^6 trophozoites suspended in fresh PEHPS medium (0.1 mL), finally the abdominal wall was sutured, and the animals were allowed to recuperate under the standard conditions of stable room temperature (24 ± 3°C), 12 h light/12 h dark cycles, with access to commercial rodent pellets and water *ad libitum* for 7 days, after which they were sacrificed under an anaesthetic. The liver lesions were dissected, and the presence of amoebae along with their effect on the liver was recorded. Briefly, small portions of liver were removed, minced and placed in culture tubes containing 11 mL of PEHPS medium, sodium penicillin G (200 units mL^{-1}) and streptomycin (50 μg mL^{-1}); the preparations were incubated at 37°C for 4 days and observed with an inverted microscope to detect the presence of trophozoites. Furthermore, we recorded the percentage of animals that had developed amoebic abscesses.

All animal procedures were performed in accordance with the guiding principles for the production, care and use of laboratory animals of the Mexican Official Norm (NOM-062-ZOO-1999, 2001), and the protocols used in this study were approved by the ethics committee of our institution.

Statistics

All of the experiments were performed in triplicate, and the arithmetic average of each determination was used to build plots, of the growth, dose, haemolysis and PLA activity; they were analysed by linear regression. The differences were identified by Mann–Whitney *U* test. The percentage of animals that developed amoebic abscesses was compared using the χ^2 test. For all *P* values < 0.05 were considered statistically significant.

Results

Growth of the *E. histolytica* trophozoites cultured with or without vitamins showed a similar growth rate (Fig. 1). The following equations were used to define the exponential growth phase

$$\text{PEHPS} + \text{Vamoebas/mL} = 0.04974 \times (120) + 7.00836, (r^2 = 0.987)$$

$$\text{PEHPS-Vamoebas/mL} = 0.04302 \times (120) + 6.9592, (r^2 = 0.983)$$

PEHPS + V the doubling time was 8.75 h; the generation time, 13.7 h and the growth rate, 0.049 h^{-1}. **PEHPS − V** the doubling time was 8.11 h, the generation time, 14.79 h, and the growth rate, 0.043 h^{-1} (PEHPS + V *vs* PEHPS − V, $P > 0.05$).

Effect of vitamins on the haemolytic activity

P30 obtained from PHEPS + V and PEHPS − V were capable of lysing *Sprague-Dawley* rat erythrocytes at pH 8.0. Haemolysis increased as a function of incubation time (Fig. 2A) and the P30 protein concentration (Fig. 2B). The time–haemolytic activity curve showed a more pronounced steep slope between 0 and 10 min than that between 10 and 60 min of incubation ($r^2 = 0.99$). For PEHPS + V, DH_{50} was 53.5 μg, and to PEHPS − V 130 μg. PEHPS + V preparations exhibited 2.65 times more haemolytic activity at 60 min than the PEHPS − V (98 *vs.* 48%, $P < 0.05$). This haemolytic activity was restored after supplementation with A and D vitamins (Fig. 2A and B).

Phospholipase A_2 activity

P30 fraction obtained from PHEPS + V and PEHPS − V increased the levels of [^{14}C]-LPC as a function of incubation time, and the amount of P30 tested. Following incubation for 30 min, the P30 obtained from PEHPS + V showed significantly more phospholipase A_2 activity than the P30 for the amoebas grown without vitamins. Moreover, after 150 min of incubation, the amount was 2.5 times greater. The phospholipase A_2 activity lost by the absence of vitamins was restored following A and D vitamin supplementation (Fig. 3A and B). The phospholipase A_2 activity was similar for the inoculum under 150 μg. However, above this amount, the activity was greater for P30 from the culture media with vitamins (Fig. 3B).

Hepatic abscesses

Amoebae grown on PEHPS + V produced liver abscesses that averaged 2.8 cm by 2 cm, while the abscesses produced by amoebas grown on PEHPS − V were 0.85 cm by 0.60 cm long. Interestingly, 88% ($n = 22$) of the hamsters inoculated with trophozoites from PEHPS + V cultures developed abscesses, while abscesses occurred in only 60% ($n = 15$) of the amoebas grown without vitamins (χ^2: $P < 0.05$).

Discussion

In this article, the effect of vitamins on the growth and virulence of amoebas grown in the PEHPS medium was evaluated. This study found that amoebic growth was not dependent on the addition of vitamins to the culture medium. However, vitamins A and D were found to be essential for the recovery of virulence lost by axenic growth, increasing the ability to form liver abscesses, and haemolytic or phospholipase A_2 activity.

Amoebae are unicellular eukaryotes that consume microbial prey through phagocytosis, many species of amoeba can be cultivated axenically in rich media or monoxenically with a single bacterial prey species (Ma *et al.*, 2017). Currently, at least 13 vitamins

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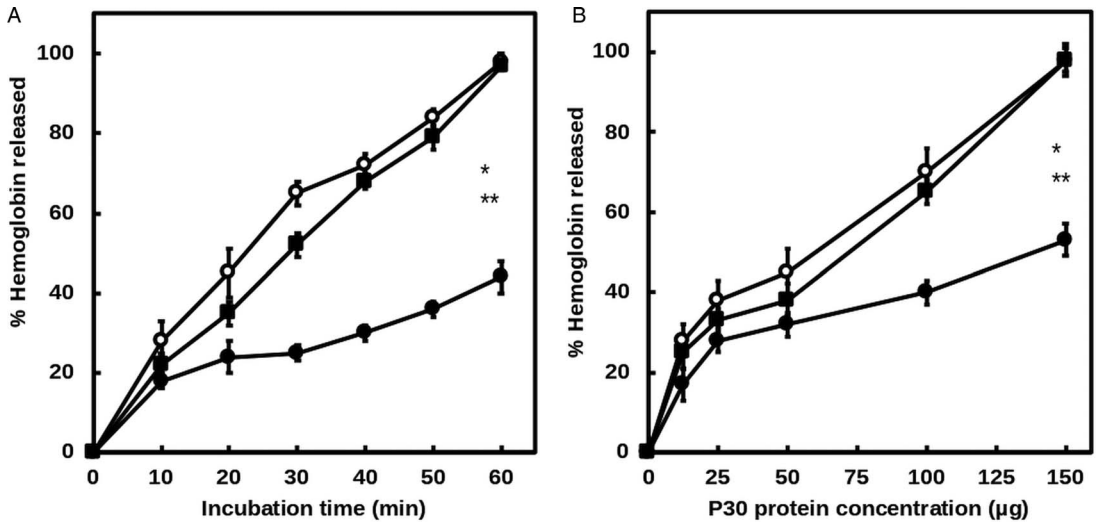

Fig. 2. Haemolytic activity of HMI: IMSS amoebae. (A) Time-related haemolytic activity of 150 μg of P30; (B) P30 protein concentration-related haemolytic activity at 60 mins of incubation. Each point corresponds to arithmetic average from three determinations, bars represent standard deviation. ○, PEHPS culture media with vitamins. •, PEHPS culture media without vitamins. ■, PEHPS culture media supplemented only with A and D vitamins. *$P < 0.05$, PEHPS + V *vs* PEHPS – V. **$P < 0.05$, supplemented with A and D vitamins *vs* PEHPS – V.

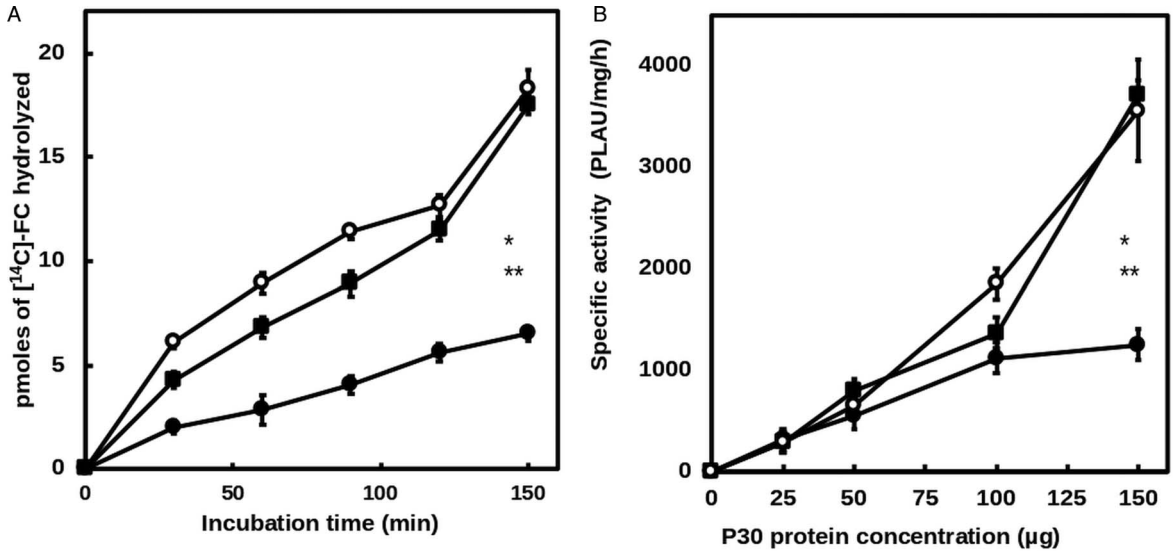

Fig. 3. Phospholipase A_2 activity of HMI: IMSS amoebae; (A) Time-related phospholipase A_2 activity of 150 μg of P30; (B) P30 protein concentration-related phospholipase A_2 activity at 150 min of incubation. Each point corresponds to arithmetic average from three determinations, bars represent standard deviation. ○, PEHPS culture media with vitamins. •, PEHPS culture media without vitamins. ■, PEHPS culture media supplemented only with A and D vitamins. *$P < 0.05$, PEHPS + V *vs* PEHPS – V. **$P < 0.05$, supplemented with A and D vitamins *vs* PEHPS – V.

have been studied in relation to amoebas, some are added to the culture media as antioxidants or to maintain a hypoxic environment in the amoebic cultures. Some reports have described that the following are essential for sustained multiplication of *E. histolytica* iron, glucose, biotin, folic acid, niacinamide, pantothenate, pyridoxal, riboflavin, thiamine, cysteine, an ammonium moiety, bovine serum albumin, lipoprotein-cholesterol and casein peptone dialysate (Diamond and Cunnick, 1991). However, Mata-Cardenas and Said-Fernandez (1990) reported that they had cultivated *E. histolytica* without vitamins.

The loss of pathogenicity was reported as a consequence of axenic growth (Bos and Van de Griend, 1977), and can be recovered *in vitro*, by adding the following to the culture media cholesterol, bacteria, pieces of the hamster liver or *in vivo* with the development of the strain in the liver of hamsters (Bos and Van de Griend, 1977; Gomes *et al.*, 1993). Notably, the liver plays an important role in the transport and storage of fat-soluble vitamins (A, D, E, K) (Andrade *et al.*, 2018). Our results showed that the addition of vitamins A and D resulted in the recovery of virulence. In eukaryote cells, vitamins A and D triggered fundamental biological processes, such as growth arrest, differentiation, apoptosis, autophagy and lysosomal activity. This highly pleiotropic effect is primarily mediated by the combinatorial action of the nuclear receptors that modulate DNA transcription ("Vitamin A and lysosomes", 1966; Wu and Sun, 2011; Chatagnon *et al.*, 2015). These receptors had not yet been described in amoebas, and their lysosomes are different from those of other eukaryotic cells; however, a similar role could be attributed to the effects observed for *E. histolytica*.

In nucleated cells like those found in mammals, the restoration of vitamin A deficiency has a dramatic and rapid effect on genomic expression and stimulates protein synthesis (Omori and Chytil, 1982). Our results did not evaluate the amount of protein, but the increase of phospholipase A_2 and haemolytic activity

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suggested that this must be confirmed experimentally in the future. The virulence of an amoeba depends on adhesion, the secretion of proteolytic enzymes and pore-forming proteins (amebopore); phospholipase A and haemolysin III are considered as amebopores (Trejos-Suárez and Castaño-Osorio, 2009).

The vitamin D receptor, an ancient nuclear hormone receptor, was originally involved in the recognition and detoxification of xenobiotic marine biotoxins that exhibited planar sterol ring scaffolds, which were involved in both immunity and Ca^{++} regulation in some modern organisms (Newmark *et al*., 2017).

We showed that restoration of the capability to form hepatic abscesses in golden hamsters and the haemolytic and phospholipase A_2 activity were associated with vitamin A and D supplementation. This likely occurred because the amoeba, which is a protozoan, lacks a deposit or reserve of metabolites and vitamins essential to life, and therefore must absorb them from the media (Laughlin and Temesvari, 2005).

Thus, our results supported the hypothesis that the loss of virulence for *E. histolytica* could be attributed to a deficit of vitamins that can be restored *via* the addition of vitamins to the media.

Conclusion

Vitamins A and D were essential for the recovery of amoeba virulence, lost by axenic growth.

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Conflict of interest. None.

Ethical standards. Not applicable.

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