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Original Article

In vitro culture of parasitic stages of *Haemonchus contortus*

Cultivo *in vitro* de estádios parasitários de *Haemonchus contortus*

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Abstract

Haemonchus contortus is a constraint to sheep production. Seeking to reduce the use of hosts and produce parasitic stages in large-scale, a 42-day *in vitro* culture protocol of *H. contortus* third-stage larvae was optimized using Dulbecco's modified Eagle's medium (DMEM). In cell-free culture, larvae were maintained at 39.6°C, in acidic media (pH 6.1) for 3 or 6 days with Δ4-dafachronic acid followed by DMEM pH 7.4 supplemented or not with Fildes' reagent. In DMEM pH 7.4 at 37°C, supplementation with Caco-2 cells was compared to Fildes. On Day 14, fourth-stage larvae (L_4) development rates in acidic media supplemented (86.8–88.4%) or not (74.4–77.8%) with Fildes and in Caco-2 cell co-culture (92.6%) were similar, and superior to DMEM pH 7.4 with Fildes (0.0%). On Day 21, Caco-2 cell co-culture resulted in higher larvae differentiation (25.0%) and lower degeneration (13.9%) compared to acidic media (1.5–8.1% and 48.6–69.9%, respectively). This is the first report of prolonged *in vitro* culture of *H. contortus* larvae using commercial media in co-culture with Caco-2 cells. Although no progression to the adult stage, Caco-2 cell co-culture resulted in morphological differentiation of *H. contortus* L_4 and larval viability for up to 28 days.

Keywords: Fourth-stage larvae, prolonged *in vitro* culture, gastrointestinal nematodes, sheep, replacement of *in vivo* methods.

Resumo

Haemonchus contortus provoca grandes prejuízos à ovinocultura. Visando reduzir a utilização de ovinos hospedeiros e produzir estágios parasitários em larga escala, um protocolo para o cultivo *in vitro* por 42 dias de larvas de terceiro estádio de *H. contortus* foi realizado em meio Eagle, modificado por Dulbecco (DMEM). No cultivo sem células, as larvas foram mantidas a 39,6°C e incubadas em DMEM ácido (pH 6,1) por 3 ou 6 dias com 4Δ-ácido dafacrônico seguido por DMEM pH 7,4 suplementado ou não com reagente de Fildes. Em DMEM pH 7,4 a 37°C, a suplementação com células Caco-2 foi comparada à suplementação com Fildes. No Dia 14, as taxas de desenvolvimento até o quarto estádio larvar (L_4) foram similares em meio ácido sem células suplementado (86,8–88,4%) ou não (74,4–77,8%) com Fildes e em co-cultura com células Caco-2 (92,6%), e superiores ao desenvolvimento em DMEM pH 7,4 com Fildes (0,0%). No Dia 21, o co-cultivo com células Caco-2 resultou em maior diferenciação (25,0%) e menor degeneração (13,9%) das larvas em comparação ao meio ácido (1,5–8,1% e 48,6–69,9%, respectivamente). Este é o primeiro relato de cultivo *in vitro* prolongado de *H. contortus* em meio comercial em co-cultivo com células Caco-2. Apesar da ausência de progressão até o estágio adulto, o co-cultivo com células Caco-2 resultou em diferenciação de L_4 e manutenção da viabilidade das larvas de *H. contortus* por até 28 dias *in vitro*.

Palavras-chave: Larva de quarto estádio, cultivo *in vitro* de longa duração, nematoides gastrintestinais, ovinos, substituição de metodologias *in vivo*.

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Introduction

Sheep production provides meat, milk, and wool for consumers, takes advantage of areas unsuitable for other forms of agriculture (Sargison, 2012), and is predicted to increase in scenarios of global warming in South America (Seo et al., 2010). The challenges faced by small ruminant producers in tropical regions include adverse climate conditions, scarcity or competition for water and food resources, and a high prevalence of gastrointestinal nematodes (McManus et al., 2011). Gastrointestinal nematodes are responsible for annual losses estimated at AUD$ 369–436 million in Australia (Hosking et al., 2009; Emery et al., 2016) and US$ 107.5 million in Brazil (Chagas et al., 2022). Economic losses are usually secondary to health issues and productive deficits resulting from nutrient spoliation, anemia, reduced weight gain, and deaths triggered by parasites, mainly when ineffective anthelmintics are used in flocks (Miller et al., 2012).

Haemonchus contortus is the most pathogenic parasite in small ruminants in tropical and subtropical climates. *H. contortus*, a hematophagous nematode of the abomasum, can remove up to 30 µL of blood per day and has a short pre patency period of 18–21 days. In addition, each adult female can reproduce with four to eight males and shed 1,300 eggs per day (Redman et al., 2008; Emery et al., 2016; Naeem et al., 2021), resulting in high mutation rates and genetic diversity in this species (Gilleard, 2013; Doyle et al., 2018). Anthelmintic treatment is the main approach used to control nematodes in sheep to reduce production and economic losses. However, treatments select helminths with genetic polymorphisms that confer resistance, and this subsequently leads to treatment failure (Barnes et al., 1995).

Therefore, the development of alternative and sustainable measures to control gastrointestinal helminths in flocks is of great interest. Parasite cultivation *in vitro* could provide large-scale production of parasites across diverse life cycle stages, which may be useful for vaccine development (Preston et al., 2015; Naeem et al., 2021), parasitic crosses to map resistance-related polymorphisms (Redman et al., 2012; Niciura et al., 2019), *in vitro* assays of new anthelmintic products, and gene silencing (Kotze & Bagnall, 2006). In addition, parasite culture improves understanding of antigenic molecules in excretory and secretory products, which induce increased systemic humoral responses and protection against *H. contortus* (Arunkumar, 2012; Lu et al., 2021) and serve as potential targets for immune or chemical control (Gamble & Mansfield, 1996). Additionally, since current methods used to extract native proteins for immunization against *H. contortus* rely on host infection and slaughter (González-Sánchez et al., 2018), the development of *in vitro* methods is desirable.

The free-living *H. contortus* stages (eggs, L_1, L_2, and L_3) can be easily collected from sheep host feces or fecal cultures, whereas parasitic stages (L_3 in hosts, L_4, and adult L_5) are only recovered after the host has been slaughtered. An alternative host model for *H. contortus* growth, using immunosuppressed jirds, resulted in slower and incomplete development (Conder et al., 1992). Thus, the lack of a protocol for obtaining all life stages of *H. contortus* by *in vitro* culture (Rufener et al., 2009) or by using laboratory animals as hosts (Gilleard, 2013) leads to dependency on small ruminants and limits the development of new control strategies. Nevertheless, there is a global demand for research methods that replace, reduce, or refine the use of animals in experiments (Liebsch et al., 2011), and models need to be developed and adapted to ensure the replacement of *in vivo* methods by *in vitro* methods (Chagas, 2015; Shivam et al., 2021).

Studies investigating *H. contortus in vitro* development resulting in male and female adults were conducted in the 1980s, and rely on complex and homemade media (Stringfellow, 1984, 1986), but these proved difficult to replicate. Considering recent advances in culture techniques, attempts to improve culture conditions and achieve development of *H. contortus in vitro* without the use of sheep hosts are sought (Geary, 2016). Thus, to improve culture conditions and large-scale *in vitro* cultures of parasitic stages of *H. contortus*, the objective of this study was to optimize an *in vitro* protocol using commercial media to obtain larvae of a more advanced developmental stage than other studies have achieved, which could be maintained *in vitro* for longer periods than currently possible. We sought to evaluate *H. contortus in vitro* development, differentiation, and viability by comparing cell-free cultures to co-cultures with Caco-2 cells using different supplements, pH, temperatures, and atmospheres.

Material and Methods

Sheep hosts and parasitological tests

Two Santa Ines ewes were housed in barns with no access to pastures and fed corn silage *ad libitum*. Natural gastrointestinal nematode infections were eliminated using a combination of three anthelmintics (9.4 mg/kg levamisole, 20 mg/kg albendazole, and 2.5 mg/kg monepantel) for three consecutive days, according to a protocol

used to eliminate infection with multiresistant populations (Almeida et al., 2020). Nematode elimination was confirmed by fecal egg count (FEC) using the McMaster technique with a sensitivity of 50 eggs per gram (Ueno & Gonçalves, 1998) at 7 and 14 days after the last treatment. Ewes were experimentally infected by oral administration of 4,000 third-stage larvae (L_3) of the *H. contortus* Echevarria isolate, which is susceptible to albendazole, ivermectin, and levamisole (Echevarria et al., 1991). FEC was assessed weekly up to the detection of eggs in feces on Day 28. Feces were cultured in glasses (Roberts & O'Sullivan, 1950) at 27°C for 7 days, and *H. contortus* L_3 migrating into water were recovered, kept at 4°C and used for *in vitro* culture within 15 days.

H. contortus third-stage larvae (L_3) decontamination

To ensure decontamination prior to *in vitro* culture, *H. contortus* L_3 were washed five times by centrifugation (1,100 × g for 5 min) in saline solution (0.85% NaCl). Larvae were then exsheathed with sodium hypochlorite (0.15% for 5 min) followed by four washes in saline with 1% antibiotics-antimycotic (100 units penicillin, 0.1 mg streptomycin, and 0.25 µg amphotericin B; Sigma A5955). Approximately 1,000–5,000 larvae in 10 mL of media were transferred to 25 or 75 cm^2 flasks with non-treated (for cell-free suspension culture) or treated (for co-culture with cells) surfaces, and flasks were laid in an incubator. Sterile conditions were maintained by manipulation under laminar flow and media filtering through 0.22 µm PES membranes.

Media and *H. contortus* larvae *in vitro* culture

Based on a successful protocol for *H. contortus in vitro* culture (Stringfellow, 1986), several media (Dulbecco's modified Eagle's medium – DMEM, Earle's balanced salt solution – EBSS, and Medium 199), supplements (Fildes' reagent and Caco2-cells), pH (4.6, 6.1, and 7.4), temperatures (37.0 and 39.6°C), and atmospheric conditions (5% CO_2 in air; 10% CO_2 in air; and 85% N_2, 5% O_2, and 10% CO_2) were investigated.

H. contortus lack an endogenous pathway for heme biosynthesis (Toh et al., 2010); therefore, this compound needs to be provided in the media through the addition of hemoglobin, hemin, tissue extracts, or blood cell lysates (Bolla, 1979). Thus, two supplements were tested: Fildes' reagent, which is a pepsin-digested defibrinated sheep blood medium, and Caco-2 cells (human colorectal adenocarcinoma-derived intestinal epithelial cells). Furthermore, acidic media (or media without bicarbonate) was used considering that the pH in the sheep abomasum varies from 1 to 6 (Harder, 2016) and that pH 4–4.5 resulted in maximum egg production by *H. contortus* (Honde & Bueno, 1982). Additionally, larvae were incubated at 39.6°C to simulate sheep average temperature, which is 39.9°C in the rumen, 39.8°C in the aorta, 39.6°C in the rectum, and 39.1°C in the abomasum (Bailey et al., 1962; Sommerville, 1966). In addition, the CO_2 concentration in the incubator was set to 10% considering that CO_2 pressure (pCO_2) is higher in the rumen (380 mmHg), where *H. contortus* L_3 can remain for approximately 12 h after ingestion before moving to the abomasum (50 mmHg pCO_2) (Sommerville, 1966), and considering the beneficial effect of 40% CO_2 injected into the media (Sommerville, 1977).

Media used (composition presented in Supplementary Table 1) were DMEM (Sigma D5648) with or without 44 mM bicarbonate (Sigma S5761), EBSS (Sigma E7510) without bicarbonate, and Medium 199 (Sigma M5017) with 26.2 mM bicarbonate. For prolonged culture, media were variously supplemented with 1% antibiotics-antimycotic (100 units penicillin, 0.1 mg streptomycin, and 0.25 µg amphotericin B; Sigma A5955), 10% fetal calf serum (FCS; Cripion), 1 mM sodium pyruvate (Sigma P4562), 1% non-essential amino acids (MEM; Sigma M7145; Supplementary Table 1), 2 mM L-glutamine (Sigma G3126), and 10 mM HEPES (Sigma H4034) (Table 1). Every 7 days for up to 42 days, all media were removed by centrifugation and replaced with freshly prepared media. Each culture condition was tested once, and all combinations tested for prolonged culture are shown in Table 1.

Dafachronic acid (DA), a steroid hormone discovered in *Caenorhabditis elegans* (Motola et al., 2006), is upregulated in *H. contortus* L_3 in the first 24h following exsheathment (Ma et al., 2019) and exogenous supplementation of DA to *H. contortus* L_3 increased the percentage and speed of molting to L_4 (Ma et al., 2019; Marks et al., 2019). Therefore, in cell-free cultures, 2.3 µM Δ4-dafachronic acid (Δ4-DA; Cayman Chemicals 14100-1) was used for 6 days, and acidic media (EBSS pH 4.6 or DMEM without bicarbonate pH 6.1) were used for 3 or 6 days as follows: (1) acidic DMEM for 6 days, (2) acidic DMEM for 3 days followed by DMEM with bicarbonate (pH 7.4) for an additional 3 days, and (3) EBSS for 6 days. After 6 days, larvae cultured in conditions (1) and (2) were transferred to DMEM with bicarbonate (pH 7.4) and cultured for up to 42 days in prolonged culture protocols with or without Fildes' reagent. Fildes' reagent was produced as described by Stringfellow (1984) with 100 mL defibrinated sheep blood (Cecon), 300 mL 0.85% NaCl, 21 mL HCl (Sigma 258148), and 2 g pepsin (Sigma P7000), and added to the media at 2.5% v/v concentration. Stringfellow (1986) estimated 2 mg/mL hemin in Fildes' reagent stock solutions.

Table 1. Rates of *Haemonchus contortus* fourth-stage larvae (L_4) development (Develop) on Days 6–7, 14, and 21, and differentiation (Diff) and degeneration (Degen) on Day 21 after *in vitro* culture under different media, supplement (Suppl), temperature (Temp), and atmosphere conditions.

Media + antibiotics-antimycotic	T°C	Atmosphere	pH	Δ4–ΔA	Suppl	D6–7	D14	D21		
						Develop	Develop	Develop	Diff	Degen
DMEM (D0–3); DMEM + FCS + pyr + AA + glut (D4–6); DMEM + FCS + pyr + AA + glut + HEPES (D7–42)	39.6	10% CO_2	6.1 (D0–3); 7.4 (D4–42)	D0–3	-	63/116 (54.3%)[a]	29/39 (74.4%)[b]	59/62 (95.2%)[a,b]	fev/62 (3.2%)[b,c]	39/62 (62.9%)[c,d]
					Fildes (D7–42)	63/116 (54.3%)[a]	84/95 (88.4%)[a]	129/133 (97.0%)[a]	2/133 (1.5%)[c]	93/133 (69.9%)[d]
DMEM (D0–6); DMEM + FCS + pyr + AA + glut + HEPES (D7–42)	39.6	10% CO_2	6.1 (D0–6); 7.4 (D7–42)	D0–6	-	29/97 (29.9%)[b]	28/36 (77.8%)[a,b]	61/62 (98.4%)[a]	mai/62 (8.1%)[b]	35/62 (56.5%)[c,d]
					Fildes (D7–42)	53/140 (37.9%)[b]	66/76 (86.8%)[a,b]	30/35 (85.7%)[b,c]	jan/35 (2.9%)[b,c]	17/35 (48.6%)[c]
Medium 199 + FCS (D0–42)	37	5% CO_2	7.4 (D0–42)	-	Fildes (D0–42)	0/24 (0.0%)[c]	fev/33 (6.1%)[d]	jul/48 (14.6%)[e]	0/48 (0.0%)[c]	jan/48 (2.1%)[a]
DMEM + FCS + pyr + AA + glut + HEPES (D0–42)	37	5% CO_2	7.4 (D0–42)	-	Fildes (D0–42)	0/21 (0.0%)[c]	0/18 (0.0%)[d]	28/87 (32.2%)[d]	abr/87 (4.6%)[b,c]	15/87 (17.2%)[b]
					Caco-2 cell (D0–42)	jun/14 (42.9%)[a,b]	25/27 (92.6%)[a,b]	35/36 (97.2%)[a,b]	set/36 (25.0%)[a]	mai/36 (13.9%)[b]
DMEM + FCS + pyr + AA + glut + HEPES (D0–42)	37	85% N_2, 5% O_2, 10% CO_2	7.4 (D0–42)	-	Fildes (D0–42)	jan/53 (1.9%)[c]	4/100 (4.0%)[d]	17/76 (22.4%)[d,e]	jan/76 (1.3%)[b,c]	jun/76 (7.9%)[a,b]
					Caco-2 cell (D0–42)	4/100 (4.0%)[c]	51/129 (39.5%)[c]	67/83 (80.7%)[c]	fev/83 (2.4%)[b,c]	15/83 (18.1%)[b]

DMEM: Dulbecco's modified Eagle's medium; pyr: sodium pyruvate; FCS: fetal calf serum; AA: non-essential amino acids; glut: L-glutamine. [a,b,c,d,e]Values with different superscripts in the same column differ at a significance level of 0.05.

For co-cultures, a fresh 33-passage Caco-2 cell line obtained from Banco de Células do Rio de Janeiro (BCRJ 0059, lot 001783) was incubated in DMEM with bicarbonate, FCS, amino acids, pyruvate, L-glutamine, and HEPES at 37°C under 5% CO_2, which are optimal conditions for culturing Caco-2 cells. Subcultures were performed when the cells reached 80% confluency. *H. contortus* larvae were co-cultured with confluent Caco-2 cells from passages 37–39 in the same conditions used for Caco-2 cell cultures.

Larvae development assessment and statistical analysis

Every 7 days, simultaneously to the media replacement after centrifugation, an aliquot of 50–100 µL from the pellet was removed, and all larvae recovered in the aliquot (varying from 14 to 140 larvae) were assessed under an optical microscope. Progression to L_4 and sexual differentiation were evaluated according to the morphological classification by Veglia (1915). Mouth development and asymmetrical tail with dorsal curvature indicated progression to L_4. Furthermore, in early differentiated L_4, thick posterior end with the tail being short, conical, smooth, and slightly curved posteriorly, with no detection of bursa, was observed in males, while longer tail tapered slightly and bent dorsally was present in females. From the total number of recovered larvae, the number of larvae in the L_4 stage was counted (developmental rates). Additionally, on Days 21, 28, 35, and 42, the number of L_4 larvae showing sexual differentiation (differentiation rates) and the number of degenerated L_3- and L_4-stage larvae (degeneration rates) were counted from the total number of recovered larvae.

The purpose was to achieve an *in vitro* culture protocol resulting in higher developmental and differentiation rates with lower degeneration rates. Thus, larvae development, differentiation, and degeneration rates were compared by chi-square test at a significance level of 5%.

Results and Discussion

H. contortus larvae recovery and *in vitro* culture

Most larvae recovered from fecal cultures were L_3 (Figure 1B); however, L_2 were also retrieved (Figure 1A). After *in vitro* culture, L_4 were observed, based on mouthpart development and asymmetrical tail with dorsal curvature (Figure 1C).

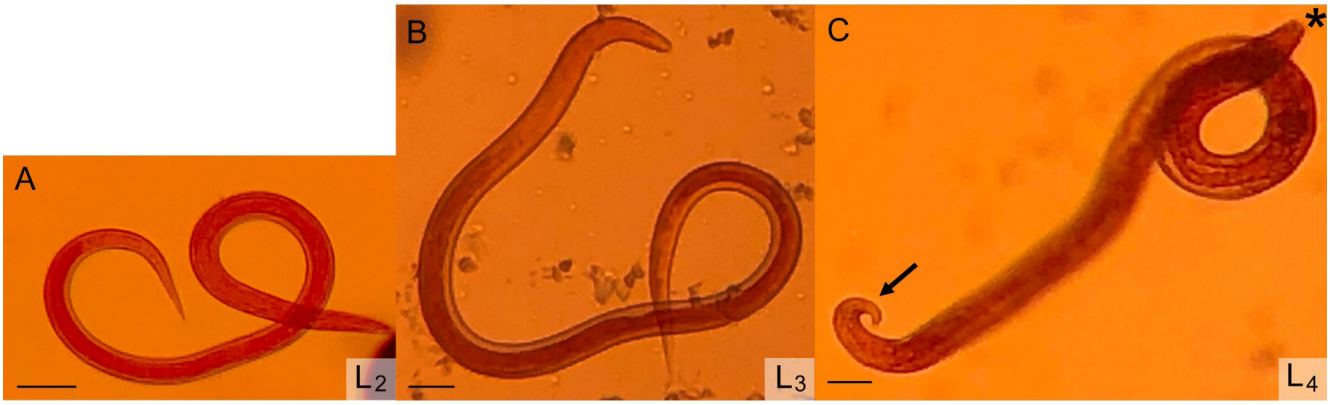

Figure 1. Stages of *Haemonchus contortus* larval development (L_2, L_3, and L_4) recovered from fecal and *in vitro* cultures. A) L_2. B) L_3. C) L_4 with developed mouth (*) and tail dorsal curvature (arrow). Optical magnification 10× and digital zoom.

No differences were observed in cultures using 25 or 75 cm^2 flasks with 1,000–5,000 larvae; however, using more than 5,000 L_3/10 mL resulted in frequent culture contamination, observed through changes in media color and turbidity and larvae death.

Results of prolonged culture protocols for up to 21 days are presented in Table 1. Due to the large number of degenerated and dead larvae recovered on Days 28, 35, and 42, these results are described in the text and not included in Table 1.

An acid pH and the use of a steroid hormone supplement promotes molting of exsheathed L_3 to L_4 *in vitro*

For the first three to six days in culture, two different media without bicarbonate supplemented with 2.3 µM Δ4-DA and antibiotics-antimycotic were tested: EBSS (pH 4.6) and DMEM (pH 6.1). These resulted in similar ($p>0.05$) L_4 development: 26.3% on Day 3 and 60–70% on Day 6. As DMEM was the media employed for Caco-2 cell cultures and there were no significant differences in L_4 development, DMEM was selected for use in further experiments to allow comparisons between cell-free and co-cultures, while EBSS was not included in prolonged culture experiments. In addition, it was observed that the Δ4-DA steroid hormone, and not only the Δ7-DA reported in the literature (Ma et al., 2019; Marks et al., 2019), can be used for *in vitro* culture supplementation leading to increased *H. contortus* L_4 development.

Prolonged culture in weak acidic media is detrimental to larvae *in vitro*

Incubation in acidic media for periods longer than 6 days was detrimental to larval development and resulted in the death of all larvae by Day 14. Exposure to more acidic conditions (pH 2) for 5 min resulted in the death of *H. contortus* larvae (Sommerville, 1977). Thus, contrary to natural acidic environment in the abomasum, prolonged culture under low pH conditions was deleterious for *H. contortus* larval development *in vitro*.

Subsequently, acidic DMEM media used for 3 or 6 days, supplemented with Δ4-DA for all 6 days, followed by culture in DMEM pH 7.4 without Fildes' reagent resulted in 74.4–77.8% L_4 on Day 14 and 95.2–98.4% on Day 21 (Table 1). Similar percentages ($p>0.05$) were observed following larval culture in DMEM pH 7.4 supplemented with Fildes' reagent (86.8–88.4% on Day 14 and 85.7–97.0% on Day 21) (Table 1). On Day 21, in all culture conditions at 39.6ºC and 10% CO_2, differentiation rates of 1.5–8.1% in L_4 were observed (Table 1), and distinct morphological patterns suggested the development of males (Figure 2A) and females (Figure 2B-2C). However, incubation in acidic conditions for 3 or 6 days resulted in high rates ($p<0.05$) of degeneration (48.6–69.6%) on Day 21 (Table 1) and by Day 35 almost all larvae were degenerated, immobile, or dead. Morphological alterations observed in degenerated larvae on Day 21 consisted of granule or vacuole formation in intestinal cells (Figure 3A), cuticle wrinkling (Figure 3B), cuticle detachment (Figure 3C), or cuticle rupture and evisceration (Figure 3D). These changes were similar to those observed in larvae exposure to anthelmintics, indicating death or imminent death (Acevedo-Ramírez et al., 2019; Nguyen et al., 2019). In addition, there was no progression to adulthood (L_5) for up to 35 days in culture.

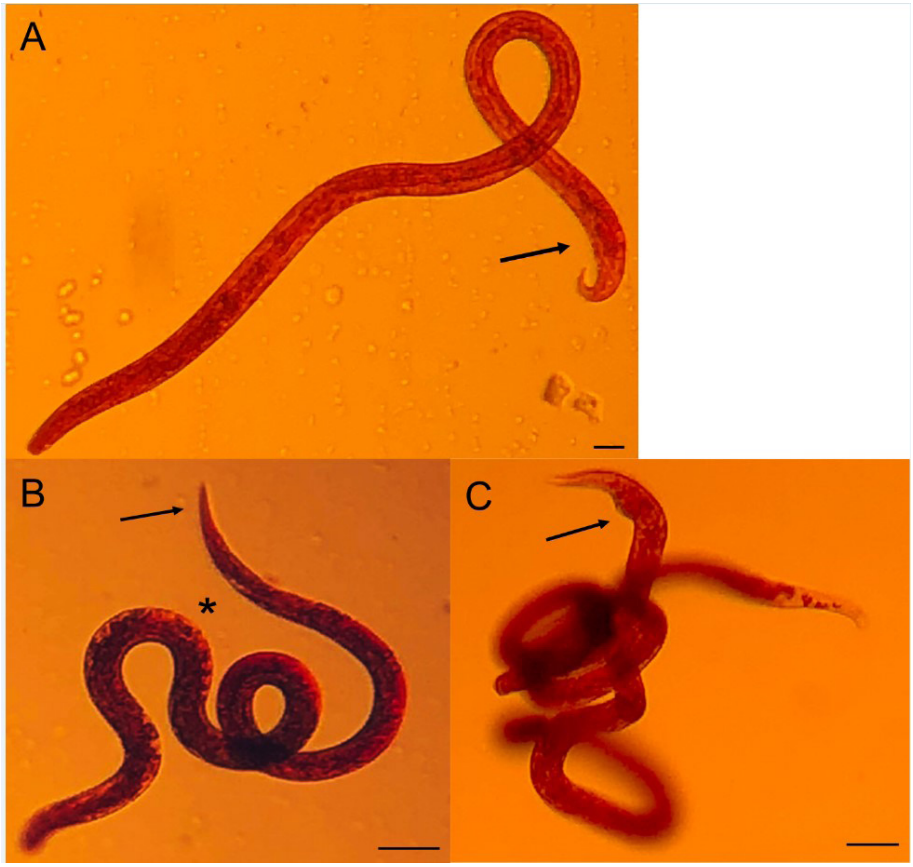

Figure 2. *Haemonchus contortus* L_4 with morphological differentiation of posterior end after 21 days in cell-free *in vitro* culture. A) Suggestive pattern of male development with thick and curved tail (arrow). B) Suggestive pattern of female development with longer and taped tail (arrow). A and B) Culture in acidic DMEM for 3 days with Δ4-DA for 6 days followed by incubation in DMEM without Fildes' reagent. C) Cuticle inflation or potential knob-shaped vulva (arrow) in differentiated female, following culture in acidic DMEM for 3 days with Δ4-DA for 6 days followed by incubation in DMEM supplemented with Fildes' reagent. *Rectum and anus. Optical magnification 10× and digital zoom.

Using additional supplementation of media promotes development, with the use of Caco-2 cells found to be of increased benefit to larval development than Fildes' reagent

In *Trichinella spiralis*, Caco-2 cell monolayers supported L_1 molting, ecdysis, adult development, and reproduction of 50% larvae *in vitro* cultured for 11 days (Gagliardo et al., 2002). Therefore, *H. contortus* co-cultures with Caco-2 cells were evaluated and compared with supplementing a cell-free DMEM culture with Fildes' reagent. In this experiment, Δ4-DA was not used and not initial period in acidic media was provided. Since Caco-2 cells remained viable only at 37°C in DMEM without Fildes' reagent, the incubation temperature for these experiments was reduced to 37°C.

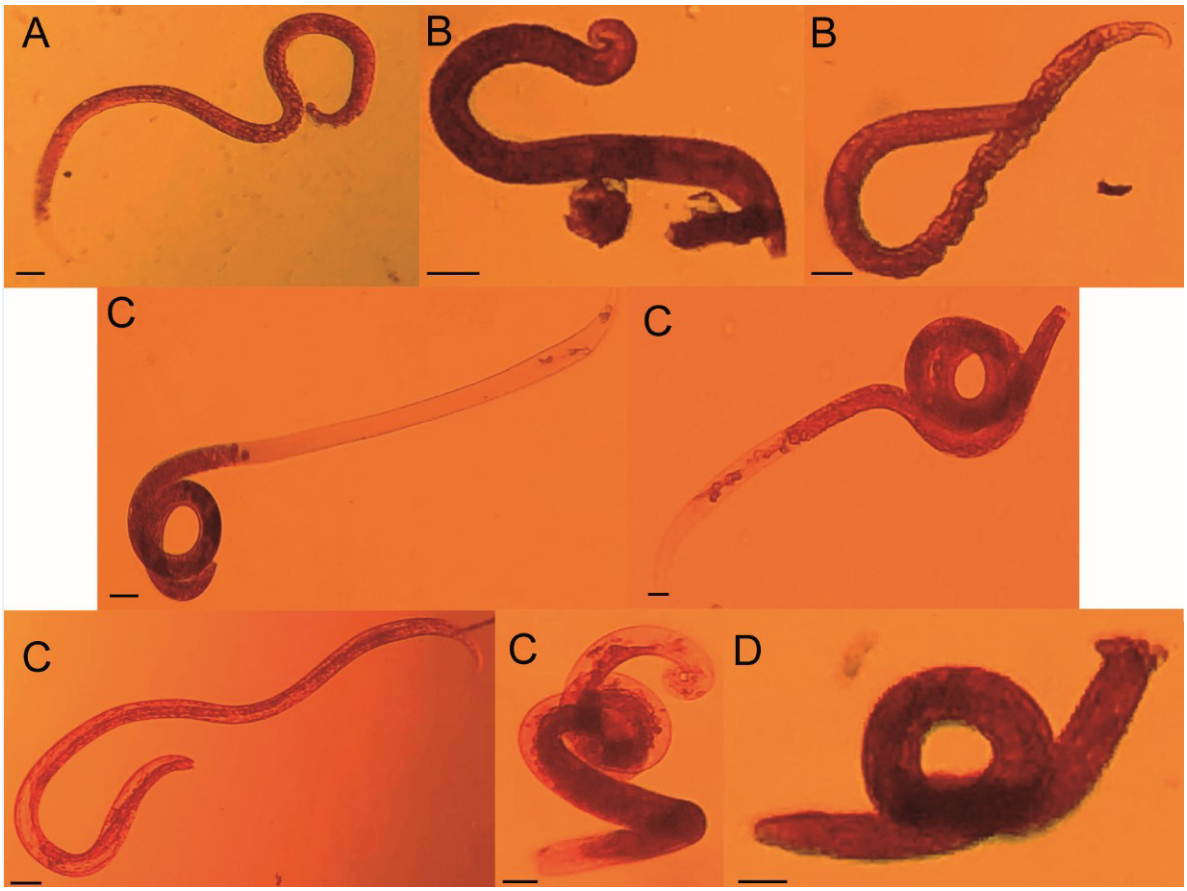

Figure 3. Morphological alterations in *Haemonchus contortus* L_4 on Day 21 after *in vitro* culture in acidic media for the first 3 or 6 days. A) Granule or vacuole formation. B) Cuticle wrinkling. C) Cuticle detachment. D) Cuticle rupture and evisceration. Optical magnification 10× and digital zoom.

At 5% CO_2, co-cultures with Caco-2 cells in DMEM resulted in higher ($p<0.05$) developmental rates on Days 7, 14, and 21 (42.9%, 92.6%, and 97.2%, respectively), compared to DMEM supplemented with Fildes (0.0%, 0.0%, and 32.2%, respectively) and Medium 199 supplemented with Fildes (0.0%, 6.1%, and 14.6%, respectively) (Table 1). Medium 199 contains more vitamins and amino acids than DMEM supplemented with non-essential amino acids (Supplementary Table 1). However, higher ($p<0.05$) rates of L_4 development were observed on Day 21 following culture in DMEM with supplements. In addition, co-culture with Caco-2 enhanced ($p<0.05$) L_4 development compared to supplementation with Fildes' reagent (Table 1). This is the first report comparing Caco-2 cells to Fildes' reagent supplementation in prolonged cultures. In the literature, *H. contortus* culture with Caco-2 cells for up to 3 weeks resulted in higher rates of development from L_3 to L_4, but no development to adults (Britton et al., 2016). Then, we tested the use of Caco-2 in co-culture for prolonged periods (up to 42 days or 6 weeks), but still no adults were obtained.

Different atmospheric conditions tested using Caco-2 cell co-culture or Fildes' reagent

Comparing the culture gas atmospheres, when using DMEM supplemented with Fildes' reagent, similar L_4 development rates on Days 7, 14, and 21 were observed under 5% CO_2 (0.0%, 0.0%, and 32.2%, respectively) and 85% N_2, 5% O_2, and 10% CO_2 (1.9%, 4.0%, and 22.4%, respectively). However, co-cultures with Caco-2 cells in DMEM resulted in higher ($p<0.05$) L_4 development rates on Days 7, 14, and 21 under 5% CO_2 (42.9%, 92.6%, and 97.2%, respectively) compared to 85% N_2, 5% O_2, and 10% CO_2 (4.0%, 39.5%, and 80.7%, respectively) (Table 1). Sommerville (1977) also reported delayed L_4 development using nitrogen for *in vitro H. contortus* culture.

In vitro co-culture of *H. contortus* larvae with Caco-2 cells at 37°C and 5% CO_2 resulted in similar ($p>0.05$) rates of L_4 development on Days 6–7, 14, and 21 to cell-free conditions using acidic media at 39.6°C and 10% CO_2, but with

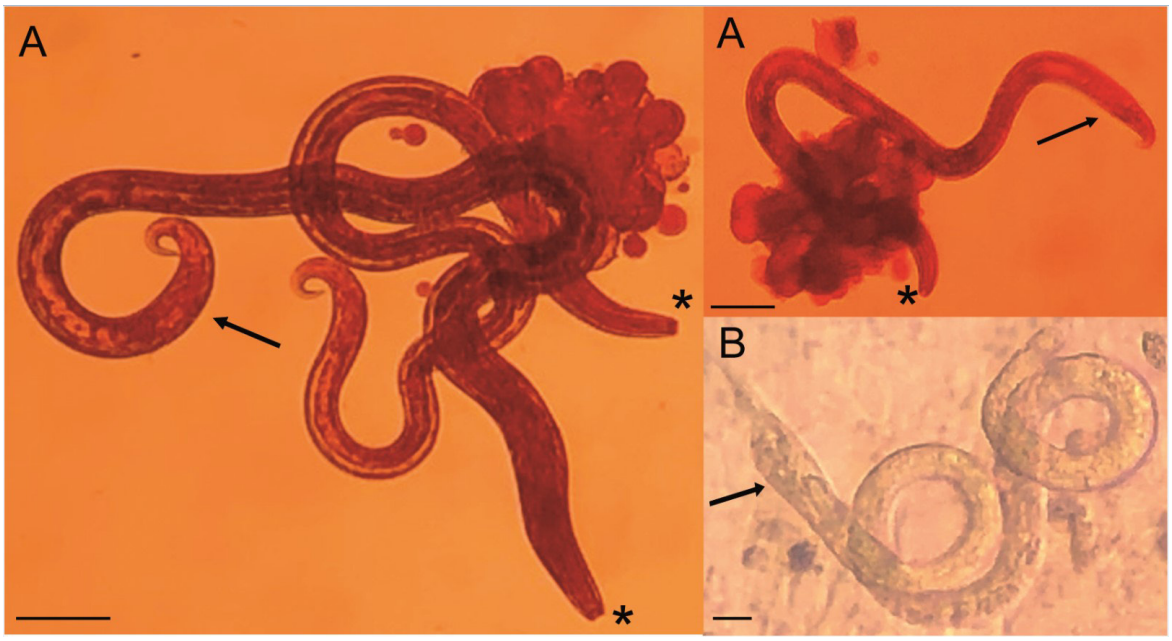

Figure 4. Progression of *Haemonchus contortus* L_4 differentiation after *in vitro* co-culture with Caco-2 cells. A) L_4 with developed mouth (*) and posterior end thickening (arrow) on Day 21. B) L_4 with posterior end differentiation, suggestive of male development on Day 35. Optical magnification 10× and digital zoom.

higher ($p<0.05$) differentiation rates (25.0%; Figure 4A) and inferior ($p<0.05$) degeneration rates on Day 21 (Table 1). In addition, in Caco-2 cell co-culture, morphologically viable larvae were recovered on Day 28 (at a degeneration rate of 31.9%), and differentiated larvae were still observed on Day 35 (Figure 4B), however most larvae were degenerated (presence of granules or vacuoles and reduced movement) or dead on Days 35 and 42. Degeneration of 50% of larvae on Day 28 in culture was reported by Stringfellow (1984), which was higher than that observed here on Day 28 in co-cultures. Thus, co-culture with Caco-2 cells resulted in viable *H. contortus* L_4 for up to 28 days *in vitro*, but no progression beyond the L_4 stage was observed.

In the literature, the development of adult *H. contortus* following *in vitro* culture has been reported by Stringfellow (1984, 1986), using API-I medium (Douvres & Malakatis, 1977; Supplementary Table 1), resulting in the development of 0.1% males on Day 28 and 0.07% females on Day 36 (Stringfellow, 1986). Stringfellow (1986) used API-I medium supplemented with Fildes' reagent and sheep gastric contents, with the pH adjusted to 6.4 for 1 week and then to pH 6.8, at 39°C under 85% N_2, 5% O_2, and 10% CO_2. In the present study, culture under acidic pH beyond Day 6 was detrimental to larval development, whereas incubation in an atmosphere with nitrogen delayed and reduced larval development in co-culture with Caco-2 cells. Considering L_4 development, the rates observed in the present study using the best protocol (co-culture with Caco-2 cells at 5% CO_2) were similar but delayed (42.9% on Days 6–7 and 92.6% on Day 14) compared to those reported previously for short-term cultures. Sommerville (1966) achieved 90% L_4 in 3 days following culture in saline solution pH 6 at 40°C ± 0.5°C under 40% CO_2 and 10% O_2. Preston et al. (2015) reported 80% L_4 after 5 days of culture in DMEM with L-glutamine, antibiotics, and antimycotics at 40°C and 10% CO_2. Nguyen et al. (2019) obtained more than 80% L_4 after 7 days using Luria-Bertani medium supplemented with antibiotics and antimycotic at 38°C and 10% CO_2. Marks et al. (2019) obtained more than 65% L_4 on Day 3 following culture in EBSS pH 5 with antibiotics, antimycotics, and 2.5 µM Δ7-DA at 37°C and 5% CO_2.

Conclusion

Despite no progression to the adult L_5 stage, co-culture with Caco-2 cells at 37ºC and 5% CO_2 was sufficient to stimulate transition to L_4 and assured maintenance of morphologically viable and differentiated L_4 for 28 days. Thus, it was considered the best protocol for *in vitro* culture of *H. contortus* from L_3 recovered from fecal cultures using commercial media.

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Ethics declaration

The Ethical Committee of Animal Experimentation (CEUA) of Embrapa Southeast Livestock (protocol number 03/2019) approved all experimental procedures in sheep.

Conflict of interest

The authors declare they have no conflict of interest.

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Supplementary Material

Supplementary material accompanies this paper.

Supplementary Table 1. Composition of API-I, DMEM, Medium 199, and EBSS media, and non-essential amino acid (MEM) supplement.

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