Biochemistry and Cell Biology
Biochimie et biologie cellulaire

Parasiticidal effect of synthetic bovine Lactoferrin peptides on the enteric parasite *Giardia intestinalis*

Journal:	*Biochemistry and Cell Biology*
Manuscript ID	bcb-2016-0079.R5
Manuscript Type:	Article
Date Submitted by the Author:	16-Nov-2016

Complete List of Authors:	Aguilar-Diaz, Hugo; Facultad de Medicina, Universidad Autónoma de Sinaloa, CIASaP Canizalez-Roman, Adrian; Facultad de Medicina, Universidad Autonoma de Sinaloa, CIASaP; Hospital de la Mujer, Departamento de Investigación Nepomuceno-Mejia, Tomas; Instituto Nacional de Salud Pública., Centro Regional de Investigación en Salud Pública Gallardo-Vera, Francisco; Facultad de Medicina. Universidad Nacional Autónoma de México, Departamento de Biologia Celular y Tisular Hornelas-Orozco, Yolanda; Universidad Nacional Autónoma de México Nazmi, Kamran; Academic Center Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, Department of Oral Biochemistry Bolscher, Jan; Academic Center Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, Department of Oral Biochemistry Carrero, Julio Cesar; Universidad Nacional Autonoma de Mexico Instituto de Investigaciones Biomedicas Leon-Sicairos, Claudia; Universidad Autónoma de Sinaloa, Programa Regional de Noroeste para el Doctorado en Biotecnología. FCQB Leon-Sicairos, Nidia; Facultad de Medicina, Universidad Autónoma de Sinaloa, CIASaP; Hospital Pediátrico de Sinaloa, Departamento de investigación

Keyword:	Lactoferrin, Peptides, LFchimera, parasiticidal, Giardiasis

SCHOLARONE™
Manuscripts

1 # Parasiticidal effect of synthetic bovine Lactoferrin peptides on
2 # the enteric parasite *Giardia intestinalis*

3

4 Hugo Aguilar-Diaz[1], Adrian Canizalez-Roman[1,2], Tomas Nepomuceno-Mejia[3], Francisco
5 Gallardo-Vera[4], Yolanda Hornelas-Orozco[5], Kamran Nazmi[6], Jan G.M. Bolscher[6], Julio
6 Cesar Carrero[7], Claudia Leon-Sicairos[8], Nidia Leon-Sicairos[1,9*]

7
8
9 [1] CIASaP, Facultad de Medicina, Universidad Autónoma de Sinaloa. Cedros y Sauces,
10 Fracc. Fresnos Culiacán 80246, Sinaloa, México
11 [2] Departamento de Investigación, Hospital de la Mujer. Boulevard Miguel Tamayo
12 Espinoza de los Monteros, S/N Col. Desarrollo Urbano Tres Ríos. Culiacán 80020, Sinaloa,
13 México
14 [3] Centro Regional de Investigación en Salud Pública, Instituto Nacional de Salud Pública.
15 Calle 4a. Avenida Norte esquina con Calle 19 Pte S/N, Centro, Tapachula 30700, Chiapas,
16 Mexico
17 [4] Laboratorio Inmunobiología, Departamento de Biología Celular y Tisular, Facultad de
18 Medicina. Universidad Nacional Autónoma de México. Ciudad Universitaria, México DF
19 04510, México
20 [5] Servicio Académico de Microscopía Electrónica de Barrido. Instituto de Ciencias del Mar
21 y Limnología, Universidad Nacional Autónoma de México, México, D. F. 04510, México
22 [6] Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam, University
23 of Amsterdam and VU University, 1081 LA, Amsterdam, The Netherlands
24 [7] Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad
25 Nacional Autónoma de México. Ciudad Universitaria, México, DF 04510, México
26 [8] Facultad de Ciencias Químico-Biológicas, Universidad Autónoma de Sinaloa, Avenida de
27 las Américas y Josefa Ortiz (Ciudad Universitaria), Culiacán 80030, Sinaloa, México
28 [9] Departamento de Investigación, Hospital Pediátrico de Sinaloa. Blvd. Constitución S/N,
29 col. Jorge Almada, Culiacan 80200, Sinaloa, México
30
31
32 *Corresponding author. Nidia León-Sicairos. Email* nidialeon@uas.edu.mx
33 *CIASaP, Facultad de Medicina, Universidad Autónoma de Sinaloa, Cedros y Sauces S/N*
34 *Fracc. Fresnos. Culiacán Sinaoa, 80246 México Telephone +52 6672278588 Fax +52 66*
35

36

37

38

Draft

39 **Abstract**

40

41 *Giardia intestinalis* is the most common infectious protozoan parasite in children. Despite
42 the effectiveness of some drugs, the disease remains a major worldwide problem.
43 Consequently, the search for new treatments is important for disease eradication. Biological
44 molecules with antimicrobial properties represent a promising alternative to combat
45 pathogens. Bovine lactoferrin (bLF) is a key component of the innate host defense system,
46 and its peptides have exhibited strong antimicrobial activity. Based on these properties, we
47 evaluated the parasiticidal activity of these peptides on *G. intestinalis*. Trophozoites were
48 incubated with different peptide concentrations for different periods of time, and the growth
49 or viability was determined by carboxyfluorescein-succinimidyl-diacetate-ester (CFDA)
50 and propidium iodide (PI) staining. Endocytosis of peptides was investigated by confocal
51 microscopy, damage was analyzed by transmission and scanning electron microscopy, and
52 the type of programmed cell death was analyzed by flow cytometry. Our results showed
53 that the LFpeptides had giardicidal activity. The LFpeptides interacted with *G. intestinalis*
54 and exposure to LFpeptides correlated with an increase in the granularity and vacuolization
55 of the cytoplasm. Additionally, the formation of pores, extensive membrane disruption, and
56 programmed cell death was observed in trophozoites treated with LFpeptides. Our results
57 demonstrate that LFpeptides exhibit potent *in vitro* antigiardial activity.

58

59 **Keywors: Lactoferrin; LFchimera; parasiticidal; peptides; Giardia; Giardiasis**

60

61

62

Draft

63 **Introduction**

64

65 *Giardia intestinalis* (also known as *Giardia lamblia* or *Giardia duodenalis*), is a flagellated
66 unicellular eukaryotic parasite that causes giardiasis, a diarrheal disease, throughout the
67 world (Watkins and Eckmann 2014). Giardiasis is the most common cause of waterborne
68 outbreaks of diarrhea in the United States and is occasionally considered a cause of food-
69 borne diarrhea (Furness et al. 2000). In certain areas of the world, water contaminated
70 with *G. lamblia* commonly causes travel-related giardiasis in tourists (Painter et al. 2015;
71 Watkins and Eckmann 2014). This parasite is particularly problematic in developing
72 countries, where a very high prevalence and incidence of infection has been reported. Data
73 suggest that long-term growth retardation in children can result from chronic giardiasis, in
74 part due to the parasite attaching itself to the lining of the small intestine in humans, where
75 it interferes with the body's absorption of fats and carbohydrates from digested foods
76 (Eckmann 2003). Giardiasis is reported more frequently in young children and
77 immunocompromised or chronically ill individuals, and *G. intestinalis* infection is
78 particularly significant for people with malnutrition, immunodeficiencies, or cystic fibrosis
79 (Painter et al. 2015; Watkins and Eckmann 2014).

80

81 Giardia species have two major stages in their lifecycle. Infection with *G. intestinalis*
82 initiates when the cysts are ingested with contaminated water or, less commonly, food or
83 through direct fecal-oral contact. The cyst is relatively inert, allowing prolonged survival in
84 a variety of environmental conditions (Adam 2001; Carranza and Lujan 2010). After
85 exposure to the acidic environment of the stomach, cysts excyst into trophozoites in the
86 proximal small intestine. The trophozoite (the vegetative form) replicates in the small

Draft

87 intestine, causing symptoms of diarrhea and malabsorption. After exposure to biliary fluid,
88 some of the trophozoites form cysts in the jejunum and are passed in the feces, allowing for
89 completion of the transmission cycle by infecting a new host (Adam 2001; Carranza and
90 Lujan 2010).
91
92 Standard treatment for giardiasis consists of antibiotic therapy. Metronidazole is the most
93 commonly prescribed drug for this condition. However, metronidazole use has been
94 associated with significant failure rates in clearing parasites from the gut and with poor
95 patient compliance (Watkins and Eckmann 2014). In addition, an increasing incidence of
96 nitroimidazole-refractory giardiasis has been reported in travelers from India (Nabarro et al.
97 2015). Appropriate fluid and electrolyte management is critical, particularly in patients with
98 large-volume diarrheal losses and in children with acute or chronic diarrhea who manifest a
99 failure to thrive, malabsorption, or other gastrointestinal tract symptoms in whom Giardia
100 organisms have been identified (Dominguez-Lopez et al. 2011; Hill 1993; Vesy and
101 Peterson 1999).
102
103 Innate-immunity mechanisms play a role in the control and/or severity of the infection;
104 however, little is known about the mechanisms involved in this immune response
105 (Roxstrom-Lindquist et al. 2006). Breastfeeding protects infants from *G. intestinalis*
106 infection. Breast milk contains detectable titres of secretory IgA, which is protective for
107 infants, especially in developing countries (Eckmann 2003; Morrow et al. 1992). A study
108 from Egypt showed breast-fed infants had a lower incidence of symptomatic and
109 asymptomatic infection. Furthermore, infected infants who were exclusively breast-fed had
110 fewer clinical manifestations than those who were not exclusively breast-fed (Abdel-Hafeez

https://mc06.manuscriptcentral.com/bcb-pubs

111 et al. 2013; Gendrel et al. 1989). Apart from IgA, a milk protein called lactoferrin (LF) has
112 been reported as an immunological factor that kills trophozoites *in vitro* and *in vivo*: both
113 hLf and bLF and peptides derived from their N-terminals (LfcinH and LfcinB) have
114 microbicidal activity against Giardia (Gillin et al. 1983; Ochoa et al. 2008; Turchany et al.
115 1995; Turchany et al. 1997). However, it is not known if synthetic bLF peptides share this
116 activity and the mechanism of action of Lf-derived peptides is unknown. Thus, the aim of
117 this work was to study the possible microbicidal activity of synthetic bLF-based peptides
118 against *Giardia intestinalis* and to explore the mechanism involved in parasitical effects
119 against Giardia *in vitro*.
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134

Draft

135 **Materials and methods**

136

137 **Bovine lactoferrin, synthetic peptides and chemotherapeutic agents**

138 Bovine LF (bLF, 20% iron saturated) was kindly donated by Morinaga Milk Industries Co
139 (Tokyo, Japan). The purity of bLF (>98%) was checked by SDS-PAGE stained with silver
140 nitrate. Lactoferrin concentration was assessed by UV spectroscopy on the basis of an
141 extinction coefficient of 15.1 (280 nm, 1% solution). The bLF iron saturation was about
142 20% as detected by optical spectroscopy at 468 nm on the basis of an extinction coefficient
143 of 0.54 (100% iron saturation). LPS contamination of bLf, estimated by Limulus
144 Amebocyte assay (LAL Pyrochrome kit, ThermoFicherScientific, Waltham, MA, USA),
145 was equal to 0.7±0.06 ng/mg of bLF. Synthetic peptides LFcin17-30, LFampin265-284 and
146 LFchimera were obtained by solid-phase peptide synthesis using Fmoc chemistry, as
147 described previously (Bolscher et al. 2012; Bolscher et al. 2009). The chemotherapeutic
148 agents used were metronidazole and albendazole (Sigma Chemical Co., St. Louis, MO,
149 USA). Stock solutions were prepared in phosphate-buffered saline for metronidazole and
150 dimethyl sulphoxide (DMSO) for albendazole. The final DMSO concentration in the
151 culture tubes was <0.5% (v/v).

152 ***Giardia intestinalis*** **cyst isolation**

153 Human fecal samples from different patients containing abundant *G. intestinalis* cysts were
154 obtained from children at Hospital Pediatrico de Sinaloa in Culiacan City. *G. intestinalis*
155 cysts were purified and concentrated from feces using a combined sucrose flotation and
156 simplified sucrose gradient method (Hautus et al. 1988). The cysts, after being washed
157 twice in distilled water, were resuspended in distilled water and stored at 4°C for a

Draft

maximum of 3 days prior to use. The *G. intestinalis*-positive cysts were confirmed by light microscopy and PCR (Elsafi et al. 2013; Stojecki et al. 2014).

Excystation and axenization

G. intestinalis cysts were purified and concentrated from feces by combining the sucrose flotation method with a simplified sucrose gradient method. The excystation procedure was a modification of the Bingham and Meyer technique performed by Schupp et al. (1988). Briefly, the isolation procedure involved three steps: the concentration and cleaning of cysts by centrifugation in sucrose gradients performed 1 to 3 days after collection, the induction of excystation performed in acid solution from 1 to 5 days after cleaning cyst suspensions, and the culture and axenization in modified TYI-S-33 medium (Schupp et al. 1988).

Draft

Viability and growth inhibition assays

The effect of bLF, LFpeptides and chemotherapeutic agents on the long-term viability or permabilization of *G. intestinalis* trophozoites was determined by the inclusion of carboxyfluorescein-succinimidyl-diacetate-ester (CFDA) (St. Louis, MO, USA), or the exclusion of the dye propidium iodide (PI).

In one set of experiments, cultures were initiated by the addition of 2.5 x 10^4 trophozoites in 0.1 ml of medium to vials (15 x 45 mm) containing 3.9 ml of medium containing none (optimal viability) or one of the following agents: 1, 5, 10, 20, or 40 μM of bLF, LFcin17-30, LFampin265-284, or LFchimera. As control of growth inhibition, treatments with metronidazole (1, 5, 10, 20, and 40 μM) were used. The vials were incubated at 37°C for 12 h, chilled on ice to detach trophozoites, and centrifuged at 500 g for 10 min, and the pellet was resuspended in 1 ml of medium. Long-term viability was determined using the fluorescent probe carboxyfluorescein-succinimidyl-diacetate-ester

182 (CFDA-SE) (10 μg/ml) and visualized by epi-fluorescense microscopy (dos Santos et al.
183 2015). Experiments were performed at least three times in triplicate, and the mean and
184 standard deviations are indicated. Comparison of means was done by using a two-tailed t-
185 test for independent samples. A value of $P < 0.05$ was considered statically significant.

186 In other experiments, *G. intestinalis* trophozoites (10^6) were placed in tubes with
187 TYI-S-33 and were then incubated alone (optimal viability) or with 100 μM metronidazole,
188 5 μM albendazole, or 40 μM LFcin17-30, LFampin265-284 or LFchimera for 2 h at 37º C.
189 Membrane permeabilization by propidium iodide (PI) was used as a measure of trophozoite
190 viability. The total number of organisms per vial was counted and compared to that of
191 parallel untreated cultures. Experiments were performed at least three times in triplicate,
192 and the mean and standard deviations are indicated. Comparison of means was done by
193 using a two-tailed t-test for independent samples. A value of $P < 0.05$ was considered
194 statically significant.

195 Tubes with trophozoites were also incubated with combinations of metronidazole ±
196 LFpeptides or albendazole ± LFpeptides or LFpeptides alone. Membrane permeabilization
197 of propidium iodide (PI) measured by flow cytometry was used as a measure of trophozoite
198 viability.

199

200 **Confocal microscopy**

201 Trophozoites (10^6/ml in TYI-S-33 medium) were incubated with 2 μM FITC-LFcin17-30,
202 FITC-LFampim265-284 or FITC-LFchimera for 0, 5, 15, 30, 45 or 60 min at 37ºC. After
203 washing twice in cold PBS, trophozoites were collected by centrifugation, fixed with 4% *p*-
204 formaldehyde (30 min at 37ºC), permeabilized with 0.5% triton X-100, counterstained with
205 PI, washed and processed for analysis by confocal microscopy.

Draft

206 **Transmission electron microscopy**

207 *G. intestinalis* trophozoites (approximately 10^6 cells) were untreated or treated with 40 µM
208 of bLF-peptides for 2 h at 37 ºC. Trophozoites were collected by centrifugation and
209 processed for standard transmission electron microscopy (Vázquez-Nin and Echeverría
210 2000). Briefly, samples were fixed using 2.5% glutaraldehyde in PBS for 2 h at 4ºC, post-
211 fixed in 1% osmium tetroxide for 1 h, dehydrated in a graded series of ethanol, and
212 embedded in Epon resin. Semi-thin sections (approximately 300-400 nm) were stained with
213 toluidine blue and observed with bright-field microscopy. Sections were mounted on
214 copper grids and contrasted with uranyl acetate and lead citrate. Samples embedded in the
215 resin were cut with an ultramicrotome. Serial thin sections (50-60 nm width) were obtained,
216 and the samples were then stained with uranyl acetate and lead citrate. The sections were
217 observed with a JEOL 1010 transmission electron microscope at 80 kV.

Draft

218 **Scanning electron microscopy**

219 *G. intestinalis* trophozoites (5 x 10^6) were incubated with 40 µM of LFpeptides for 2 h at
220 37 ºC, washed and fixed with 10% formaldehyde for 72 h. Samples were washed and then
221 dehydrated in a graded series of ethanol. Dehydrated samples were placed in acetone and
222 then collocated in desiccator for 30 min. Finally samples were mounted in glass chambers
223 and bombarded with gold particles for 20 min (Perez-Rangel et al. 2013). Processed
224 specimens were observed with a scanning electron microscope JEOL LSM6360LV.

225 **Flow cytometry**

226 To detect early apoptosis or programmed cell death, *G. intestinalis* trophozoites (5 x 10^6)
227 treated with 40 µM LFpeptides for 2 h at 37ºC were stained with Allophycocyanin-Annexin
228 and 7-Amino-Actinomicyn D (BD Pharmingen, San José, CA), following the
229 manufacturer's instructions. Annexin V (also known as Annexin A5) and 7-AAD (7-amino-

230 actinomycin D) are indicators of early apoptosis or programmed cell death in Giardia and
231 necrosis or late programmed cell death, respectively. Both fluorescent dyes were measured
232 in *G. intestinalis* using Acoustic Focusing Cytometer AttuneTM Blue/Red (Life
233 Technologies).
234

235 **Results**

236 **Effects of bLF, LFpeptides and chemotherapeutics agents on the viability and long-**
237 **term growth of *Giardia intestinalis***

238 The effect of LFcin17-30, LFampin265-284 and LFchimera on *G. intestinalis* trophozoites
239 was analyzed using the membrane permeabilization probe PI, as a measure of viability.
240 When the *G. intestinalis* trophozoites were incubated with 40 µM LFpeptides for 2 h, the
241 trophozoites showed a marked PI uptake, indicating a drastic effect on the viability (Figure
242 1, panel A). LFchimera had the best killing effect (more than 98% of the trophozoites were
243 stained with PI) followed by the other LFpeptides (70-80% of the trophozoites were
244 stained) compared with untreated cells. Furthermore, the parasiticidal effect of LFchimera
245 was higher than the drugs albendazole and metronidazole (controls of viability inhibition)
246 (Figure 1, panel A).

247 Concentrations of 100 µM of metronidazole or 5 µM of albendazole were needed to
248 permeate more than 95% of *G. intestinalis* cultures. However, only 20 µM and 3 µM of
249 these drugs were needed to reach the same level of membrane permeation when 20, 30 or 5
250 µM of LFcin17-30, LFampin254-284 or LFchimera, respectively, were added, (Table 1).
251 Thus, the combined effect of lower concentrations of metronidazole or albendazole with
252 LFcin17-30, LFampin265-284 or LFchimera had the best initial parasiticidal activity (Table
253 1).

Draft

254 Next, the ability of different concentrations of LFcin17-30, LFampin265-284 and
255 LFchimera to inhibit long-term growth was tested using the live-stain CFDA. After 12 h of
256 interaction, the growth of *G. intestinalis* trophozoites in the presence of 5, 10, 20 or 40 µM
257 of LF and LFpeptides was lower than that found in the untreated trophozoites (Figure 1,
258 panel B). Interestingly, LFchimera had better giardicidal activity than the drug
259 metronidazole: 40 µM LFchimera inhibited the growth of cultures with more efficacy than
260 40 µM metronidazole. Similar results were obtained when *G. intestinalis* cultures were
261 incubated with 40 µM of each treatment for longer periods of time (24, 36 and 48 h, data
262 not shown). Therefore, at 40 µM LFchimera had the best parasiticidal activity, followed by
263 40 µM of the drug metronidazole and LFcin17-30, LFampin 265-284, and bLF.
264

Draft

265 **LFcin17-30, LFampin265-284 and LFchimera interact with *Giardia intestinalis***
266 The majority of live trophozoites incubated with 2 µM FITC-Labeled LFcin17-30,
267 LFampin265-284 or LFchimera showed bright green fluorescence (Figure 2, panels B, C
268 and D, respectively). Trophozoites were counterstained with PI, which exhibits red
269 fluorescence (Figure 2, panels A-D). At 30 min, all FITC-LFpeptides were visible in the
270 trophozoites (green fluorescence, arrowheads), suggesting that the LFpeptides were
271 endocytosed or internalized by *G. intestinalis*. Additionally, it would appear that degraded
272 RNA and DNA are present in the images of trophozoites treated with the peptides (B-D).
273 The controls (untreated trophozoites) were negative for the green fluorescence (A).
274

275 **Morphologic effects of LFcin17-30, LFampin265-284 and LFchimera on *Giardia***
276 ***intestinalis* trophozoites**

https://mc06.manuscriptcentral.com/bcb-pubs

277 We examined cells after relatively short times of exposure to observe early and more direct
278 changes, as well as those accompanying or secondary to cell lysis, by transmission electron
279 microscopy (TEM). Untreated cells had a smooth cellular membrane (cm, double-headed
280 arrow), peripheral vacuoles (pv, lines) near the cellular membrane, three pairs of flagella
281 (F, discontinuous arrow), adherent disk (ad), and two nuclei (N) and two nucleoli (no)
282 (Figure 3, panel A). Magnification of the picture shows granules of electron-dense material
283 (asterisk) distributed in the cytoplasm and the arrangement of the microtubules belonging to
284 the adherent disk (ad) (panel B). Exposure to LFcin17-30 led to profound intracellular
285 changes, such as an increase in electron-dense material in the cytoplasm (asterisk),
286 reorganization of the flagella (F, discontinuous arrow) and displacement of the adherent
287 disk (ad, arrow), Figure 3, panel C. In the magnified picture, there are no peripheral
288 vacuoles (pv) near the cellular membrane (cm, double-headed arrow), Panel D. Treatment
289 with LFampin265-284 also caused intracellular damage, including an increase in electron-
290 dense material (asterisk), reorganization of the flagella (F, discontinuous arrow), a large
291 hole in the cytoplasm (arrowhead), and also disruption in the cellular membrane (cm,
292 double-headed arrow) (Figure 3, panel E). The magnified picture shows the large hole
293 induced by treatment with LFampin265-284 (arrowhead, panel F). Trophozoites treated
294 with LFchimera produced the most significant changes and damage in the trophozoites.
295 There were marked changes in the electron-dense material in the cytoplasm (asterisks), the
296 flagella (F, discontinuous arrow) were disrupted and reorganized, and the cytoplasm
297 showed large holes (arrowheads) in which some electron-dense material is visible (Figure
298 3, panel G). In the magnified picture the larges holes (arrowheads) with aggregates inside
299 them (asterisks) can be seen in more detail (Panel H). Shrunken and distorted peripheral
300 vacuoles (pv, lines) were also observed.

Draft

301 *G. intestinalis* trophozoites treated with LFpeptides also were analyzed under scanning
302 electron microscopy (SEM). Treated trophozoites exhibited damage on the cell surface
303 (Figure 4). By SEM it was found that *G. intestinalis* cultures treated with LFcin17-30 (B),
304 LFampin265-284 (C), or LFchimera (D) showed alterations in size, irregular form and
305 perforations (arrows) compared with untreated trophozoites (which had the typical structure
306 of *G. intestinalis* trophozoites) (A). Additionally, the large hole in the membrane of the
307 trophozoite treated with Lfchimera shown in panel D revealed the presence of unusual
308 aggregates.
309

310 **LFcin17-30, LFampin265-284 and LFchimera induced programmed cell death in**
311 ***Giardia intestinalis* trophozoites**

Draft

312 LFcin17-30, LFampin265-284 and LFchimera induced early programmed cell death in *G.*
313 *intestinalis* trophozoites treated with 40 μM of these peptides for 2 h (Figure 5, panels A, B,
314 and C, respectively). The quadrants represent Q1: Necrotic cells, Q2: Necrosis and apototic
315 cells, Q3: Earlier apoptotic cells, Q4: live cells. Cells treated with LFchimera and
316 LFampin265-284 induced early programmed cell dead as measured by the liberation of
317 phosphatidylserine (PS) (Q3. 27.9 and 25.5%, respectively), and, to a lesser extent, early
318 programmed cell death was also induced by treatment with LFcin17-30 (Q3. 7.66%).
319 Although the total of the analyzed population were not undergoing programmed cell death,
320 this result and the images observed under electron microscopy reinforce the idea that *G.*
321 *intestinalis* is induced to undergo programmed cell death after treatment with LFpeptides.
322
323
324

325 **Discussion**

326 Although giardiasis has been a threat to mankind for thousands of years, this parasitic
327 infection has been, until recently, relatively neglected. *G. intestinalis* is a major cause of
328 parasite-induced diarrhea in humans and animals and is currently an important public health
329 problem, mostly in developing countries but also in developed countries (Escobedo et al.
330 2010; Painter et al. 2015). Nearly 33% of people in developing countries have had
331 giardiasis, and nearly 2% of adults and 6% to 8% of children have giardiasis worldwide
332 (Escobedo et al. 2010; Furness et al. 2000; Painter et al. 2015). Although giardiasis is
333 considered by most medical practitioners to be an easily treatable infection, prolonged
334 symptoms due to, or following, *G. intestinalis* infection can significantly impact the quality
335 of life (Painter et al. 2015; Vesy and Peterson 1999; Watkins and Eckmann 2014).
336 Symptom recurrence, including abdominal symptoms and fatigue, can result from re-
337 infection, treatment failure, and disturbances in the gut mucosa or post-infection syndromes
338 (Watkins and Eckmann 2014). In developed countries, these sequelae can have an
339 enormous impact on the quality of life; in developing countries, particularly in children,
340 they add yet another burden to populations that are already disadvantaged. Infection with
341 *G. intestinalis* remains latent because only a handful of agents have been used in therapy,
342 and the agents that are available may have adverse effects or be contraindicated in certain
343 clinical situations. Additionally, resistance may play a role in some infections (Vesy and
344 Peterson 1999; Watkins and Eckmann 2014). Thus, research on the development of new
345 compounds to combat giardiasis is needed.

346

347 In this work, we demonstrated that synthetic, bLF-derived LFcin17-30, LFampin265-284,
348 and LFchimera have parasiticidal activity against *G. intestinalis* trophozoites *in vitro. In*

Draft

349 *vitro* giardicidal activity of native human and bovine LF, as well as their derived N-
350 terminal peptides, has been observed previously (Turchany et al. 1995). Treated
351 trophozoites showed ultrastructural damage, with lactoferrin and its N-terminal peptides
352 causing striking and complex morphologic changes in the trophozoite plasmalemma,
353 endomembrane and cytoskeleton and increasing the electron density of the lysosome-like
354 peripheral vacuoles (Turchany et al. 1997). The synthetic peptides used in this work are
355 different from those reported by Turchany et al. (1995), but the giardicidal activity, the
356 binding and endocytosis of the peptides by the trophozoites and the damage induced at the
357 ultrastructural level were similar. Neither Fe^{3+}, Fe^{2+}, nor other compounds such as $MgCl_2$,
358 or $CaCl_2$, diminished or prevented the parasiticidal activity of LFpeptides against *G.*
359 *intestinalis* trophozoites (unpublished results). While we cannot directly compare our
360 results with Turchany et al. (1995, 1997) because we used cultures from cysts of *G.*
361 *intestinalis* directly isolated from patients that were forced to excyst in the laboratory, their
362 results support our conclusion that LFpeptides exhibit potent *in vitro* antigiardial activity.

363

364 LFchimera presented greater giardicidal activity in the viability assays and induced more
365 pronounced damage to *G. lamblia* trophozoites at the ultrastructural level compared to its
366 individual constituent peptides LFcin17-30 and LFampin265-284. All of the peptides at
367 low concentrations had a dramatic parasiticidal effect when they were combined with the
368 pharmacological drugs metronidazole or albendazole (used to treat giardiasis).

369

370 Regarding differences found with the two methods to estimate viability (PI and CFDA) in
371 *G. intestinalis* treated with LFpeptides, LF and drugs (Figures 1 A and 1 B, respectively), it
372 is known that the fluorogenic dye PI is unable to traverse intact cell membranes; therefore,

Draft

373 only cells with disrupted or broken membranes are counterstained by PI. Consequently, PI
374 is an indirect indicator of cellular viability. On the other hand, it has been established that
375 an intact lipid bilayer slows the leakage of the fluorochrome CFDA from within intact cells,
376 while injured or stressed cells cannot retain or accumulate the fluorochrome CFDA (dos
377 Santos et al. 2015; Schupp et al. 1988). Additionally, the replication time is different in the
378 distinct genotypes of *G. intestinalis*; consequently, the drug sensitivity data from *in vitro*
379 studies in Giardia varies as a function of the replication time and the methodological
380 techniques employed (Hahn et al. 2013). Therefore, both factors (different replication times
381 and methodologies) could explain the differences of results obtained using PI and CFDA
382 (Figures 1 A and 1B). Despite the differences in these methodologies, it is clear that LF and
383 LFpeptides have a parasiticidal effect on *G. intestinales* isolates, and data obtained by
384 electron microscopy (Figures 3 and 4, respectively) corroborate our conclusion that LF and
385 LF peptides exhibited giardicidal activity.
386
387 Regarding the mechanism of action, our confocal microscopy observations demonstrated
388 that LFpeptides were bound and internalized by *G. intestinalis* trophozoites (Figure 2). We
389 speculate that the localization of peptides inside *G. intestinalis* is an event involved in the
390 mechanism of action of LF peptides against this protozoan. It is likely that LFpeptides
391 caused damage to membranes, including internal membranes. In agreement with this idea,
392 LFpeptides appeared to trigger an early programmed cell death or apoptosis-like event in *G.*
393 *intestinalis* (Figure 5).
394
395 In higher eukaryotes, programmed cell-death (PCD) is the death of a cell in any form
396 mediated by an intracellular program. Programmed cell death is a genetically regulated

https://mc06.manuscriptcentral.com/bcb-pubs

Draft

397 process that is central to the development, homeostasis and integrity of multicellular
398 organisms (Ameisen 2002). Interestingly, several molecules or pathways that regulate PCD
399 in higher eukaryotes have been implicated in the death of unicellular organisms (Bruchhaus
400 et al. 2007), and apoptosis-like programmed cell death (PCD) has been described in
401 multiple primitive eukaryotes and protists, including unicellular parasites, meaning that
402 unicellular organisms can commit suicide in response to various stimuli (Bruchhaus et al.
403 2007; Kaczanowski et al. 2011; van Zandbergen et al. 2010). *G. intestinalis* is a divergent
404 amitochondrial eukaryote with a unicellular, bi-nucleated flagellated structure, but even this
405 organism undergoes PCD (Bagchi et al. 2012). However, this is a pathway of autophagy
406 and differs from the classical apoptosis of higher eukaryotes. Annexin-V and 7-AAD
407 staining was used to analyze early programmed cell death in *G. intestinalis* (Bagchi et al.
408 2012; Ghosh et al. 2009). Annexin V (or Annexin A5) is a member of the annexin family of
409 intracellular proteins and binds to phosphatidylserine (PS). Externalization of PS is an
410 indicator of early apoptosis-like or programmed cell death in Giardia (Ghosh et al. 2009).
411 7-AAD (7-amino-actinomycin D) has a high DNA binding constant and is efficiently
412 excluded by intact cells and bound by cells in necrosis or late programmed cell death. Both
413 fluorescent dyes were measured in *G. intestinalis* using Acoustic Focusing Cytometer
414 AttuneTM Blue/Red (Life Technologies). Our results demonstrate that all of the peptides
415 induced early programmed cell death in *G. intestinalis* (Figure 5). These data are
416 corroborated by the type of damage observed at the ultrastructural level (Figure 3, Panels
417 C-H, Figure 4, panels B-D).
418
419 There is no previous evidence of apoptosis-like or programmed cell death induced by bLF
420 in a parasite, but it has been reported that LF triggered programmed cell death in cells

Draft

421 infected with influenza virus (Pietrantoni et al. 2010), echovirus (Tinari et al. 2005), and
422 *Listeria monocytogenes* (Valenti et al. 1999). Additionally, LF triggers apoptosis or
423 apoptosis-like activity in microorganisms such as *Saccharomyces cerevisiae* (Acosta-
424 Zaldivar et al. 2016) and *Candida albicans* (Andres et al. 2008).
425
426 Further studies are needed to determine if LF and LFpeptides have an effect against *G.*
427 *intestinalis* in *in vivo* models. However, all data to date suggest that LFpeptides are active
428 compounds with the potential to combat giardiasis, alone or when combined with
429 chemotherapeutic drugs.
430
431 **Acknowledgments**
432 The authors thank Dr. Martha Ponce-Macotela from LPE-Instituto Nacional de Pediatría for
433 her generous donation of clinical isolates of *Giardia intestinalis,* Dr. Luis Felipe Jimenez
434 from Laboratorio MET-Facultad de Ciencias UNAM for his efficient technical support, and
435 Jorge A. Canizalez-León for his support in the preparation of images. This work was
436 supported by CONACyT México grant CB-2014-236546 (NLS). Aguilar-Diaz was a PhD
437 student from the Programa Regional del Noroeste para el Doctorado en Biotecnología de la
438 Universidad Autónoma de Sinaloa, and was supported by a PhD fellowship CONACyT-
439 México (Grant 169048). Kamran Nazmi and Dr. Jan Bolscher were supported through a
440 grant from the University of Amsterdam for research into the focal point Oral Infections
441 and Inflammation.
442
443
444

https://mc06.manuscriptcentral.com/bcb-pubs

Draft

445 ## References

446 Abdel-Hafeez, E.H., Belal, U.S., Abdellatif, M.Z., Naoi, K., and Norose, K. 2013. Breast-feeding
447 protects infantile diarrhea caused by intestinal protozoan infections. The Korean journal of
448 parasitology **51**(5): 519-524. doi: 10.3347/kjp.2013.51.5.519.
449 Acosta-Zaldivar, M., Andres, M.T., Rego, A., Pereira, C.S., Fierro, J.F., and Corte-Real, M. 2016.
450 Human lactoferrin triggers a mitochondrial- and caspase-dependent regulated cell death in
451 *Saccharomyces cerevisiae.* Apoptosis : an international journal on programmed cell death **21**(2):
452 163-173. doi: 10.1007/s10495-015-1199-9.
453 Adam, R.D. 2001. Biology of *Giardia lamblia*. Clinical microbiology reviews **14**(3): 447-475. doi:
454 10.1128/CMR.14.3.447-475.2001.
455 Ameisen, J.C. 2002. On the origin, evolution, and nature of programmed cell death: a timeline of
456 four billion years. Cell death and differentiation **9**(4): 367-393. doi: 10.1038/sj/cdd/4400950.
457 Andres, M.T., Viejo-Diaz, M., and Fierro, J.F. 2008. Human lactoferrin induces apoptosis-like cell
458 death in *Candida albicans*: critical role of K+-channel-mediated K+ efflux. Antimicrobial agents and
459 chemotherapy **52**(11): 4081-4088. doi: 10.1128/AAC.01597-07.
460 Bagchi, S., Oniku, A.E., Topping, K., Mamhoud, Z.N., and Paget, T.A. 2012. Programmed cell death
461 in Giardia. Parasitology **139**(7): 894-903. doi: 10.1017/S003118201200011X.
462 Bolscher, J., Nazmi, K., van Marle, J., van 't Hof, W., and Veerman, E. 2012. Chimerization of
463 lactoferricin and lactoferrampin peptides strongly potentiates the killing activity against *Candida*
464 *albicans.* Biochem Cell Biol **90**(3): 378-388. doi: 10.1139/o11-085.
465 Bolscher, J.G., Adao, R., Nazmi, K., van den Keybus, P.A., van 't Hof, W., Nieuw Amerongen, A.V.,
466 Bastos, M., and Veerman, E.C. 2009. Bactericidal activity of LFchimera is stronger and less sensitive
467 to ionic strength than its constituent lactoferricin and lactoferrampin peptides. Biochimie **91**(1):
468 123-132. doi: 10.1016/j.biochi.2008.05.019.
469 Bruchhaus, I., Roeder, T., Rennenberg, A., and Heussler, V.T. 2007. Protozoan parasites:
470 programmed cell death as a mechanism of parasitism. Trends in parasitology **23**(8): 376-383. doi:
471 10.1016/j.pt.2007.06.004.
472 Carranza, P.G., and Lujan, H.D. 2010. New insights regarding the biology of *Giardia lamblia.*
473 Microbes and infection / Institut Pasteur **12**(1): 71-80. doi: 10.1016/j.micinf.2009.09.008.
474 Dominguez-Lopez, M.E., Gonzalez-molero, I., Ramirez-Plaza, C.P., Soriguer, F., and Olveira, G.
475 2011. [Chonic diarrhea and malabsorption due to common variable immunodeficiency,
476 gastrectomy and giardiasis infection: a difficult nutritional management]. Nutricion hospitalaria
477 **26**(4): 922-925. doi: 10.1590/S0212-16112011000400037.
478 dos Santos, S.R., Branco, N., Franco, R.M., Paterniani, J.E., Katsumata, M., Barlow, P.W., and Gallep
479 Cde, M. 2015. Fluorescence decay of dyed protozoa: differences between stressed and non-
480 stressed cysts. Luminescence : the journal of biological and chemical luminescence **30**(7): 1139-
481 1147. doi: 10.1002/bio.2872.
482 Eckmann, L. 2003. Mucosal defences against Giardia. Parasite immunology **25**(5): 259-270.
483 Elsafi, S.H., Al-Maqati, T.N., Hussein, M.I., Adam, A.A., Hassan, M.M., and Al Zahrani, E.M. 2013.
484 Comparison of microscopy, rapid immunoassay, and molecular techniques for the detection of
485 *Giardia lamblia* and *Cryptosporidium parvum.* Parasitology research **112**(4): 1641-1646. doi:
486 10.1007/s00436-013-3319-1.
487 Escobedo, A.A., Almirall, P., Robertson, L.J., Franco, R.M., Hanevik, K., Morch, K., and Cimerman, S.
488 2010. Giardiasis: the ever-present threat of a neglected disease. Infectious disorders drug targets
489 **10**(5): 329-348.

Draft

490 Furness, B.W., Beach, M.J., and Roberts, J.M. 2000. Giardiasis surveillance--United States, 1992-
491 1997. MMWR. CDC surveillance summaries : Morbidity and mortality weekly report. CDC
492 surveillance summaries / Centers for Disease Control **49**(7): 1-13.
493 Gendrel, D., Richard-Lenoble, D., Kombila, M., Gendrel, C., and Baziomo, J.M. 1989. Giardiasis and
494 breast-feeding in urban Africa. The Pediatric infectious disease journal **8**(1): 58-59.
495 Ghosh, E., Ghosh, A., Ghosh, A.N., Nozaki, T., and Ganguly, S. 2009. Oxidative stress-induced cell
496 cycle blockage and a protease-independent programmed cell death in microaerophilic *Giardia*
497 *lamblia*. Drug design, development and therapy **3**: 103-110.
498 Gillin, F.D., Reiner, D.S., and Wang, C.S. 1983. Killing of *Giardia lamblia* trophozoites by normal
499 human milk. Journal of cellular biochemistry **23**(1-4): 47-56. doi: 10.1002/jcb.240230106.
500 Hahn, J., Seeber, F., Kolodziej, H., Ignatius, R., Laue, M., Aebischer, T., and Klotz, C. 2013. High
501 sensitivity of *Giardia duodenalis* to tetrahydrolipstatin (orlistat) in vitro. PloS one **8**(8): e71597. doi:
502 10.1371/journal.pone.0071597.
503 Hautus, M.A., Kortbeek, L.M., Vetter, J.C., and Laarman, J.J. 1988. In vitro excystation and
504 subsequent axenic growth of *Giardia lamblia.* Transactions of the Royal Society of Tropical
505 Medicine and Hygiene **82**(6): 858-861.
506 Hill, D.R. 1993. Giardiasis. Issues in diagnosis and management. Infectious disease clinics of North
507 America **7**(3): 503-525.
508 Kaczanowski, S., Sajid, M., and Reece, S.E. 2011. Evolution of apoptosis-like programmed cell
509 death in unicellular protozoan parasites. Parasites & vectors **4**: 44. doi: 10.1186/1756-3305-4-44.
510 Morrow, A.L., Reves, R.R., West, M.S., Guerrero, M.L., Ruiz-Palacios, G.M., and Pickering, L.K. 1992.
511 Protection against infection with *Giardia lamblia* by breast-feeding in a cohort of Mexican infants.
512 The Journal of pediatrics **121**(3): 363-370.
513 Nabarro, L.E., Lever, R.A., Armstrong, M., and Chiodini, P.L. 2015. Increased incidence of
514 nitroimidazole-refractory giardiasis at the Hospital for Tropical Diseases, London: 2008-2013.
515 Clinical microbiology and infection: the official publication of the European Society of Clinical
516 Microbiology and Infectious Diseases **21**(8): 791-796. doi: 10.1016/j.cmi.2015.04.019.
517 Ochoa, T.J., Chea-Woo, E., Campos, M., Pecho, I., Prada, A., McMahon, R.J., and Cleary, T.G. 2008.
518 Impact of lactoferrin supplementation on growth and prevalence of Giardia colonization in
519 children. Clinical infectious diseases : an official publication of the Infectious Diseases Society of
520 America **46**(12): 1881-1883. doi: 10.1086/588476.
521 Painter, J.E., Gargano, J.W., Collier, S.A., and Yoder, J.S. 2015. Giardiasis surveillance -- United
522 States, 2011-2012. MMWR supplements **64**(3): 15-25.
523 Perez-Rangel, A., Hernandez, J.M., Castillo-Romero, A., Yepez-Mulia, L., Castillo, R., Hernandez-
524 Luis, F., Nogueda-Torres, B., Luna-Arias, J.P., Radilla, G., and Leon-Avila, G. 2013. Albendazole and
525 its derivative JVG9 induce encystation on *Giardia intestinalis* trophozoites. Parasitology research
526 **112**(9): 3251-3257. doi: 10.1007/s00436-013-3521-1.
527 Pietrantoni, A., Dofrelli, E., Tinari, A., Ammendolia, M.G., Puzelli, S., Fabiani, C., Donatelli, I., and
528 Superti, F. 2010. Bovine lactoferrin inhibits influenza A virus induced programmed cell death in
529 vitro. Biometals: an international journal on the role of metal ions in biology, biochemistry, and
530 medicine **23**(3): 465-475. doi: 10.1007/s10534-010-9323-3.
531 Roxstrom-Lindquist, K., Palm, D., Reiner, D., Ringqvist, E., and Svard, S.G. 2006. Giardia immunity--
532 an update. Trends in parasitology **22**(1): 26-31. doi: 10.1016/j.pt.2005.11.005.
533 Schupp, D.G., Januschka, M.M., Sherlock, L.A., Stibbs, H.H., Meyer, E.A., Bemrick, W.J., and
534 Erlandsen, S.L. 1988. Production of viable Giardia cysts in vitro: determination by fluorogenic dye
535 staining, excystation, and animal infectivity in the mouse and Mongolian gerbil. Gastroenterology
536 **95**(1): 1-10.

Draft

537 Stojecki, K., Sroka, J., Karamon, J., Kusyk, P., and Cencek, T. 2014. Influence of selected stool
538 concentration techniques on the effectiveness of PCR examination in *Giardia intestinalis*
539 diagnostics. Polish journal of veterinary sciences **17**(1): 19-25.
540 Tinari, A., Pietrantoni, A., Ammendolia, M.G., Valenti, P., and Superti, F. 2005. Inhibitory activity of
541 bovine lactoferrin against echovirus induced programmed cell death in vitro. Int J Antimicrob
542 Agents **25**(5): 433-438. doi: S0924-8579(05)00060-9 [pii] 10.1016/j.ijantimicag.2005.02.011.
543 Turchany, J.M., Aley, S.B., and Gillin, F.D. 1995. Giardicidal activity of lactoferrin and N-terminal
544 peptides. Infection and immunity **63**(11): 4550-4552.
545 Turchany, J.M., McCaffery, J.M., Aley, S.B., and Gillin, F.D. 1997. Ultrastructural effects of
546 lactoferrin binding on *Giardia lamblia* trophozoites. J Eukaryot Microbiol **44**(1): 68-72.
547 Valenti, P., Greco, R., Pitari, G., Rossi, P., Ajello, M., Melino, G., and Antonini, G. 1999. Apoptosis of
548 Caco-2 intestinal cells invaded by *Listeria monocytogenes*: protective effect of lactoferrin.
549 Experimental cell research **250**(1): 197-202. doi: 10.1006/excr.1999.4500.
550 van Zandbergen, G., Luder, C.G., Heussler, V., and Duszenko, M. 2010. Programmed cell death in
551 unicellular parasites: a prerequisite for sustained infection? Trends in parasitology **26**(10): 477-
552 483. doi: 10.1016/j.pt.2010.06.008.
553 Vázquez-Nin, G.H., and Echeverría, O.M. 2000. Introducción a la Microscopía Electrónica Aplicada
554 a las Ciencias Biológicas. Universidad Nacional Autónoma de México Fondo de Cultura Económica.
555 **México City**.
556 Vesy, C.J., and Peterson, W.L. 1999. Review article: the management of Giardiasis. Alimentary
557 pharmacology & therapeutics **13**(7): 843-850.
558 Watkins, R.R., and Eckmann, L. 2014. Treatment of giardiasis: current status and future directions.
559 Current infectious disease reports **16**(2): 396. doi: 10.1007/s11908-014-0396-y.

560
561
562
563
564
565
566
567
568
569
570
571

Draft

572 **Figure legends**

573

574 Figure 1. *LFcin17-30, LFampin265-284 and LFchimera have parasiticidal activity against*
575 *Giardia intestinalis trophozoites. G. intestinalis* cultures were incubated with 100 µM
576 Metronidazole or 5 µM Albendazole (used as negative controls of viability) or 40 µM
577 LFpeptides for 2 h at 37ºC (Panel A), or with different concentrations of Metronidazole
578 (used as a negative control of culture growth), LF, or LFpeptides for 12 h at 37ºC (Panel
579 B). Then, the samples were washed and viability or culture growth was determined by the
580 exclusion of the dye PI (A) or inclusion of CFDA (B). In both cases (A and B), untreated
581 cultures were used as positive controls of viability or growth. Experiments were performed
582 at least three times in triplicate. The means and standard deviations are indicated in
583 percentages. A value of $P<0.05$ was considered statistically significant (*).

Draft

584

585 Figure 2. *LFcin17-30, LFampin265-284 and LFquimera are internalized by Giardia*
586 *intestinalis.* Trophozoites were untreated (A) or treated with 2 µM FITC-LFpeptides for 30
587 min at 37 ºC: LFcin17-30 (B), LFampin265-284 (C), and LFchimera (D). Then, samples
588 were fixed, washed and permeabilized with 0.5% triton X-100 and counterstained with PI
589 (red color). Finally, samples were processed for analysis by confocal microscopy. Arrows
590 show the green fluorescence due to the FITC-LFpeptides (B-D). Bars 20 and 50 µm.

591

592 Figure 3. *LFcin17-30, LFampin265-284 and LFchimera cause damage to Giardia*
593 *intestinalis.* Trophozoites were untreated (A, B) or treated with 40 µM LFcin17-30 (C, D),
594 LFampin265-284 (E, F), or LFchimera (G, H), for 2 h at 37 ºC. Then, samples were

595 processed for analysis by transmission electron microscopy. The sections were analyzed
596 and photographed using a JEOL 1010 transmission electron microscope at 80 kV.
597 Untreated cells had a smooth cellular membrane (cm, double-headed arrow), peripheral
598 vacuoles (pv, lines) near the cellular membrane, three pairs of flagella (F, discontinuous
599 arrow), adherent disk (ad), and two nuclei (N) and two nucleoli (no) (Figure 3, panel A).
600 Magnification of the picture shows granules of electron-dense material (asterisk) distributed
601 in the cytoplasm and the arrangement of the microtubules belonging to the adherent disk
602 (ad) (panel B). Exposure to LFcin17-30 led to profound intracellular changes, such as an
603 increase in electron-dense material in the cytoplasm (asterisk), reorganization of the flagella
604 (F, discontinuous arrow) and displacement of the adherent disk (ad, arrow), Figure 3, panel
605 C. In the magnified picture, there are no peripheral vacuoles (pv) near the cellular
606 membrane (cm, double-headed arrow), Panel D. Treatment with LFampin265-284 also
607 caused intracellular damage, including an increase in electron-dense material (asterisk),
608 reorganization of the flagella (F, discontinuous arrow), a large hole in the cytoplasm
609 (arrowhead), and also disruption in the cellular membrane (cm, double-headed arrow)
610 (Figure 3, panel E). The magnified picture shows the large hole induced by treatment with
611 LFampin265-284 (arrowhead, panel F). Trophozoites treated with LFchimera produced the
612 most significant changes and damage in the trophozoites. There were marked changes in
613 the electron-dense material in the cytoplasm (asterisks), the flagella (F, discontinuous
614 arrow) were disrupted and reorganized, and the cytoplasm showed large holes (arrowheads)
615 in which some electron-dense material is visible (Figure 3, panel G). In the magnified
616 picture the larges holes (arrowheads) with aggregates inside them (asterisks) can be seen in
617 more detail (Panel H). Shrunken and distorted peripheral vacuoles (pv, lines) were also
618 observed.

Draft

619 Figure 4. *LFcin17-30, LFampin265-284 and LFchimera cause damage* to *Giardia*
620 *intestinalis.* Trophozoites were untreated (A) or treated with 40 µM of LFcin17-30 (B),
621 LFampin265-284 (C), or LFchimera (D) for 2 h at 37 ºC. Then, samples were processed for
622 analysis. Specimens were observed under a scanning electron microscope JEOL
623 LSM6360LV. Trophozoites treated with LF peptides show alterations in size, irregular
624 form and perforations (arrows, B-C), compared with untreated trophozoites (which had the
625 typical structure of *G. intestinalis* trophozoites, A). Trophozoites treated with LFchimera
626 exhibited aggregates or vesicles and several had a large hole in their membranes (arrow,
627 panel D).
628
629 Figure 5. *Programmed cell death in Giardia intestinalis induced by LFpeptides.*
630 Trophozoites of *G. intestinalis* were treated with 40 µM of LFcin17-30 (A), LFampin265-
631 284 (B) or LFchimera (C) for 2 h at 37 ºC. Samples were stained with Allophycocyanin-
632 Annexin (Annexin-APC, to stain Annexin V) and 7-Amino-Actinomicyn D (7-AAD),
633 processed and analyzed by flow cytometry. Q1: Necrotic cells, Q2: Necroptotic cells, Q3:
634 Earlier apoptotic cells. Q4: live cells. One of three representative experiments is shown.
635
636
637
638
639
640
641
642

Draft

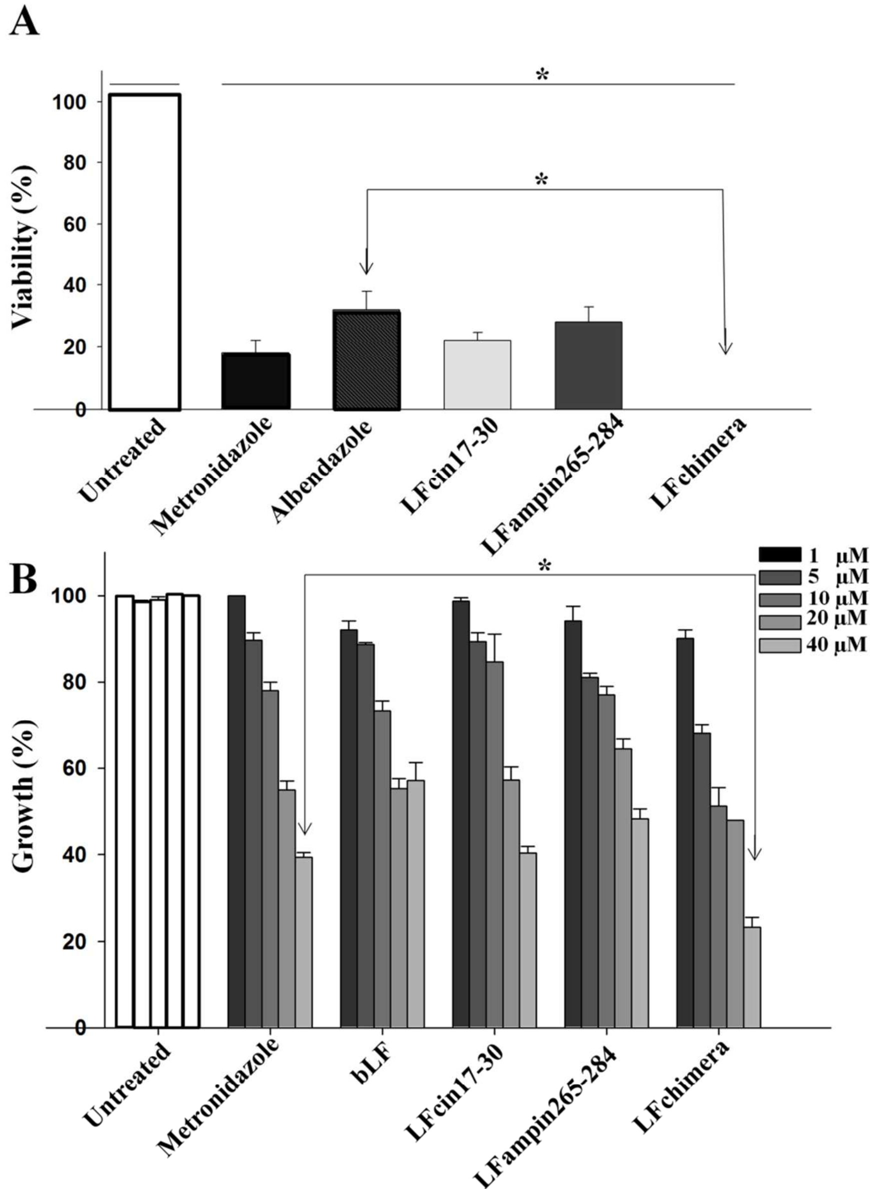
643 **Table I. Metronidazole, albendazole and LFpeptides needed alone or in combination**
644 **to kill >95% of *Giardia intestinalis* trophozoites**
645
646

Compounds	Concentrations (µM)	LFcin 17-30 (µM)	LFampin 265-284 (µM)	LFchimera (µM)
Metronidazole	**100**	-	-	-
Metronidazole	20	20	-	-
Metronidazole	20	-	30	-
Metronidazole	20	-	-	5
Albendazole	**5**	-	-	-
Albendazole	3	20		
Albendazole	3		30	
Albendazole	3			5
LFcin17-30	**80**	-	-	-
LFampin265-284	**80**	-	-	-
LFchimera	**40**	-	-	-

Draft

647
648
649
650
651
652
653
654
655
656
657
658
659
660

661 Figure 1



662

663

664

665

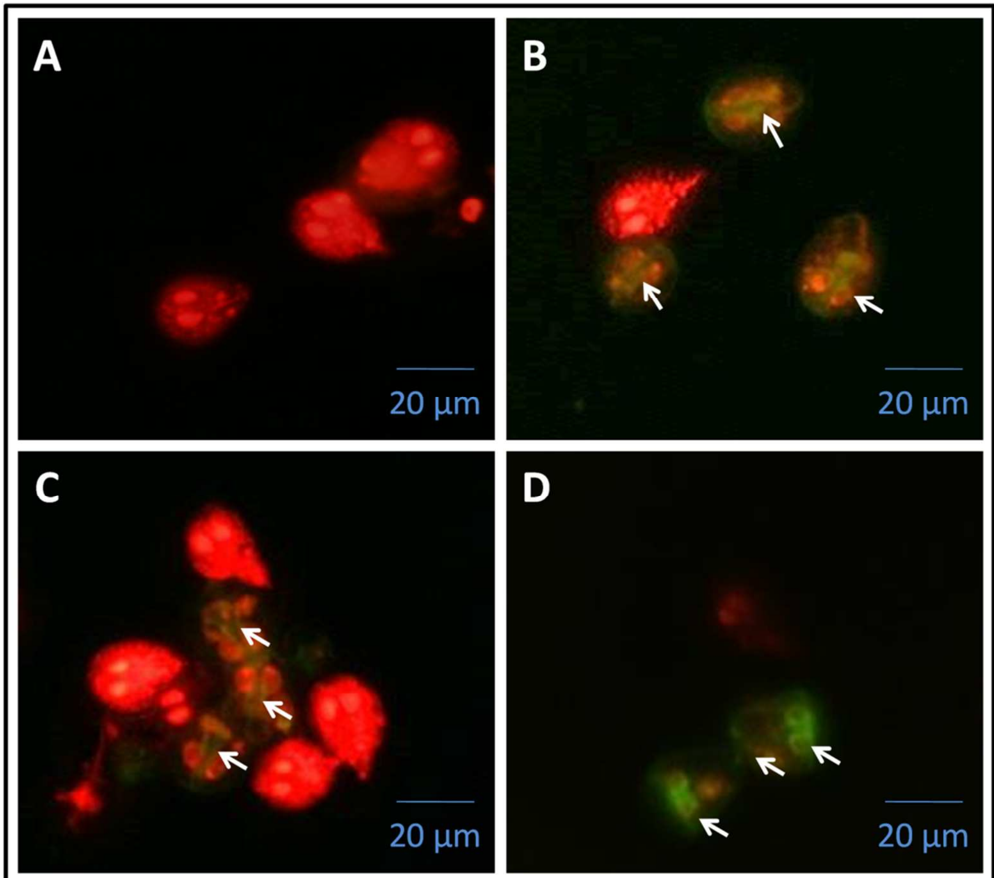
666

667

668

669

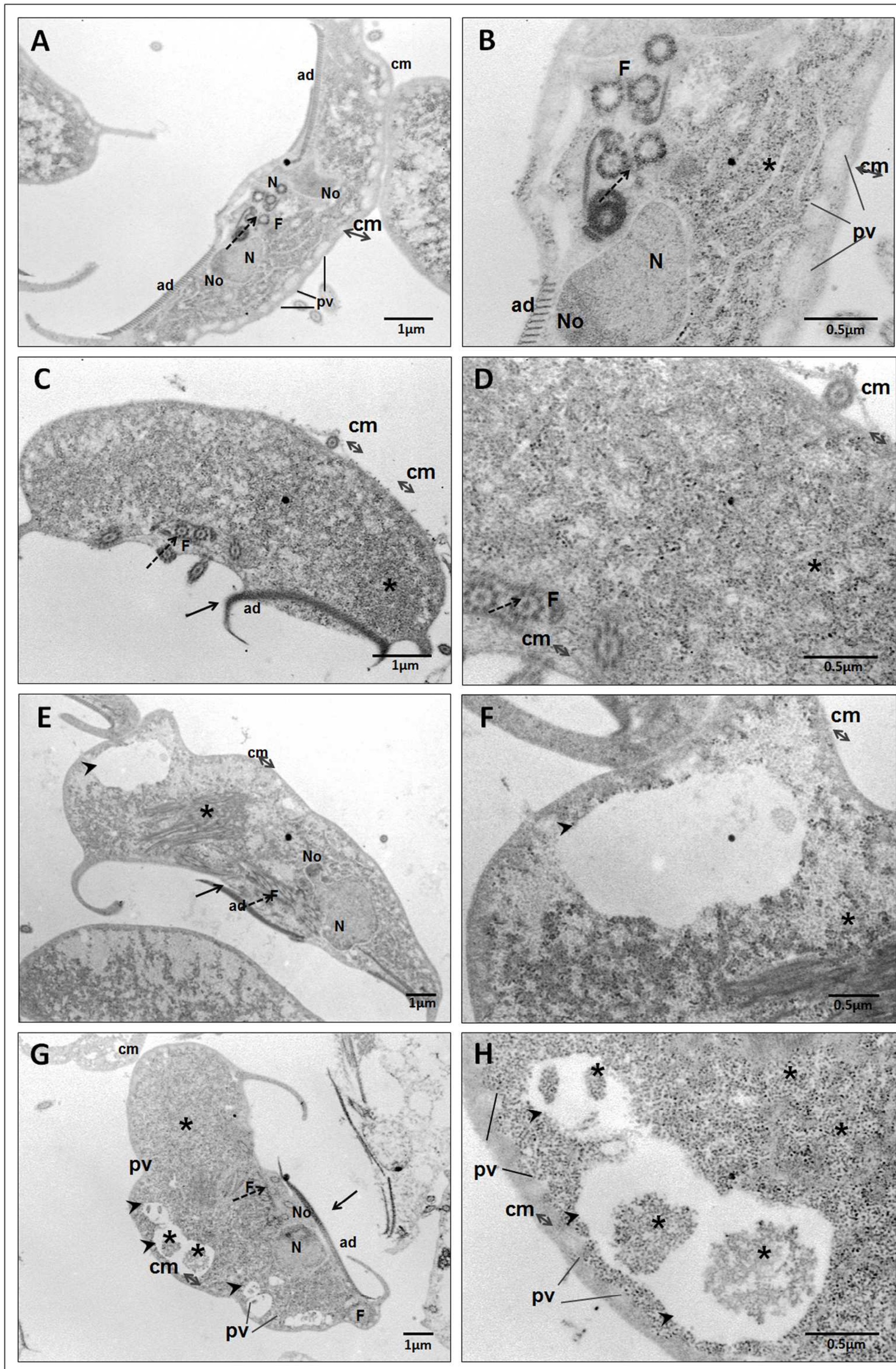
670 Figure 2



671
672
673
674
675
676
677
678
679
680
681

682

683 Figure 3



684

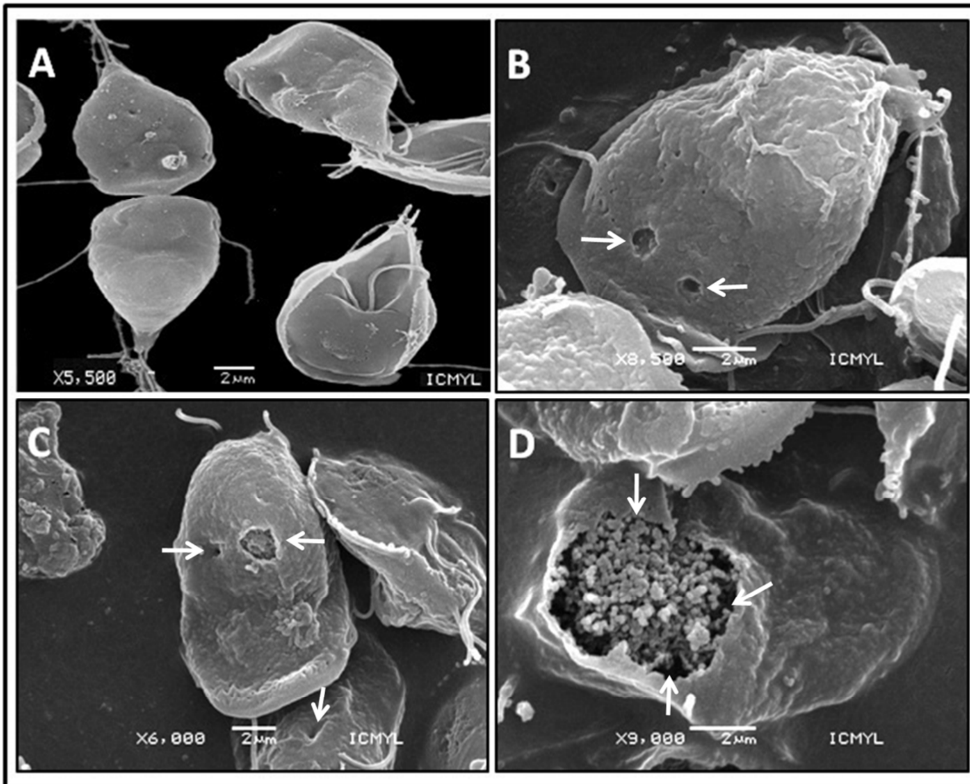
685

686

687

688 Figure 4

689



690

691

692

693

694

695

696

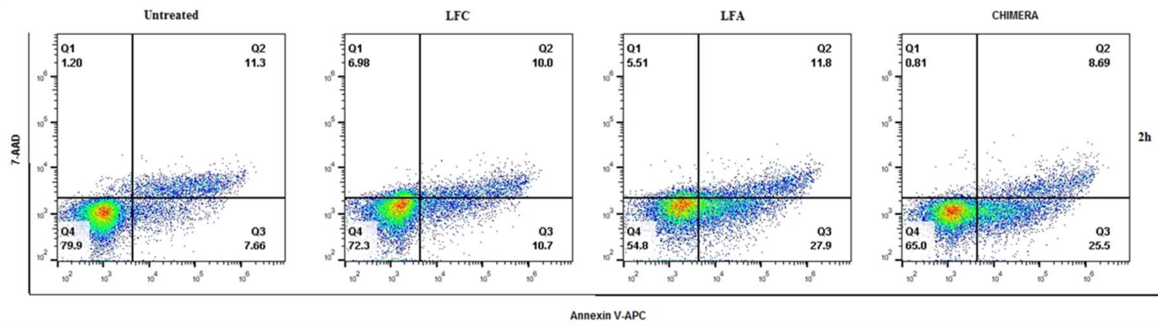
697

698

699

700

701 Figure 5



702

Draft