

EFFECT OF LACTOBACILLUS POSTBIOTICS ON *ENTAMOEBIA HISTOLYTICA* TROPHOZOITES

FABIOLA L. CUELLAR-GUEVARA, MARÍA P. BARRÓN-GONZÁLEZ*, AND JORGE L. MENCHACA-ARREDONDO

Department of Cell Biology and Genetics, Faculty of Biological Sciences, Universidad Autónoma de Nuevo León, Nuevo León, México

ABSTRACT

Background: Amebiasis is an infectious disease caused by *Entamoeba histolytica*. It represents one of the three worldwide leading causes of death by parasites and a public health problem due to its frequency, morbidity, mortality, and easy dispersion. **Objective:** The study was aimed to evaluate the *in vitro* effect of *Lactobacillus spp.* postbiotics on *E. histolytica* trophozoites (HM1-IMSS strain) and to determine morphometric changes in trophozoite membrane by atomic force microscopy (AFM). **Methods:** Bioassays on trophozoites were conducted with lyophilized postbiotics at 0.1, 0.3, and 0.5 mg/mL concentrations, and trophozoite samples were obtained for AFM analysis. **Results:** Results indicated postbiotic inhibitory activity; the highest percentage inhibition was 89.63% at 0.5 mg/mL. Trophozoites nanomechanical analysis showed 28.32% increase in ruggedness and 56% decrease in size with treatments compared to the control. **Conclusion:** Our study showed that the synergy of *Lactobacillus* postbiotics inhibited *E. histolytica* HM1-IMSS *in vitro* growth under axenic conditions, inducing morphometric alterations in trophozoites' cell membrane. These results would allow designing strategies or treatments aimed at *E. histolytica* control in the future. (REV INVEST CLIN. 2019;71:402-7)

Key words: Protozoa. Amoebiasis. Probiotic. Metabolites. Antimicrobial. Atomic force microscopy.

INTRODUCTION

Amebiasis is an infection that may have or not clinical manifestations; 90% of amebic infections are asymptomatic and self-limiting (estimated, 50 million cases per year). Only 10% of infected persons develop an acute intestinal or extraintestinal disease¹. According to the World Health Organization (WHO), *Entamoeba*

histolytica is the third leading parasitic cause of death (100,000 deaths/year)², and after malaria, amebiasis is the second most lethal disease caused by a protozoan parasite. This infection has a worldwide distribution, with a higher incidence in developing countries and high prevalence in countries with poor sanitary and socioeconomic conditions³. The risk groups include male homosexuals, travelers and

*Corresponding author:
María P. Barrón-González
E-mail: maria.barrongn@uanl.edu.mx

Received for publication: 07-06-2019
Approved for publication: 23-07-2019
DOI: 10.24875/RIC.19003134

recent immigrants, and institutionalized populations⁴. Food and water contamination combined with a poor hygiene are the main sources of transmission. Water-associated outbreaks of *E. histolytica* disease as well as sexually transmitted infections have been reported⁵. Metronidazole is the first-line drug for the treatment of invasive amebiasis, although it has undesirable side effects (nausea, headache, and among other adverse manifestations) and is potentially carcinogenic⁶. Unfortunately, *E. histolytica*, as many other parasites, has developed mechanisms to evade the drug pressure⁷ and became resistant^{8,9}. Due to the medical importance of *E. histolytica*, it is necessary to search for alternative compounds that inhibit this parasite. Investigations have been conducted with probiotic bacteria to find therapeutic alternatives for amebiasis. Prebiotics, probiotics, and synbiotics are appealing as preventive and therapeutic agents for human medical disorders. Their efficacy depends on disease etiology and the probiotic strain used¹⁰. Probiotics are living microorganisms that confer health benefits to the host when administered in adequate amounts; however, dead bacteria and their components can also exhibit probiotic properties¹¹. Recently, increasing evidence supports the notion that certain probiotic-derived components, such as bacteriocins, lipoteichoic acids, surface layer protein, and secreted protein, have a similar protective role on intestinal barrier function as that of live probiotics. These bioactive components have been named “postbiotics” in the most recent publications¹². Postbiotics are “soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis,” and they provide physiological benefits to the host^{13,14}. However, most investigations in this field are focused on infections of bacterial origin and not on those of parasitic origin.

Typical characteristics of *E. histolytica* are observed by specific stains and the optical, fluorescence, transmission electron, or scanning microscopes have been used to examine this material¹⁵. However, these cells can be currently analyzed using new microscopic tools such as atomic force microscopy (AFM), which allows to observe structures at a nanometric resolution and analyze physical-mechanical properties to study biological material¹⁶. In the present study, the *in vitro* effect of lyophilized postbiotics on *E. histolytica* trophozoites was determined, and cell membranes were analyzed by AFM to evaluate morphometric changes.

METHODS

E. histolytica culture medium

The TYI-S-33 medium for *E. histolytica* (HM1-IMSS strain) culture and maintenance was prepared according to Diamond et al.¹⁷

Probiotic bacteria culture and obtention of postbiotics

Lactobacillus plantarum (229 strain) and *Lactobacillus casei* (CCRC 10697 strain) were cultivated in Man-Rogosa-Sharpe broth (Difco, Becton Dickinson Co., Sparks, MD, USA) and incubated (LabNet[®]) for 24–48 h at 37°C. After incubation, the extracellular factor supernatant was obtained by centrifugation (IEC CL30 Centrifuge Thermo SCIENTIFIC[®]) at 2500 rpm for 20 min. The recovered material was filtered using Millipore container (0.22 µm, GP Millipore Express[®] PLUS Membrane, Massachusetts, USA). Then, 140.26 g of ammonium sulfate were added to 80% saturation, stirring during 24 h at 4°C in an ice bath. The mixture was centrifuged at 2547 rpm for 1 h. The obtained protein precipitate was resuspended with 0.2 M phosphate buffer at pH 7.2 and was dialyzed into membranes (spectra/Por membrane tubing). A protein qualitative determination was made to dialyzed material by Biuret test. The dialyzed material was placed in glass containers (LABCONCO[®]) for lyophilization (LABCONCO Corporation, Kansas City, Missouri 63132, USA) at 0.133 mBar pressure and –40°C collector temperature for 12 h.

Bioassay

The *in vitro* effect of lyophilized postbiotics on *E. histolytica* trophozoites was evaluated. *E. histolytica* trophozoites (2×10^4 cell/mL) were axenically grown in 13 × 150 mm borosilicate tubes containing 5 mL of TYI-S-33 medium and 0.55 mL of adult bovine serum-antibiotic mixture (400 IU penicillin and 4 mg streptomycin). Different concentrations (0.1, 0.3, and 0.5 mg/mL) of lyophilized postbiotics from each strain were added; 5 mm coverslips were placed inside the tubes and incubated for 72 h at 37°C. Nisin, at the same concentrations as the postbiotics, and metronidazole (0.1 mg/mL) were also incubated in separate assays with trophozoites. Each coverslip was removed for AFM analysis. Tubes were chilled in an

Table 1. Effect of *Lactobacillus* postbiotics on cell growth of *E. histolytica* trophozoites after 72 h incubation

Treatment	Concentration (mg/mL)	Growth (1×10^4 cell/mL)	Growth inhibition (%) ^a at 72 h
<i>Lactobacillus casei</i> postbiotics	0.1	3.68	63.65
	0.3	8.1	20
	0.5	4.71	53.48
<i>Lactobacillus plantarum</i> postbiotics	0.1	9.01	11
	0.3	5.1	49.63
	0.5	8.05	20.49
Postbiotics synergy	0.1	6.37	37
	0.3	4	60.49
	0.5	1.05	89.63*
Nisin	0.1	2	80.25
	0.3	1.7	83.21
	0.5	1.05	89.63*
Metronidazole	0.1	0	100
Control		10.13	–

^aMean values determined in three independent events done in triplicate.

*The data are highly significant according to the ANOVA and Tukey test.
ANOVA: Analysis of variance.

ice-water bath for 20 min at 4°C. The total number of cells per mL was determined using a hemocytometer (Neubauer chamber, American®).

Preparation of trophozoite samples and AFM analysis

The trophozoites adhered to the coverslips surface were fixed with 2.5% glutaraldehyde tempered at 37°C during 30 min. Coverslips were rinsed with de-ionized water to remove glutaraldehyde excess and placed with double-sided tape on the special sample holder to observe by AFM. An AFM – Ntegra Prima NT-MDT sitting on an active damping table was used to observe the samples employing a silicon nitride (Si₃N₄) rectangular cantilever (RTESPA-300, tip radius 8 nm) with a length of 125 µm and nominal spring constant of 40 N/m. AFM images (height, amplitude, and phase) were obtained under normal conditions while operating the instrument in semi-contact mode (tapping). On the other hand, roughness analysis was performed comparing the obtained images using a cantilever as mentioned above with a nominal resonant frequency of 300 kHz. The analysis was carried out using the software package NOVA version 1.1.0.1921 and WSxM v5.0 Develop 8.0.

Statistical analysis

An analysis of variance test was performed to determine the *in vitro* effect of postbiotics on *E. histolytica* trophozoites with $p < 0.05$ using the statistical software package SPSS version 17.0. Tukey multiple comparison test with a significance level of <5% was then performed.

RESULTS

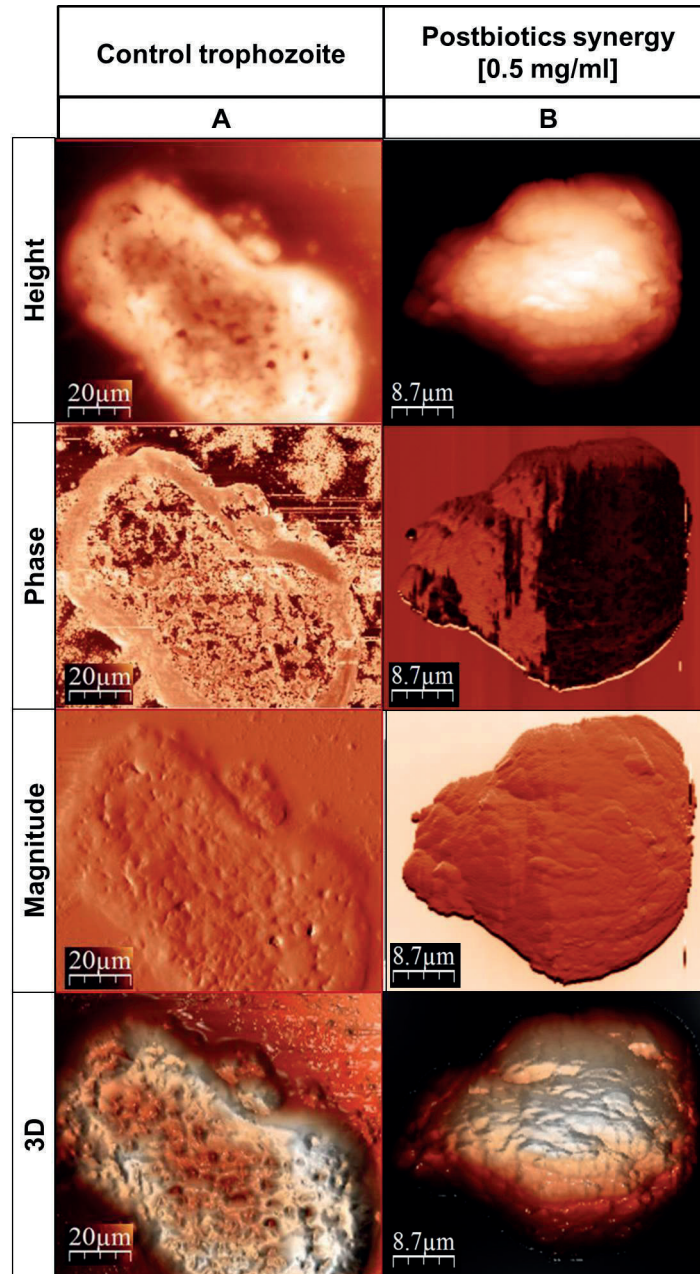
Bioassay

Table 1 shows the *in vitro* effect of postbiotics on *E. histolytica* trophozoites. The lyophilized postbiotics showed variable percentages of inhibition for each strain evaluated independently. The percentage inhibition of postbiotics synergy at a concentration of 0.5 mg/mL was significantly higher than the control and similar to metronidazole and was the same as Nisin. Significant difference between treatments was found.

AFM analysis

We analyzed the *in situ* cell morphology of *E. histolytica* trophozoites using tapping mode (Fig. 1). The

Figure 1. *E. histolytica* images by atomic force microscopy. (A) Control trophozoite: the cell membrane's porosity and pleomorphic structure can be appreciated. (B) Trophozoites' size decreased with postbiotics synergy treatment at 0.5 mg/mL concentration.



typical pleomorphic structure and a measure range of 10-60 µm in control trophozoites were observed, as reported in literature⁴ (Column A). Furthermore, phase and magnitude images showed homogeneity in cell membrane composition, with a magnitude of 40 nA. The three-dimensional (3D) micrograph of control trophozoite showed a pleomorphic structure, maximum height of 5 µm and a membrane topography with indentations and pores (Column A), while

the trophozoite with treatment in the 3D micrograph presented a rounded and smaller structure and a uniform membrane with less pores (Column B).

Table 2 shows the AFM analysis where there was an increase in roughness of 28.32% and a decrease in size of 56% after postbiotic synergy treatment at 0.5 mg/mL concentration compared to the control.

Table 2. Morphometric analysis of *Entamoeba histolytica* trophozoites using software package NOVA version 1.1.0.1921 and WSxM v5.0 Develop 8.0

<i>E. histolytica</i> treatment	Morphometric properties		
	Morphology	Size (µm)	Roughness (nm)
Control trophozoite	Pleomorphic structure	74.14	359.5462
With postbiotics synergy (0.5 mg/mL)	Round and small structure	32.6	461.3557

^aMean values determined in three independent events done in triplicate.

*The data are highly significant according to the ANOVA and Tukey test. ANOVA: Analysis of variance.

DISCUSSION

To provide a safer, non-toxic, and effective alternative to antiprotozoal drugs, recent studies have been aimed to evaluate the effect of probiotics against intestinal diseases. The WHO suggested using probiotic metabolites with microbial interference capacity to prevent pathogen intestinal colonization, producing antagonistic substances¹⁴. Many investigations have determined the inhibitory activity or microbial interference of probiotic metabolites against bacteria of medical importance. However, in few studies, this activity has been evaluated on medically important protozoa¹⁸. A recent study¹⁹ reported *in vitro* coculture of *E. invadens* in the presence of *L. casei* and *Enterococcus faecium*. The percentage of survival reduced gradually up to 80%, similar to what we found in this work. Another study using *Lactobacillus fermentum* and *Lactobacillus delbrueckii* on *in vitro* growth of *E. histolytica* reported the effective inhibiting parasite proliferation²⁰. Some probiotic-derived components (postbiotics) such as bacteriocins have antimicrobial action on microorganisms of medical importance without toxicity to eukaryotic cells. A recent study reported the *in vitro* and *in vivo* activity of *L. salivarius* bacteriocins and supernatant on *E. histolytica*²¹. In the present work, we report the inhibitory activity of postbiotics on *in vitro* growth of *E. histolytica* trophozoites. Results showed that postbiotics synergy produced a higher percentage of inhibition than postbiotics separately, suggesting that *Lactobacillus* postbiotics could inhibit proliferation of *E. histolytica* trophozoites. These results support considering the use of postbiotics as a therapeutic alternative against amebiasis, additionally decreasing metronidazole consumption, due to current undesirable side effects in patients. As perspectives, we suggest to continue *in vivo* studies of the effect of

Lactobacillus postbiotics on *E. histolytica* to demonstrate whether the *in vitro* inhibitory activity observed in the laboratory can be reproduced in an animal model and may have a future application on disease control in patients with amebiasis. These metabolites could be considered a natural alternative, safe, and without toxicity in the future as a substitute compound for the first-choice drugs for treatment, such as metronidazole, avoiding adverse clinical effects. On the other hand, there are few investigations on *E. histolytica* analysis by AFM. In the first report, our research group studied *E. histolytica* trophozoite, precyst, and cyst *in situ* characterization and made clear some differences (roughness, composition, elasticity, and size) between each stage^{16,22}. In this study, the surviving cells were analyzed by AFM, and we observed a decrease in cell size and a modified typical cell morphology using postbiotics synergy treatment. This agrees with bacteriocin properties causing cellular alterations in sensitive cells according to each bacteriocin type. This is the first report demonstrating inhibition of *E. histolytica* trophozoites using a synergy of *Lactobacillus* postbiotics, analyzing parasite membrane morphometric alterations by AFM.

In summary, postbiotic synergy derived from *L. casei* and *L. plantarum* exerts significant antiamebic activity on *E. histolytica* HM1-IMSS *in vitro* growth under axenic conditions and induce morphometric alterations in the cell membrane of trophozoites.

REFERENCES

1. Varet H, Shaulov Y, Sismeiro O, Trebicz M, Legendre R, Coppée J, et al. Enteric bacteria boost defences against oxidative stress in *Entamoeba histolytica*. *Sci Rep*. 2018;8:9042.
2. Solaymani-Mohammadi S, Petri WA. Intestinal Invasion by *Entamoeba histolytica*. In: Burleigh B, Soldati-Favre D, editors. *Molecular Mechanisms of Parasite Invasion*. Subcell Biochemistry. New York: Springer; 2008. p. 221-32.

3. World Health Organization. Amoebiasis: an Expert Consultation. Weekly Epidemiological Record. Vol. 72. Geneva: World Health Organization; 1997. p. 97-9.
4. Centers for Disease Control and Prevention. DPDx-Laboratory Identification of Parasites of Public Health Concern. Amebiasis. Available from: <https://www.cdc.gov/dpdx/amebiasis/index.html>. [Last accessed on 2017 Oct 30].
5. Bayoumy A, Elkeiy M, Zaalok T, Gad H, Abd Elhamid W. Copro-antigen versus classical microscopy as a diagnostic tool for *Entamoeba histolytica* infection in the Egyptian patients. Egypt J Hosp Med. 2019;74:1423-7.
6. Adil M, Iqbal W, Adnan F, Wazir S, Khan I, Khayam MU, et al. Association of metronidazole with cancer: a potential risk factor or inconsistent deductions? Curr Drug Metab. 2018;19:902-9.
7. García C, Marchat L, López L, Ishiwara D, Rodríguez M, Orozco E. Drug resistance mechanisms in *Entamoeba histolytica*, *Giardia lamblia*, *Trichomonas vaginalis*, and opportunistic anaerobic protozoa. In: Mayers D, Sobel J, Ouellette M, Kaye K, Marchaim D, editors. Antimicrobial Drug Resistance. Berlin: Springer; 2017. p. 613-28.
8. Wassmann C, Hellberg A, Tannich E, Bruchhaus I. Metronidazole resistance in the protozoan parasite *Entamoeba histolytica* is associated with increased expression of iron-containing superoxide dismutase and peroxiredoxin and decreased expression of ferredoxin 1 and flavin reductase. J Biol Chem. 1999;274:26051-6.
9. Ohnishi K, Sakamoto N, Kobayashi K, Iwabuchi S, Nakamura-Uchiyama F, Ajisawa A, et al. Subjective adverse reactions to metronidazole in patients with amebiasis. Parasitol Int. 2014; 63:698-700.
10. Patel R, DuPont HL. New approaches for bacteriotherapy: prebiotics, new-generation probiotics, and synbiotics. Clin Infect Dis. 2015;60 Suppl 2:S108-21.
11. Plaza-Díaz J, Ruiz-Ojeda FJ, Gil-Campos M, Gil A. Mechanisms of action of probiotics. Adv Nutr. 2019;10:S49-66.
12. Gao J, Li Y, Wan Y, Hu T, Liu L, Yang S, et al. A novel postbiotic from *Lactobacillus rhamnosus* GG with a beneficial effect on intestinal barrier function. Front Microbiol. 2019;10:477.
13. Reynés B, Palou M, Rodríguez AM, Palou A. Regulation of adaptive thermogenesis and browning by prebiotics and postbiotics. Front Physiol. 2018;9:1908.
14. Aguilar J, García R, García H, Mata V, González A, Vallejo B, et al. Postbiotics: an evolving term within the functional foods field. Trends Food Sci Technol. 2018;75:105-14.
15. Aguilar-Díaz H, Díaz-Gallardo M, Lacleste JP, Carrero JC. *In vitro* induction of *Entamoeba histolytica* cyst-like structures from trophozoites. PLoS Negl Trop Dis. 2010;4:e607.
16. Menchaca J, Barrón M, León A, Ortega J, Villarreal J, Hernandez J, et al. *Entamoeba histolytica*: trophozoite, precyst and cyst studied by atomic force microscopy. In: Mendez A, editors. Microscopy: advances in Scientific Research and Education. Mexico: Méndez; 2014. p. 153-60.
17. Diamond LS, Harlow DR, Cunnick CC. A new medium for the axenic cultivation of *Entamoeba histolytica* and other Entamoeba. Trans R Soc Trop Med Hyg. 1978;72:431-2.
18. Travers M, Florent I, Kohl L, Grellier P. Probiotics for the control of parasites: an overview. J Parasitol Res. 2011;2011:11.
19. Sarjapuram N, Mekala N, Singh M, Tatu U. The potential of *Lactobacillus casei* and *Enterococcus faecium* combination as a preventive probiotic against *Entamoeba*. Probiotics Antimicrob Proteins. 2017;9:142-9.
20. Shafeek Z, Ruzzuki N. The effect of different concentrations of the run and stuck bacteria (*Lactobacillus fermentum*, *Lactobacillus delbrueckii*) in trophozoite growth of *Entamoeba histolytica in vitro*. Int J Curr Microbiol Appl Sci. 2016;5: 924-30.
21. Mohammed S, Khalaf K, Sulaiman N. *Lactobacillus salivarius* bacteriocin and supernatant activity against *Entamoeba histolytica in vitro* and *in vivo*. Br J Biol Health Med Sci Res. 2015; 3:19-28.
22. Ortega J, Barrón M, Gracia M, Perez E, Lopez A, Menchaca J. Biological samples observed *in vitro* by atomic force microscopy: morphology and elastic properties. In: Mendez A, editors. Microscopy: advances in Scientific Research and Education. Bada-joz: Formatex Research Center; 2014. p. 131-40.