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Enzymatic, antimicrobial, and leishmanicidal bioactivity of gram-negative bacteria strains from the midgut of *Lutzomyia evansi*, an insect vector of leishmaniasis in Colombia

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ABSTRACT

Knowledge regarding new compounds, peptides, and/or secondary metabolites secreted by bacteria isolated from the intestine of phebotominae has the potential to control insect vectors and pathogens (viruses, bacteria, and parasites) transmitted by them. In this respect, twelve Gram-negative bacteria isolated from the intestine of *Lutzomyia evansi* were selected and screened for their enzymatic, antimicrobial, and leishmanicidal activity. *E. cancerogenus, E. aerogenes, P. otitidis, E. cloacae, L. soli*, and *P. ananatis* exhibited enzymatic activity. 83.3% of the isolates displayed lipolytic and nitrate reductase activity and 58.3% of the isolates displayed protease activity. Hemolytic activity (17%) was identified only in *E. hormaechei*, and *P. ananatis. E. cancerogenus, A. calcoaceticus*, and *P. otitidis* showed cellulolytic activity. *A. gyllenbergii*, *P. aeruginosa*, and *E. hormaechei* showed amylolytic activity. In general, the totality of methanolic extracts exhibited antimicrobial activity, where *E. hormaechei*, *A. calcoaceticus*, and *E. cancerogenus* presented the highest activity against the evaluated reference bacteria strains. Cell-free supernatants (CFS_S) of the Gram-negative bacteria showed higher growth inhibitory activity against the reference Gram-positive bacteria. The CFS of *A. gyllenbergii* was the most active antimicrobial in this study, against *S. aureus* (AA_{ODs} = 95.12%) and *E. faecalis* (AA_{ODs} = 86.90%). The inhibition percentages of CFS against Gram-positive bacteria showed statistically significant differences (repeated measure ANOVA *df*= 2; *F*= 6.095; *P*= 0.007832). The *E. hormaechei* methanolic extract showed leishmanicidal activity (CE-50 μg/ml = 47.7 + 3.8) against metacyclic promastigotes of *Leishmania braziliensis* (UA301). Based on this finding, we discuss the possible implications of these bacteria in digestion and physiological processes in the *Lu. evansi* intestine. *P. ananatis, E. cloacae, E. hormaechei*, and *P. otitidis* were considered the most promising bacteria in this study and they could potentially be used for biological control.

1. Introduction

The control of insect vectors and etiological agents that cause tropical illnesses needs alternative strategies that impact transmission and propagation in endemic areas in a more significant way [1,2]. A determining source of natural products for biologic control is the intestinal lumen of some insect vectors. Recent studies have established that entire bacteria or their secondary metabolite molecules, as well as peptides secreted by intestinal tract bacteria, may possess the ability to modulate vector competence, impacting the life cycle of the vector species, generating reproductive alterations, and altering the immune system due to various antimicrobial activities. Some species have even demonstrated leishmanicidal activity [3–6].

During interactions such as symbiosis, mutualism, and parasitism between the microbiota and the intestine of the insect vector, biologically active substances may be generated. Among these, proteolytic enzymes can be found (e.g., aminopeptidases), cytotoxins, thioesters, galectins, antimicrobial peptides, and signaling factors [3,5,7–9]. The insect also possesses an innate

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immune defense system against pathogens (defensins, antioxidant enzymes, caspases, peroxidases) which maintains the balance of this complex behavior [10,11]. Additionally, it has been established that Gram-negative bacteria are responsible for activation via Toll, IMD, and Jak-STAT to control Gram-positive bacteria, fungi, viruses, and some parasites [12,13].

Furthering the study of the insect intestinal microbiota is of the utmost importance due to the fact that it has been described that they can accomplish functions associated with physiological and/or metabolic processes, and in the production of amino acids and in the secretion of inhibiting substances that prevent pathogen bacteria colonization [6]. Nevertheless, few studies have described their enzymatic, antimicrobial or leishmanicidal bioactivities. For this reason, it is necessary to generate relevant information that allows us to understand the microorganism interactions in the intestinal lumen of phlebotominae and to design control strategies to interrupt the transmission cycle of the disease [5,14,15].

Recent studies have identified bacterial genera such as *Serratia*, *Enterobacter,* and *Pseudomonas*, with the ability to synthesize molecules with excellent antiparasitic potential; cytotoxic metalloproteases, hemolysins, antibiotics, prodigiosin, and hemagglutinins have been widely described [7]. Additionally, leishmanicidal activity against *Le. infantum* and *Le. mexicana* has been shown with entire bacteria such as *Ochrobactrum intermedium, Asaia* spp., *Pantoea ananatis, Enterobacter cloacae*, and *O. anthropi* [4,16].

To summarize, some substances synthesized by Gram-negative bacteria have been characterized for being biologically active and functionally important compounds with metabolic activities as well as defensive roles against pathogens [6,12]. Increased knowledge of the extracellular substances produced by these bacteria represents an important source for antibiotic and enzyme production.

In this study, bacterial strains isolated from the intestinal microbiota of *Lu. evansi* were evaluated for their antimicrobial activity from methanolic extracts and cell-free supernatants against pathogenic bacterial strains. Extracellular hydrolase activity (protease, lipase, hemolysis, amylase, cellulase, nitrate reduction) was qualitatively evaluated using entire bacteria. Finally, leishmanicidal activity was estimated against promastigotes of *L. infantum* and *L. braziliensis*. Increased knowledge of the bioactivities of the intestinal microbiota of phlebotominae could be useful to understand their interactions and lead to the formulation of new compounds with biotechnological potential.

2. Materials and methods

2.1. Gram-negative bacteria selection

In this study, we used 12 Gram-negative bacteria isolates previously obtained from the intestinal microbiota of wild populations of *Lu. evansi* collected in Sucre, Colombia [4] (Table 1). These isolates were selected for having leishmanicidal activity, and for previously reported biotechnological potential from other environmental sources. Taxonomical and molecular identification was carried out [4].

2.2. Bacteria and culture conditions

Isolates were reactivated using 100 μL of the cellular suspension, in LB liquid medium (Luria-Bertoni, LAB, United Kingdom) and subsequent growth in an LB-solid medium. Pure colonies were selected and incubated all night at 37 °C with shaking at 180 rpm, until a final concentration of 1.2×10^8 UFC/mL (OD_{600} = 0.125) [4].

2.3. Detection of hydrolyzing extracellular enzymes

2.3.1. Assay for proteolytic activity

Proteolytic activity was determined using skim milk-supplemented media (100 g L^{-1}), according to [17] and Hossain (2015). Pure isolates were grown on agar milk plates and incubated at 30 °C for 48 h. The presence of a clear zone around the colony indicated casein hydrolysis [18]. Additionally, proteolytic activity was evaluated by gelatin liquefaction (120 g L^{-1}, in distilled H_2O; Merck), incubated at room temperature for 24 h. For this assay, a deep culture of each bacterial isolate was carried out. Their activity was evaluated in triplicate. *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* ATCC 14579™ were used as the positive and negative controls, respectively.

2.3.2. Assay for lipase activity

Bacteria were screened using medium supplemented with Tween 20 at 1% (w/v), which contained peptone 1% (10 g L^{-1}), NaCl 0.5% (5 g L^{-1}), and $CaCl_2$ 0.01% (0.1 g L^{-1}) (El-bestawy and El-masry, 2005). Isolates were streaked onto media plates and incubated at 30 °C. Each sample was evaluated in triplicate. After seven days, the activity was confirmed by the appearance precipitation zone around the colony [19]. *Serratia* sp. B006 and *Escherichia coli* ATCC 8739™strains were used as the positive and negative controls, respectively [20].

Table 1
Gram-negative bacterial selected and isolated from the digestive tract of *Lutzomyia evansi* for enzymatic, antimicrobial and leishmanicidal bioactivity assays.

Isolation source [4]	Bacterial isolate code (Accession number_GenBank)	Strain
Unfed female	Isolate 41 (KU134743)	*Enterobacter cancerogenus*
Unfed female	Isolate 43 (KU134745)	*Pseudomonas putida*
Fed Female	Isolate 44 (KU134746)	*Enterobacter aerogenes*
Fed Female	Isolate 64 (KU134748)	*Acinetobacter calcoaceticus*
Male	Isolate 70 (KU134777)	*Pseudomonas otitidis*
Unfed female	Isolate 71 (KU134741)	*Enterobacter cloacae*
Larvae L4	Isolate 102 (KU134766)	*Ochrobactrum anthropi*
Unfed female	Isolate 140 (KU134754)	*Pantoea ananatis*
Larvae L4	Isolate 154 (KU134779)	*Acinetobacter gyllenbergii*
Larvae L4	Isolate 157	*Enterobacter hormaechei*
Larvae L4	(KU134771)	*Pseudomonas aeruginosa*
Larvae L4	Isolate 173 (KU134783)	*Lysobacter soli*
	Isolate 188 KU134784)	

2.3.3. Assay for amylolytic activity

Amylolytic activity was evaluated following a previously described protocol [21]. Pure colonies of each bacterium were grown in medium supplemented with starch 1% (w/v), yeast extract 0.3% (3 g L^{-1}), and agar 1.5% (15 g L^{-1}). The isolates were streaked and incubated at 35 °C ± 2 °C for 48 h. After incubation, iodine solution (3 mL) was flooded with a dropper for five minutes onto the starch agar plate. The clear zone of hydrolysis around the colony indicates a positive result. *Escherichia coli* ATCC 9029™ and the *Bacillus cereus* ATCC 14579™ strains were used as the negative and positive controls, respectively.

2.3.4. Assay for cellulolytic activity

Pure colonies were grown in medium supplemented with carboxymethylcellulose 1% (w/v), yeast extract 0.25% (2.5 g L^{-1}), peptone 0.25% (2.5 g L^{-1}), $(NH_4)_2SO_4$ 0.05% (0.5 g L^{-1}), $CaCl_2$ 0.05% (0.5 g L^{-1}), KH_2PO_4 0.01% (0.1 g L^{-1}), K_2HPO_4 0.01% (0.1 g L^{-1}), and 1.5% agar (15 g L^{-1}). Subsequently, cellulolytic activity was assessed by means of a qualitative evaluation, adding 3 ml of Congo red reactive at 1% for 15 min, used to illustrate the clear the zone around the colony [22]. Afterwards, 2 mL of 0.1 M NaCl was added for 15 min and the excess was decanted. The Petri plates with growth were incubated at 4 °C for 24 h. After that time, the corresponding readings of the degradation halos were conducted. *Bacillus cereus* ATCC 14579™ strain was used as the positive control [23]. PBS was used as the negative control.

2.3.5. Assay for hemolytic activity

Hemolysis was determined according to [24]. Pure isolates were evaluated on blood agar plates for 30 °C for 72 h. *S. aureus* (clinical isolate), *E. coli* ATCC 9029™ and *E. faecalis* (clinical isolate) were used as controls to determine complete hemolysis (β), partial hemolysis (α), and non-hemolysis (γ), respectively [25–27].

2.3.6. Nitrate reductase and nitrite reductase activity

Nitrate reduction was evaluated following a previously described protocol [21]. Pure colonies of each bacterial isolate were grown in medium supplemented with potassium nitrate 1% (w/v) broth, meat extract 0.3% (3 g L^{-1}), and peptone 0.5 (5 g L^{-1}), and incubated at 35 °C ± 2 °C for 24 h [21]. Nitrate and nitrite reduction was assessed by adding Griess reagent. *Escherichia coli* ATCC 9029™ and *Acinoteobacter baumanni* ATCC 17978™were used as the positive and negative controls, respectively [21].

2.4. Extraction of secondary metabolites and antimicrobial activity

Secondary metabolite extraction was performed according to the described method [28]. An Erlenmeyer with 50 mL of LB broth was inoculated with 0.5 mL of each of the selected strains culture for 18 h at 30 °C, then amberlite XAD-16 (AMRESCO®) 2% (w/v) was added. After seven days of incubation at 30 °C and 180 rpm, the metabolites absorbed by the amberlite were eluted with 20 mL of methanol (100%) for 60 min [29]. The methanolic fraction of each extract was concentrated up to 1.0 mL in a rotary evaporator at 40 °C (Heidolph Laborota 4001 Efficient Rotary Evaporator) [4].

Antimicrobial screening was carried out by means of the agar diffusion test on Mueller-Hinton medium (Becton Dickinson) [30] with some modifications. Sterile Whatman filter paper discs (No. 1, 6 mm in diameter) were impregnated with each extract. The discs were placed over the Mueller-Hinton agar (Becton Dickison) in Petri dishes, evenly inoculating a liquid culture of the reference *Pseudomonas aeruginosa* ATCC 9027, *E. coli* ATCC 8739, *Enterococcus faecalis* ATCC 51299, and *S. aureus* ATCC 29213 strains and two clinical isolates, which corresponded to *S. marcescens* and *B. cereus*. The determination of antimicrobial activity was evaluated following the procedure described by the Clinical and Laboratory Standards Institute (CLSI, 2014). The dishes were incubated at 37 °C for 18 h, and then the diameter of the inhibition halo of the growth surrounding each of the discs was measured. The antibacterial activity assays were performed in triplicate in six independent experiments.

2.5. Assessment of antimicrobial activity of cell-free supernatants

Strains were inoculated in an LB broth until reaching their exponential growth. The supernatants were recovered by centrifugation at 10,000 g, at 4 °C for 10 min, and were neutralized by pH (7.0) modification. Finally, they were filtered through a 0.2 μm membrane [31].

Antimicrobial activity of cell-free supernatants was done by means of an agar diffusion test and serial microdilution [30]. This assay was done using 96-well microplates on the previously described reference strains (1.0×10^6 CFU/ml with serial dilutions) [32]. As negative control, 100 μL of BMH along with 100 μL of reference strain were added to each well. The positive control was evaluated with 20 μL of chloramphenicol, 80 μL of BMH in 100 μL of the pathogen. Additionally, cyclophenicol (30 μg/mL) in BMH was used as a negative control. Each assay was carried out in triplicate, and the microplates were incubated at 37 °C for 18 h with 600 nm photometric readings every hour [30]. Bacterial inhibition percentages based on absorbance were calculated using the formula: antibacterial activity (% OD) = ((DC-Ds)/DC) x 100, where DC is the control (cellular concentration of reference strains more LB broth) and Ds is the final growth of reference strains with CFS in the plate.

2.6. Leishmanicidal activity of secondary metabolites

Roto-evaporation was used to eliminate the solvent from the methanolic extracts. Afterwards, they were weighed and solubilized with DMSO. The assay for the effectiveness on metacyclic promastigotes of *L. infantum,* (BCN- strain) and *L. braziliensis* (UA301 strain) promastigotes was done in 96-well plates with six serial dilutions to a maximum concentration of 400 μg/mL. Subsequently, the promastigotes were added at 1,200,000 parasites/mL and incubated at 26 °C for 72 h. Amphotericin B was used as the control. Each concentration of the compound (the extracts), as well as the compound-free control, were tested in triplicate in two different experiments. The viability of the parasites was determined by measuring mitochondrial dehydrogenase activity by the MTT method [33].

2.7. Statistical analysis

The statistical analysis of the antibacterial activity of the extracts was estimated by repeated measures ANOVA using XLSTAT software (version 2018.5), considering the inhibition-halo diameter comparison. The same analysis was done for the cell-free supernatants in terms of the inhibition percentages. Antimicrobial activity is shown in a graph with the standard error using PAST (Version 3.21) software.

3. Results

3.1. Extracellular hydrolase detection

Out of the 12 Gram-negative isolates, seven showed light halo formation around the colony in the supplemented milk medium (Table 2). The isolates with the highest activity proteolytic in milk were *E. hormaechei* and *P. ananatis* (Appendix A). Proteolytic activity by gelatin liquefaction was also positive for these two bacteria (Table 2). A wide number of isolates (n = 10; 83.3%) that

Table 2
Enzymatic activity of Gram-negative bacteria isolated from the intestinal tract of *Lutzomyia evansi.*

Gram-negative bacteria - Code	Enzymatic activity						
	Proteolytic *	**Proteolytic ****	**Lipolytic**	**Hemolytic**	**Cellulolytic**	**Amylolytic**	**Nitrate reductase and nitrite reductase**
*E. cancerogenus*_41	+	–	++	–	+	–	+
*P. putida*_43	–	–	+	–	–	–	–
*E. aerogenes*_44*	++	–	++	–	–	–	+
*A. calcoaceticus*_64*	–	–	++	–	+	–	+
*P. otitidis*_70	+	–	++	–	+	+	+
*E. cloacae*_71*	+	–	+	–	–	–	+
*O. anthropi*_102*	–	–	–	–	–	–	+
*P. ananatis*_140*	+++	+	+++	+	–	–	–
*A. gyllenbergii*_154	–	–	+	–	–	–	+
*E. hormaechei*_157*	+++	+	–	++	.	+	+
*P. aeruginosa*_173**	–	–	+++	–	.	+	+
*L. soli*_188	++	–	+	–	–	–	+

Proteolytic activity of Gram-negative bacteria on agar supplemented with milk * and gelatin**.-: no activity (absence of clear areas around the colony); **+**: positive activity, presence of clear areas around the colony. Degradation zone on specific supplements (cm): - (0); **+** (0.1-0.3); **++** (0.4-0.6); **+++** (0.7-1.0). * Reported positive for nitrate reduction (Gitaitis et al., 2003; Villalobo et al., 1977; Brenner et al., 2015); * Reported positive for amylase (genome.jp/kegg-bin/show_pathway?pae00500).

showed lipolytic activity were found (Table 2, Appendix A). *P. ananatis* and *E. hormaechei* were the only bacterial isolates with complete hemolytic activity (hemolysis β) (Table 2, Appendix A).

One of the enzymatic tests of great interest, and which is associated with complex carbohydrate hydrolysis, was the amylolytic activity test where *E. hormaechei*, *P. aeruginosa* and *P. otitidis* isolates were positive (Table 2, Appendix A). With regard to the qualitative test of cellulolytic activity, *P. otitidis, A. calcoaceticus*, and *E. cancerogenus* were biologically active (Table 2, Appendix A). Finally, *P. putida* and *P. ananatis* isolates were the only species in which nitrate reductase activity and molecular nitrogen production were not evidenced (Table 1, Appendix A).

3.2. Antimicrobial activity of secondary metabolites extracts

Antibacterial activity was showed by all strains tested against at least six strains used as the target (Table 3). *E. hormaechei*, *A. calcoaceticus*, and *E. cancerogenus* showed the greatest inhibitory activity with a standard inhibition halo average of 19 mm in diameter (Table 3). Statistically significant differences were found when comparing the measurements of the inhibition zone produced against *S. marcescens* (Repeated Measure ANOVA *df*= 2; *F*= 12.18 *P*= 0.01432) with regards to *E. coli* and *P. aeruginosa*. In contrast to this, the antimicrobial activity does not reflect statistically significant differences in the size of the halos generated by the methanolic extracts activities against Gram-positive bacteria (Repeated Measure ANOVA *df*= 2; *F*= 2.598 *P*= 0.08157). No antimicrobial activity of bacterial isolates was determined for *L. soli* against some bacteria like *E. coli* (ATCC 8739TM) and *E. faecalis* (ATCC 51299TM).

3.3. Antimicrobial activity of cell-free supernatants (CFS)

The inhibition percentage of CFS against three Gram-negative and three-Gram positive reference strains are listed in Table 4. An example of the bioassay is shown in Fig. 1 (a representation of the growth of *B. cereus* against the CFS of *P. otitidis*). *P. aeruginosa, P. ananatis*, and *E. aerogenes* CFSs showed strong antimicrobial activity against *S. marcescens* (inhibition of bacterial growth > 60%), while *O. anthropi* showed the lowest activity against this

Table 3
Evaluation of antimicrobial activity using metabolic extract of 12 Gram-negative isolates of gut microbiota of *Lu. evansi* against Gram negative y Gram positive bacteria.

metabolic extracts of bacteria isolates_Code	Diameter of growth or zone of inhibition					
	Gram-negative bacteria			**Gram-positive bacteria**		
	E. coli (ATCC 8739^{TM})	*P. aeruginosa* (ATCC 9027^{TM})	*S. marcescens* (Clinical isolated)	*B. cereus* (Enviromental)	*E. faecalis* (ATCC 51299^{TM})	*S. aureus* subsp *aureus* (ATCC 29213 ^{TM})
*E. cancerogenus*_41	++	+++	++	++	++	++
*P. putida*_43	+	++	+	++	+	+
*E. aerogenes*_44	++	++	++	++	++	++
*A. calcoaceticus*_64	+	+++	+	++	+++	++
*P. otitidis*_70	++	++	++	++	++	+
*E. cloacae*_71	+	++	++	++	+	+++
*O. anthropi*_102	++	++	+	++	++	++
*P. ananatis*_140	++	++	+	++	++	++
*A. gyllenbergii*_154	++	++	++	++	++	++
*E. hormaechei*_157	++	+++	++	+++	+++	++
*P. aeruginosa*_173	++	++	+	++	+	++
*L. soli*_188	–	++	++	+	–	+
E. cloacae (C+)	11 ± 2	12 ± 1	12 ± 2	12 ± 2	12 ± 3	13 ± 2
Cloranfenicol (C+)	**22 ± 2**	**8 ± 1**	**25 ± 2**	**23 ± 1**	**12 ± 2**	**23 ± 2**
Metanol (C-)	**0**	**0**	**0**	**0**	**0**	**0**

Determination of diameter of growth (measured in mm) for the antimicrobial activity showing different degrees of inhibition for the twelve metabolic extracts. (-): Absence zone of inhibition, (+): zone of inhibition between 8–11 mm, (++): zone of inhibition between 12–15 mm, (+++): zone of inhibition ≥16 mm. C +: Positive control, C-: Negative control. The results represent the average of the triplicates.

Table 4
Growth bacterial inhibition of reference strains using cell-free supernatants (CFS) of Gram-negative bacteria by serial microdilution test.

CFS of bacteria isolates_Code	Inhibition of bacterial growth (% OD600)					
	Bacterial reference strains					
	E. coli ATCC 8739	*P. aeruginosa* ATCC 9027	*S. marcescens* aislado clínico	*B. cereus*	*S. aureus*	*E. faecalis*
*E. cancerogenus*_41	35.11 ± 0.56	26.16 ± 1.18	17.76 ± 1.18	34,37 ± 0,12	31,46 ± 0,06	54,55 ± 0,03
*P. putida*_43	2.08 ± 0.94	11.88 ± 1.19	44.66 ± 0.92	**63,33**±0,19*	28,01± 0,14	31,86± 0,07
*E. aerogenes*_44	28.39 ± 0.72	19.89 ± 1.0	**61.34 ± 0.97***	50,00 ± 0,07	55,50 ± 0,08	**78,05** ±0,03*
*A. calcoaceticus*_64	3.92 ± 0.81	25.62 ± 1.29	16.22 ± 1.59	9,49 ± 0,13	26,46 ± 0,14	45,46 ± 0,05
*P. otitidis*_70	34.41 ± 0.57	25.37 ± 1.19	18.02 ± 1.80	53,72 ± 0,06	53,63 ± 0,09	**67,10** ±0,04*
*E. cloacae*_71	24.85 ± 0.83	16.12 ± 1.33	9.41 ± 2.16	17,65 ± 0,12	44,28 ± 0,06	**76,89** ±0,03*
*O. anthropi*_102	17.48 ± 0.87	20.25 ± 1.11	0.28 ± 2.14	30,81± 0,09	**86,53**±0,01*	73,11±0,04*
*P. ananatis*_140	26.43 ± 0. 70	27.68 ± 0.98	**75.89 ± 0.56***	23,66 ± 012	46,28 ± 0,05	**81,24** ±0,03*
*A. gyllenbergii*_154	32.02 ± 0.67	7.87 ± 1.24	13.54 ± 1.96	50,50 ±0,06	**95,12**±0,01*	86,90±0,04*
*P. aeruginosa*_173	3.02 ± 0.97	18.03 ± 1.18	**69.71 ± 0.81***	7,89 ± 0,08	45,90 ± 0,07	52,36 ± 0,04
*L. soli*_188	36.41 ± 0.54	25.16 ± 1.17	16.22 ± 1.75	**61,82**±0,05*	48,61±0,05	**70,23**±0,04*

Antibacterial activity (% OD) = ((DC-Ds)/DC) x 100, where DC is the control (cellular concentration of reference strains more LB broth) and Ds is the final growth of reference strains with CFS in the plate. *: Inhibition of CFS > 60%. The standard deviation (SD) is represented as the mean of the triplicates. Inhibitory activity above 60% are showed in bold.

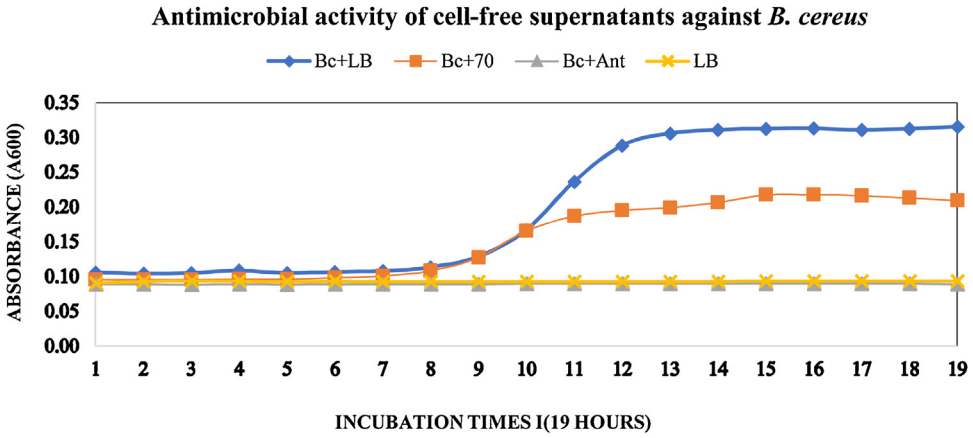

Fig. 1. Growth curves of *B. cereus* (clinical isolate) against the exposure of cell-free supernatants (CFSs) of *Pseudomonas otitidis*. **Bc + LB:** *B. cereus* in LB culture medium; **Bc+70**: *B. cereus* in presence of CFSs of *Pseudomonas otitidis* (isolate 70); **Bc + Ant:** *B. cereus* in LB medium supplemented with antibiotic (Chloramphenicol 30 μg/ml).

bacterium (Table 4). In general, most of the CFS shows less activity with regard to the three Gram-negative reference strains isolate (Table 4). The inhibition percentages of CFS against Gram-negative bacteria do not show any statistically significant differences (repeated measured ANOVA *df*= 2; *F*= 0.7936; *P*= 0.4647).

In contrast, the CFS of the Gram negative bacteria showed greater growth inhibitory activity against the reference Gram positive bacteria, specifically against *E. faecalis* (Table 4). The most active CFS were from *E. aerogenes* (which also showed activity against *S. marcescens*), *P. otitidis, E. cloacae, O. anthropi, A. gyllenbergii, P. ananatis* and *L. soli* (Table 4). The *A. gyllenbergii* CFS was the most active antimicrobial in this study against *S. aureus* (AA_{ODs} = 95.12%) and *E. faecalis* (AA_{ODs} = 86.90%) (Table 4). The inhibition percentages of CFS against Gram-positive bacteria show statistically significant differences (repeated measures ANOVA *df*= 2; *F*= 6,095; *P*= 0.007832). Only Tukey's paired test showed differences between the CFS of *S. aureus* (*P*= 0,0364) and *E. faecalis* (0,0096), when contrasted against the CFSs of *B. cereus.*

3.4. Leishmanicidal activity of secondary metabolites in the extracts

The *E. hormaechei* metabolic extract showed activity (CE-50**μg/ml = 47.7 + 3.8) against *Le. braziliensis* metacyclic promastigotes (UA301), using the MTT method to determine the viability of the parasites (Table 5). None of the evaluated extracts and drugs used in the effectiveness evaluation showed any activity against metacyclic promastigotes of the *Le. infantum* (BCN) strain, indicating that the half maximal effective concentration (EC_{50}) of these extracts is higher than 400 μg/mL against the evaluated strain, i.e., it is higher than the maximum evaluated concentration (Table 5).

Table 5
The median effective concentration (EC-50) of the bacterial extracts on metacyclic promastigotes of the *Leishmania infantum* (BCN-GFP)* and *Leishmania braziliensis* (UA301-GFP)** strains.

Leishmanicidal activity of methanolic bacteria extracts		
methanol crude extract_Code	EC-50* (μg/ml)	EC-50** (μg/ml)
*O. anthropi*_102	>400	>400
*E. aerogenes*_44	>400	>400
*P. otitidis*_70	*>400*	*>400*
*E. hormaechei*_157	*>400*	**47.7 + 3.8**
*E. cloacae*_71	*>400*	*>400*
*A. calcoaceticus*_64	>400	>400
*P. ananatis*_140	>400	>400
*A. gyllenberguii*_	>400	>400
*P. aeruginosa*_173	>400	>400
*E. cancerogenus*_41	>400	>400
*L. soli*_188	>400	>400
*P. putida*_43	>400	>400

* **(μg/ml):** micrograms per milliliter; **EC-50:** The median effective concentration. Leishmanicidal activity are showed in bold.

4. Discussion

In recent years, it has been reported an increase of microbial community studies based on sequenced amplicons from 16S rRNA gene on insect gut communities [34]. However, the knowledge of the intestinal microbiota function and its biologic potential could be useful for understand the role on metabolism, physiology, defense against pathogens, or in vector competence modulation.

In this study, we report the antimicrobial, enzymatic, and leishmanicidal activities of 12 Gram-negative isolates, obtained from the intestine of wild populations of *Lu. evansi*, a known vector of visceral leishmaniasis in Colombia. The resulting information was used to infer probable functions of these bacteria in the *Lu. evansi* gut compartment (Fig. 2), besides providing aggregate value to secondary metabolite production, due the great biotechnological and pharmacological potential use.

Proteases have a number of key roles in bacteria viability, stress response and pathogenicity., which provides them benefits over other microorganisms and enables them to survive in hostile or high pH environments into the insect intestine [35,36]. In this study, *P. ananatis, E. cancerogenus, E. cloacae, E. aerogenes*, and *E. hormaechei* displayed proteolytic activity, which suggests a possible role in the degradation of protein into amino acids or small peptides during nutrition events or cell division processes [8] (Fig. 2). The secretion into the extracellular medium of a high variety of proteolytic enzymes such as serine proteases, metalloproteases, and bacteriocins has been reported by *Enterobacter* genera [37].

It was also shown a lipolytic activity associated to *P. otitidis, P. aeruginosa, P. putida, E. cancerogenus, E. aerogenes, E. cloacae,* and *P. ananatis.* In insects, the fat body carries out multiple metabolic activities, such as storage, energy production, circulating metabolite synthesis, and protein synthesis according to requirements (Arrase and Soulages, 2010; [36]. It has been established that bacteria from *Pseudomonaceae* and *Enterobacteriaceae* families act directly on lipid hydrolysis and glycolipid degradation in insects [38]; Anand et al., 2009; [36], as well as the degradation of microbial components (lipopolysaccharides, peptidoglycans) inside the digestive tract [9,39].

A common physiological event of females insect vectors is erythrocyte lysis into the midgut that is required to produce eggs. Hematophagous arthropods are faced with a very particular situation concerned to heme metabolism, as well as the fact that they ingest many times their own mass in vertebrate blood [40]. gut isolates described in the study could participate in blood digestion, acting on haemoglobin degradation pathway, as demonstrated for *P. ananatis* and *E. hormaechei* that have shown complete hemolytic activity and probably increasing their abundance after the blood intake.

During this process, massive liberation of the heme group can occur (Fig. 2), a known pre-oxidant molecule, whose internal regulation can produce reactive oxygen species (ROS) and concomitant Fe^{2+} liberation during this process [41,42]. Reactive oxygen species are beneficial in low and moderate conditions within cellular processes [42]. Heme to biliverdin and carbon monoxide degradation (CO) definitely has a crucial role in pigmentation and can protect insects against the oxidative damage induced by light [43]. The amount and composition of blood nourishment is the main factor that affects vitellogenesis and egg production in sand flies [9,44,45]. However, feeding preferences and digestive patterns of the blood of each species can depend on the bacterial microbiota.

All isolated strains were tested for cellulolytic and aminolytic activities (Fig. 2), as shown in Table 2. However, it is necessary to highlight *P. otitidis* for having these two enzymatic activities and the reductase nitrate capacity, which suggests that it is useful in nutrient digestion in *Lu. evansi*. In general, phlebotominae have coprophagous habits, i.e. larvae forms of *Lu. evansi* develop in places with abundant decomposing organic matter [46]. We also found that most of the cellulolytic bacteria belonging to the family *Enterobacteriaceae*. In some insects, like *Bombyx mori*, it has been concluded that the number of cellulolytic bacteria increases with each urge [47].

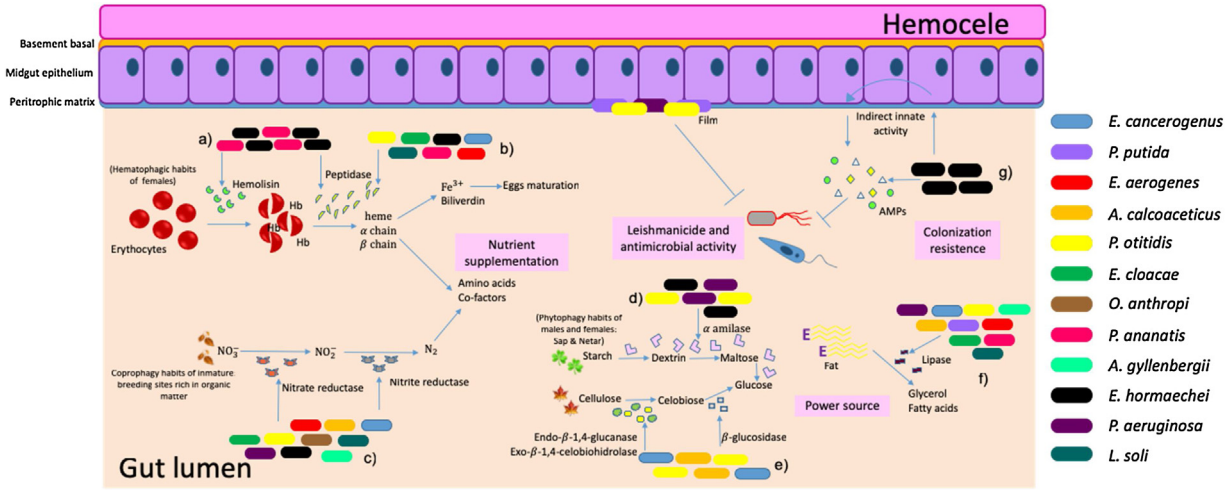

Fig. 2. Predictive model of the interactions and functions of Gram-negative bacteria in the intestine of *Lutzomyia evansi*, based on *in vitro* evaluation of enzymatic, antimicrobial, and leishmanicidal activity. a) Intestinal bacteria such as *P. ananatis* and *E. hormaechei* display hemolytic activity, suggesting that they might be related to erythrocyte lysis. b) Hemoglobin could be released and used by *P. ananatis, E. hormaechei, P. otitidis, E. cloacae, E. cancerogenus, L. soli*, and *E. aerogenes* for nutrient supplementation. Erythrocytes are also an useful source of iron for egg maturation. c) *E. aerogenes, A. calcoaceticus, E. cancerogenus, E. cloacae, P. otitidis, O. anthropi, L. soli, E. hormaechei*, and *A. gyllenbergii* are able to reduce nitrates to nitrites and/or molecular nitrogen, from decomposing organic matter, possibly during the development of the immature states of *Lu. evansi*. This process allows for the synthesis of some necessary amino acids for protein and glycoprotein synthesis, which could also permit formation of the peritrophic matrix in the adult phase. d) *P. aeruginosa*, *E. hormaechei*, and *P. otitidis* are responsible for carbohydrate hydrolysis, processing starch to obtain glucose and produce energy. e) Cellulose is hydrolyzed by *P. otitidis, E. cancerogenus*, and *A. calcoaceticus* as an additional source of glucose.) *L. soli, P. aeruginosa, E. cancerogenus, P. putida, E. aerogenes, P. otitidis*, and *A. gyllenbergii* showed lipolytic activity that might be associated with fat degradation from the fatty abdominal body, which is filled with nutrients and allows for development and/or reproduction, as fats are the precursors of the synthesis of most proteins and metabolites secreted into the hemolymph. g) *E. hormaechei* showed activity against metacyclic promastigotes of *Leishmania braziliensis.* The majority of Gram-negative bacteria show antimicrobial activity, which is a determining aspect in intestinal microbiota dynamics.

Both males and females Phlebotomine in their adult state have phytophagous habits, associated with the ingestion of sugar from sap, plant phylloplanes, or from leaves that are rich in protein and water [36,48–51]. This suggests that amylolytic bacteria play a role only in the initial stages of digestion, which includes the decomposition of complex sugar polymers into dimers or oligomers (Fig. 2).

Maladjustment in the content of nitrogen in phytophagous insects and their host plants has been recognized for many years as a critical factor that influences hematophagous insect success in their adult state when blood sources are insufficient [52,53]. In the majority of plants, nitrogen, from the sap of both the xylem and the phloem, is dominated by non-essential amino acids; essential amino acids represent less than 20% of the total. The majority of Gram-negative bacteria were nitrate reductase positive, and some generated molecular nitrogen (Fig. 2), which is important on many occasions for the formation of amino acids. Various ecological and evolutionary factors (trophic level, feeding style, body size, and phylogeny) may contribute to variations in the N content in insects that spend part of their life cycle in terrestrial substrates [54]. However, many insects obtain a nutritional advantage from persistent associations with the intestinal microbiota, which synthesizes various nutrients, digest and detoxify ingested foods, and provide essential amino acids [8,36].

A suggested hypothesis is that the nutritional contributions of the intestinal microbiota represented by Gram-negative bacteria in *Lu. evansi* might occur in different ways: 1) improved digestion efficiency, 2) optimized ability to live with a suboptimal diet, 3) acquisition of digestive enzymes, and 4) vitamin supply. Secondary metabolites extracts derived from Gram-negative bacteria exhibited similar antimicrobial activity patterns against reference bacteria. The diameters of the inhibitory halos were similar to the activity detected for complete bacteria of *P. ananatis*, *E. cloacae*, and *O. anthropi* isolated from *Lu. evansi* [4]. *E. hormaechei*, *P. ananatis*, *A. calcoaceticus*, and *E. cancerogenus* bacteria presented the strongest antimicrobial activity. It has been suggested that these bacteria might use a type 4 secretion system (T4SS) that gives them advantages in terms of microbial competence, antagonist effectors, and antibiotic resistance [55,56]. Unlike other Gram-negative isolates found in the *Pseudomonaceae* family that showed antimicrobial activity, they may use a type 2 (T2SS) or type 6 secretion system (T6SS) [57–59].

As far as we know, this is the first time that the antimicrobial activity of cell-free supernatants (CFS) of bacteria associated to the intestinal tract of phlebotominae has been evaluated. This characterization allows for a more complete spectrum of bioactive molecules produced by Gram-negative bacteria [60]. The antimicrobials produced in the CFS, presumably at variable levels and at different times depending on factors such as cellular density, intercellular signaling, and the variable composition of the intestine environments of the insect [61]. Significant antimicrobial activity was obtained regarding *P. aeruginosa*, *P. ananatis*, and *E. aerogenes* over *S. marcescens*. The inhibiting action of the CFS produced by bacteria tends to be very stable against the action of proteases [62] and some metabolites are resistant to heat degradation, i.e. non-protein molecules can also exist. This activity may confer to *Lu. evansi* the ability to adapt to several ecological niches. In general, supernatant could comprise bacteriocins, which may have inhibitory action against groups of Gram-positive and Gram-negative bacteria [62,63].

In our study, the *E. hormaechei* metabolic extract showed leishmanicidal activity against *Le. braziliensis*. This bacterium is found within the *E. cloacae* complex and is spread by horizontal transference. *E. hormaechei* metabolomics approach to identify secondary metabolites that causes leishmanicidal activity is justified as a technological prospective. *E. hormaechei* may have enzymes such as phospholipase C, cytolysins, or type IV (T4SS) and VI (T6SS) secretion systems [55,59,64,65] linked to virulence factors, with direct effects on the surface of some prokaryotes (Telleira et al., 2018). Recently, it has been found that the use of complete Gram-negative bacteria of the *P. ananatis*, *E. cloacae*, and *O. antropi* species may modulate the development of procyclic and metacyclic promastigotes of *Le. infantum* [4] in *in vitro* assays. [16], found that *Pseudozyma* sp., *Asaia* sp., and *O. intermedium* reduced the number of females infected with *Le. mexicana* promastigotes using an experimental infection model in adults of *L. longipalpis*. This effect was possibly caused by the secretion of extracellular metabolites [16,66,67].

Unfortunately, the results from methanolic extracts of the twelve Gram-negative isolates indicated that neither of these showed inhibitory activity on *Le. infantum*, which suggests that the antiparasitic mechanisms of these bacteria against this parasite species might be associated with the action of complete cells, as evidenced by [4] in a previous study. It has also been described that *L. infantum* is highly resistant to various antimonial compounds and/or pharmaceutical derivates [68,69]. In many cases, antimicrobials are also believed to be produced in subinhibitory concentrations in natural environments or used in relatively high concentrations to study the activity of antimicrobials in the laboratory [33]. A complementary analysis of cytotoxicity will be developed as a step the *in vitro* test.

Finally, we can conclude that intestinal bacteria associated with insect vectors have the ability to adapt to changes in the insect's diet due to the induction of enzymes or changes in the microbial community population. In this study, we suggest Gram-negative bacteria may provide digestive enzymes in synergy, and they may contribute to larvae growth, ingested blood digestion, complex carbohydrate breakdown, and the generation of the components required for protein synthesis, among other activities. Nevertheless, it is unclear how *in vitro* results obtained here are related to *in vivo* situation. Thus, the relative functions of the enzymes produced endogenously by *Lu. evansi* need to be studied further because they might be targets for the strategic design of control measures against pathogenic bacteria, the *Leishmania* parasite, or the insect vector. Subsequently, it may be possible to conduct studies investigating antibacterial synergy. Lastly, *P. ananatis* and *E. hormaechei* are promising strains with great biotechnological potential due to their excellent antimicrobial and enzymatic activity.

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Author's contributions

RJV, CXMH, GECR, SMR: Designed the study and contributed to writing the manuscript.

RJV, GBD: Analyzed the data, performed the experiments and contributed to writing the manuscript.

Declaration of Competing Interest

Authors have no conflict of interest to declare

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00379.

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