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Antagonistic Effect of Bacteria Isolated from the Digestive Tract of *Lutzomyia evansi* against Promastigotes of *Leishmania infantum*, Antimicrobial Activities and Susceptibility to Antibiotics

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Abstract

Lutzomyia evansi is a phlebotomine insect endemic to Colombia's Caribbean coast and is considered the main vector of visceral and cutaneous leishmaniasis in the region. Specific studies of the direct effects generated by bacteria in the digestive tract of the insect vectors, under *Leishmania infantum* using *in vitro* models, represent a novel alternative as a control strategy for the transmission of leishmaniasis and also provide the opportunity to detect natural products or antimicrobial peptides with different biological activities. In this study, we evaluate the leishmanicidal and antimicrobial activities of *Pantoea ananatis*, *Ochrobactrum anthropi* and *Enterobacter cloacae*, isolated from the digestive tract of *Lutzomyia evansi* and the susceptibility of these bacteria to commonly used antibiotics. The antagonistic effect of *Pantoea ananatis*, *Ochrobactrum anthropi* and *Enterobacter cloacae* was evaluated against six species of human pathogenic bacteria and against stationary (Metacyclic-like) and exponential promastigotes (Procyclic-like) of *Leishmania infantum* (BCN-GFP strain) by co-culture assays for 24 hours. The activity of the bacterial isolates on *Leishmania infantum* promastigotes was quantified by flow cytometry. The susceptibility of the bacterial strains to clinically used antibiotics was analyzed by antibiogram. The highest percentage of inhibition was observed against exponential promastigotes with bacterial concentrations of 10^8 CFU/ml of *Enterobacter cloacae* (77.29% ± 0.6%) and *Pantoea ananatis* (70.17% ± 1.1%). The extracts produced by three bac-

terial isolates showed similar biological activity (13 mm - 22 mm inhibition halos) against all tested bacteria; however, significant differences were observed with respect to gram-positive bacteria ($P < 0.003557$). The most active antibacterial activity was displayed against the pathogenic bacteria *Bacillus cereus*. *Ochrobactrum anthropi* was the isolate with the highest number of antibiotic resistance patterns while *Pantoea ananatis* and *Enterobacter cloacae* showed greater susceptibility to the evaluated antibiotics. The growth inhibitory activity of exponential *Leishmania infantum* promastigotes shown by extracts of *Enterobacter cloacae* and *Pantoea ananantis* suggests that the presence of these bacteria in the vector intestine may affect the parasite development to metacyclic stages, infective to human hosts. This in turn confers said bacteria, a potential in controlling the transmission of *Leishmania* spp. that deserves to be studied in depth.

Keywords

Intestinal Microbiota, Leishmanicidal Activity, Antimicrobial Activity, Antibiotic Susceptibility

1. Introduction

Leishmaniasis remains as a public health problem worldwide due to its morbidity and geographical distribution [1]. Transmission of the disease is complex and involves not only the participation of different species of *Leishmania* parasites but also sandflies vector insects [2] [3] and mammalian species that serve as reservoirs for the parasite. The infection in humans generates various clinical manifestations, being visceral leishmaniais (VL) one of the clinical forms with greater impact in the Americas specifically in countries like Colombia, Brazil and Venezuela for the possibility of causing the death of patients if not diagnosed and treated early [4].

Currently, the VL presents difficulties associated with treatment, diagnostic tests and surveillance and control strategies of insect vectors [5]. This problem is attributed mainly to the emergence of drug-resistant strains of the *L. infantum* parasite, as well as the ubiquity and adaptability of vector insects, *Lu. longipalpis*, and *Lu. evansi,* and the existence of different eco-epidemiological settings where transmission can occur [6] [7]. Therefore, it is necessary to explore alternatives aimed at interrupting the transmission of the infection and thereby reduce the impact of leishmaniasis in public health [8]. An alternative option to the chemical control of vectors or to the synthetic generation of vaccines and treatments is to understand the "intestinal microbiota" of sandflies vectors [9] [10].

From a holistic point of view, it is suggested to integrate the isolation of bacterial communities and the study of the action or activity of bacteria by generating secondary metabolites and bacterial peptides that can impact directly (antileishmanial activity) or indirectly (immune system) the development of *Leishmania* parasites, being decisive in the modulation of the transmission or vector competence of *Lutzomyia* spp [11] [12].

There are several studies on intestinal microbiota in sandflies aimed at finding molecules with antileishmanial activity. Among these, the study of lytic effects generated in *L. chagasi* (syn *L. infantum*) by its interaction with *Serratia marcescens* [13], the variability of molecules like defensins in *Phlebotomus duboscqi* induced by changes in the microbiota [14], the generation of reactive oxygen species mediated by *S. marcescens* against *L. mexicana* in the digestive tract of *Lu. Longipalpis* [15] and most recently, the *in vitro* activity of *Pseudozyma* sp., *Asaia* sp., and *Ochrobactrum intermedium* against the development of promastigotes of *L. Mexicana* [16]. In Colombia, there are not known studies that have explored the usefulness of the intestinal microbiota of insects that transmit *Leishmania* spp.

Lu. evansi, is a vector recognized species for transmitting parasites that generate cutaneous and VL in rural and urban environments of the Caribbean coast of Colombia [17] [18]. Its abundance and epidemiological importance made it an attractive biological model for the preliminary study of the microbiota using a culture dependent approach under aerobic conditions. This strategy allowed the isolation of bacterial strains *P. ananatis*, *O. anthropi* and *E. cloacae*, arousing interest either by being dominant in the digestive tract of *Lu. evansi* (*E. cloacae*), by being symbionts (*P. ananatis*) or by their reports on antitrypanosomal activity (*Ochrobactrum* sp.) [16]. Therefore, this study aimed to evaluate the leishmanicidal and antibacterial activity of extracts and whole bacteria (*P. ananatis*, *O. anthropi* and *E. cloacae*), isolated from the digestive tract of *Lu. evansi* and their susceptibility to antibiotics.

2. Methodology

2.1. Ethics Statement

Sand fly collection was performed in accordance with the parameters of Colombian decree number 1376, which regulates specimen collection of biologically diverse wild species for non-commercial research. No specific permits were required for this study. The sand flies were collected on private property and permission was received from landowners prior to sampling.

2.2. Identification of Bacterial Isolates and Estimate Cell Concentration

P. ananatis, *O. anthropi* and *E. cloacae* all Gram negative strains were isolated from the digestive tract of adults and immatures from natural populations of *Lu. evansi*, associated with a peri-urban biotype from the municipality of Ovejas (Sucre department, Caribbean coast of Colombia), classified as a tropical dry forest ecosystem. The adult specimens were collected using Shannon-type extra-domiciliary white light traps that remained active between 18:00 h and 22:00 h. Prior to gut dissection, adult specimens were washed with 50 μL of 1X PBS and Tween 20, centrifuged at 3000 g for 5 minutes, and submerged in a 70% ethanol wash for one minute to remove excess microvilli, dust and exogenous bacteria.

The guts of adult *Lu. evansi* specimens were removed aseptically with sterile stilettos under a stereoscope in 1X PBS buffer. Isolates were cultured under aerobic conditions

(33°C for 24 and 48 hours) by surface plating intestinal homogenates on Luria-Bertani (LB) agar (Merck). The selected isolates were purified, characterized by macro and microscopic appearence of the colony, Gram stained (**Figures 1(a)-(c)**) and molecularly by analyzing the spacer region (ITS) between the 23S and 16S ribosomal gene, the 16S rRNA and (**Figure 1(d)**) *gyr*B genes partial nucleotide sequences. Estimated concentrations of 10^7 CFU/ml and 10^8 CFU/ml were calculated to challenge the isolates in the *in vitro* activity test against promastigotes of *L. infantum*. The cell concentration of bacteria was estimated with commercial McFarland turbidity standard pattern (BBL McFarland Turbidity Standard No. 0.5).

2.3. Reactivation of *Leishmania infantum* Fluorescent Promastigotes, Fluorescence Emission Estimation and Calculation of Cell Concentration

The BCN-GFP strain of *L. infantum* transfected with green fluorescent protein was thawed and planted in biphasic modified Novy, Nicolle and McNeal (NNN) medium for growth of promastigotes, verifying their viability by observation with a fluorescence inverted microscope (Nikon eclipse TS100) [19]. GFP-expressing promastigotes were analyzed flow cytometrically in 10,000 gated events and the numeric data were processed by using WinMDI software. *L. infantum* promastigotes were incubated at 26°C, performing successive sub-cultures to obtain parasites with 98% of fluorescence, which allow estimating the action of bacterial isolates *in vitro* by flow cytometry.
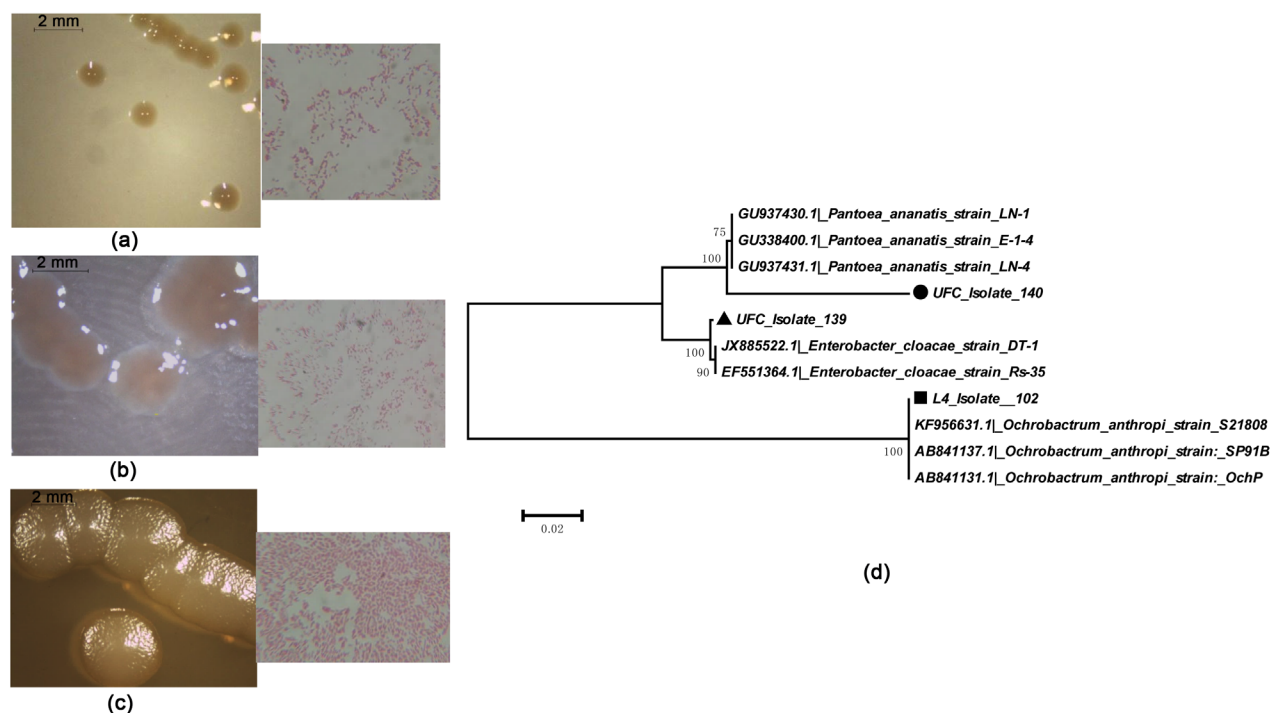

Figure 1. Colony morphology (left panel), Gram stain (right panel) of the strains *P. ananatis* (a); *E. cloacae* (b); *O. anthropi*; (c) isolated from the gut of *Lu. evansi* and NJ dendrogram (d) of partial nucleotide sequences of 16S gene, illustrating the taxonomic confirmation of the bacterial isolates.

2.4. *In Vitro* Antileishmanial Activity Assay of Bacterial against Stationary and Exponential Promastigotes of *L. infantum*

Metacyclic-like (6 days of culture, stationary) and procyclic-like (3 days of culture, exponential) promastigotes of *L. infantum* were centrifuged at 1500 g for 10 minutes, washed twice with sterile PBS buffer for carbohydrate removal and then re suspended in single phase RPMI liquid culture medium without antibiotic at a final concentration of 3×10^6 parasites/ml for each co-culture and activity assay.

Cultures of *P. ananatis*, *O. anthropi* and *E. cloacae* grown in liquid LB medium (Merk) to 10^8 CFU/ml and 10^7 CFU/ml were obtained. These concentrations have been the most used in studies that evaluate the leishmanicidal activity of bacteria obtained from the digestive tract of insects [13] [16]. The cell pellet (concentrated by centrifugation at 6500 g for 5 minutes) was washed twice with sterile PBS buffer. Bacteria were re-suspended in PBS to a final concentration of 10^7 CFU/ml and incubated for 24 hours at 27°C with metacyclic-like or procyclic-like *L. infantum* promastigotes in RPMI. The trials were independent for each strain and in triplicate for each stage of development of the parasite. Cell viability controls consisted of PBS with promastigotes and the three bacterial strains re-suspended in PBS independently.

2.5. Quantification of the Bacterial Isolates Activity on *L. infantum* Promastigotes

The action of bacterial isolates on the viability of *L. infantum* promastigotes were determined by flow cytometry on a Cytomics FC 500MPL using an argon laser at 488nm of excitation and 525nm of emission, counting at least 10,000 events to calculate the number of fluorescent promastigotes. The acquired data was analyzed using the CXP (Beckman Coulter, Fullerton, CA, USA) software.

2.6. Evaluation of Antibacterial Activity from Extracts Secreted by *P. ananatis, O. anthropi* and *E. cloacae*

Production and evaluation of secondary metabolites secreted was performed following the method previously described [20]. An Erlenmeyer containing 50 ml of 2% LB broth (w/v), 2% Amberlite resin XAD-16 (W/V), was inoculated with 0.5 ml of each strain culture and grown overnight [20] [21]. This Amberlite, allows adsorption of organic substances of small and medium molecular weight in aqueous solutions. This is a macroreticular resin nonionic that absorbs and releases substances through hydrophobic and polar interactions [22]. These resins have been used successfully in the identification and characterization of antibiotics [20] [21] and other secondary metabolites.

After seven days of incubation at 30°C and 180 rpm, the resin was decanted from the culture medium and washed with distilled water and the absorbed products were eluted with 40 ml of 100% methanol for 30 minutes [23]. Each extract was then concentrated to 1.5 ml in a rotating evaporator at 40°C (Heidolph Efficient Rotary Evaporator Laborota 4001).

The bacteria used were reference strains: *Escherichia coli*, *Enterococcus faecalis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *S. marcescens* and *Staphylococcus aureus* subsp. *aureus* (Table 2). *Psychrobacter* sp. CP25 isolates were used as controls (positive control from Microbiop reference strain collection, National University of Colombia), Methanol (negative control) and the antibiotic chloramphenicol (10 ug/ml, positive control).

Diffusion test in agar was used [24]. Sterile Whatman No.1 filter paper discs, 6 mm diameter, were impregnated with 10, 20 and 50 ul of each extract and placed on the surface of Petri dishes containing Mueller-Hinton agar (Becton Dickinson), previously inoculated with a liquid culture of the target strains at a concentration of 1.2×10^8 CFU/ml (absorbance 600 nm = 0.1). The plates were incubated at 37°C for 18 hours and the diameter of the growth inhibition halo around each disk was measured. Determination of the antibacterial activity was performed following the procedure described by Bauer *et al.* 1966 and amended by the Clinical and Laboratory Standards Institute [25] [26]. The antibacterial activity assays were performed in duplicate in two independent experiments.

2.7. Antibiotic Susceptibility Test

The antibiotic susceptibility tests for the bacterial isolates (*P. ananatis*, *O. anthropi* and *E. cloacae*), were developed with Mueller Hilton agar plates (Bckton Dickinson). An inoculum of 10^8 CFU/ml of each bacterial isolate was used (0.5 units on the McFarland scale, McFarland Turbidity Standard BBL). All isolates were tested against 14 different antibiotics of known concentration classified as follows: Rifampicin (RD 5; Oxoid, 5 ug), Tetracycline (Te 30, Valtek, 30 mcg), Gentamicin (CN120; Oxoid; 120 ug - 10 ug); Penicillin (P 30; Comprolab; 30 FMU), Chloramphenicol (C 30; Oxoid, 30 ug), Sulbactam Cefopeazone (SFC 105; Oxoid, 105 ug), Cefepime (CEP; Oxoid, 30 ug); Cefoperazone (PIC; Oxoid, 75 ug), Cefuroxime (CXM, Oxoid; 30 ug), Cephazolin (KZ; Oxoid, 30 ug), Ceftriaxone (CRO; Oxoid, 30 ug), Cefoxitin (FOX; Oxoid, 30 ug) Ceftazidime (CAZ; Oxoid, 30 ug). The plates were incubated at 30°C for 24 hours and the bacterial growth inhibition halos were measured by the diameter in mm. The percentage inhibition was calculated with reference to the measurement of the diameter of the inhibition zone, established for gram positive and gram negative bacteria (M100-S25 protocol-Performance Standards for Antimicrobial Susceptibility Testing).

2.8. Data Analysis

Statistical analysis of the antibacterial activity was estimated by a two-way ANOVA with the GraphPad Prism version 4.0 program using the extracts and targeted pathogenic bacteria as factors. Based on the diameter of the antibiotics inhibition halos (growth inhibition), bacteria were categorized as susceptibility, moderately susceptibility, highly susceptibility and resistant according to the M100-S25 protocol (Performance Standards for Antimicrobial Susceptibility Testing).

3. Results

3.1. *In Vitro* Bacterial Test with Procyclic and Metacyclic Promastigotes

The co-culture of the three bacterial isolates with promastigotes of *L. infantum* caused inhibition of procyclic-like but not metacyclic-like promastigotes. A greater impact on the inhibition percentage of the promastigotes using the bacterial cell concentration of $1 - 2 \times 10^8$ CFU/ml (Table 1) was observed. The standard deviation calculated for triplicate assays of co-culturing bacteria and promastigotes was low SD = 0.5 and 5.3, respectively, indicating that the experimental design is robust.

A greater impact of cell concentration of $1 - 2 \times 10^8$ CFU/ml of *E. cloacae* and *P. ananatis* on the percent inhibition of procyclic-like parasites is further noted, with values of 70.17 ± 1.1 and 77.29 ± 0.6 respectively (Table 1), whereas *E. cloacae* also significantly altered the development of procyclic-like promastigotes with bacterial concentrations of $1 - 2 \times 10^7$ CFU/ml (Table 1). *O. anthropi* had the lowest inhibitory activity on procyclic-like (62.33 ± 2.0; 38.13 ± 1.4) and metacyclic-like promastigotes (32.95 ± 5.3; 36.81 ± 3.2), with respect to the other two isolates analyzed. *P. ananatis* was the only bacterial isolate that presented inhibitory activity of 50.01% of metacyclic-like promastigotes.

3.2. Antimicrobial Activity Test of Crude Methanolic Extracts

The three extracts produced by *O. anthropi*, *P. ananatis* and *E. cloacae* exhibited similar antimicrobial activity patterns against all bacteria tested, with inhibition zones between 13 mm and 22 mm (Table 2, Figure 2). Highly significant differences between the inhibition zones associated with gram-positive bacteria used were found ($P < 0.003557$). The species most susceptibility to the extracts produced by the isolates from the digestive tract of *Lu. evansi* was *B. cereus*, with inhibition halos of 22 mm with others less susceptibility to the extracts activity like *E. coli* and *E. faecalis*, with inhibition halos between 13 mm and 15 mm. Figure 2(a) shows the antibacterial activity of the *E. cloacae* extract (more active) with the clinical isolate *B. cereus*.

Table 1. *In vitro* activity of three bacterial isolates from the gut of *Lu. evansi* against procyclic and metacyclic promastigotes of *Leishmania infantum*.

Cell Concentration	*O. anthropi*		*P. ananatis*		*E. cloacae*	
	Inhibition % MP	Inhibition % PP	Inhibition % MP	Inhibition % PP	Inhibition % MP	Inhibition % PP
$1 - 2 \times 10^7$ CFU/ml	36.81 ± 3.2	38.13 ± 1.4	44.46 ± 3.2	66.41 ± 0.5	38.12 ± 0.5	71.04 ± 1.2
$1 - 2 \times 10^8$ CFU/ml	32.95 ± 5.3	62.33 ± 2.0	50.01 ± 1.3	$\mathbf{70.17 \pm 1.1}$	48.73 ± 1.5	$\mathbf{77.29 \pm 0.6}$

The data represents the average value **(X)** ± standard deviation **(SD)** of two experiments each in triplicate. Symbols: **CFU/ml** colony forming units per milliliter; **%** Percentage; **±** standard deviation; **MP** metacyclic promastigotes of *Leishmania infantum*; **PP** procyclic promastigotes of *Leishmania infantum*. Note: Number of parasites in each trial = 3×10^6 parasites/ml.

Table 2. Antibacterial activity of extracts produced by strains *O. anthropi, E. cloacae* and *P. ananatis* isolated from the gut of *Lu. evansi*.

Bacterial Group	Target microorganism/ Inhibition halo (mm)*					
	Gram negative				Gram positive	
Strain tested (Extract source)	*E. coli* (ATCC• 8739™)	*P. aeruginosa* (ATCC• 9027™)	*S. marcescens* (Clinical isolate)	*B.cereus* (Clinical isolate)	*E. faecalis* (ATCC• 51299™)	*S. aureus* subsp. *aureus* (ATCC• 29213™)
O. anthropi	14	15.5	18	22	13	19
E. cloacae	13	17.5	18	22	15	19
P. ananatis	15	15	16	22	14	17
Psychrobacter sp. C+	18.5	13.5	15	21	14	13.5
Methanol C−	0	0	0	8.5	0	0
Chloramphenicol C+	28	20	29	34.5	9	33.5

C+: positive control; **C−:** negative control; **mm:** diameter of the inhibition zones, average of the replicates per sample.
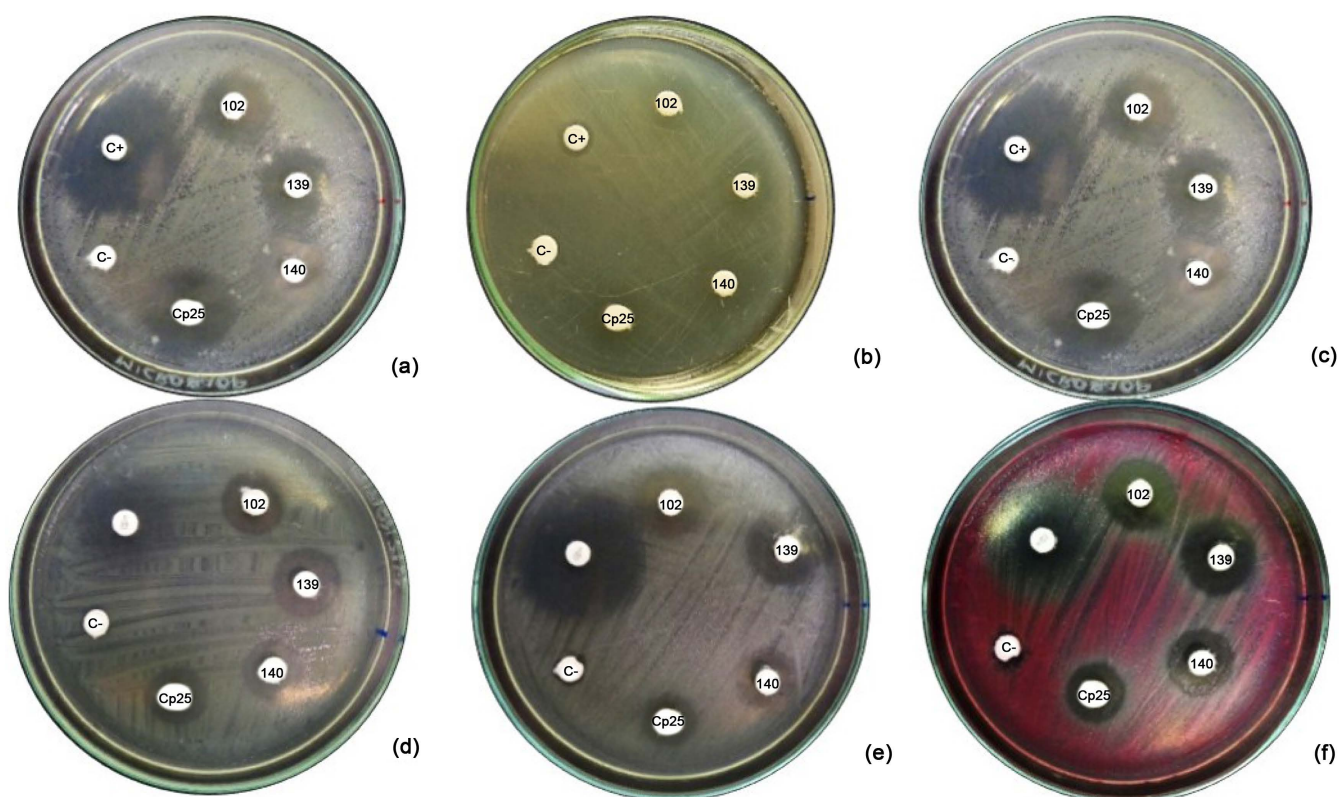

Figure 2. Agar diffusion assay of the antibacterial activity of the extracts produced by *P. ananatis* (140), *E. cloacae* (139), *O. anthropi* (102): (a) antibacterial activity against B. cereus; (b) antibacterial activity against *E. faecalis*; (c) antibacterial activity against *E. coli*; (d) antibacterial activity against *P. aeruginosa*; and. antibacterial activity against *S. aureus* subsp. *aureus*; F. antibacterial activity against *S. marcescens*; C− = methanol; C+ = Chloramphenicol; CP25 = *Psychrobacter*.

3.3. Antibiotic Susceptibility Test

The *E. cloacae* and *P. ananatis* isolates showed resistance to penicillin and rifampicin, while *O. anthropi* presented antibiotic resistance to Cephazolin and Cefoxitin (Table 3). Additionally, *O. anthropi* presented a greater number of resistance patterns to antibiotics, being resistant to penicillin, sulbactam, cefopeazone, cefuroxime, cephazolin, ceftriaxone, cefoxitin and ceftazidime (Table 3). *E. cloacae* and *P. ananatis* had higher susceptibility, mainly with Beta-lactams, cephalosporins, chloramphenicol and whereas *O. anthropi* only presented high susceptibility with tetracyclines and aminoglycosides (Table 3).

4. Discussions

The bacterial isolates *P. ananatis*, *O. anthropi* and *E. cloacae*, obtained from the intestinal microbiota of *Lu. evansi* assessed in this study exhibited differential activity against *L. infantum* as well as a differential susceptibility to antibiotics and against clinical isolates. The high inhibition percentage (72.29%) is generated by *E. cloacae* against procyclic-like promastigotes of *L. infantum*, when co-cultured under *in vitro* conditions is emphasized. This is the first study demonstrating the *in vitro* activity of *E. cloacae* against promastigotes of *Leishmania*, from studies that recognize its importance in the vector competence of some insects [11] [27]. It is suggested that the action of *E. cloacae* can be derived from the expression of peptides or molecules with lytic activity on the surface of prokaryotes, by the action of enterococcal cytolysins (hemolysin) [28]. However, this hypothesis needs further studies.

Table 3. Antibiotic sensitivity patterns of the strains *O. anthropi*, *E. cloacae* and *P. ananatis* isolated from the gut of *Lu. evansi*.

Classification	Antibiotic	Bacterial isolates (10⁸ CFU/ml)		
		E. cloacae	*P. ananatis*	*O. anthropi*
Rifamycins	Rifampicin (RD)	R	R	++
Tetracyclines	Tetracyclin (Te)	++	++	+++
Aminoglycosides	Gentamicin (CN120)	++	++	+++
Beta-Lactams	Penicillin (P)	R	R	R
Chloramphenicol	Chloramphenicol (C)	+++	+++	+
Cephalosporins	Sulbactam Cefopeazone (SFC)	+++	+++	R
Cephalosporins	Cefepime (CEP)	+++	+++	+
Cephalosporins	Cefoperazone (CFP)	+++	+++	+
Cephalosporins	Cefuroxime (CXM)	+++	++	R
Cephalosporins	Cephazolin (KZ)	R	+++	R
Cephalosporins	Ceftriaxone (CRO)	+++	+++	R
Cephalosporins	Cefoxitin (FOX)	R	+++	R
Cephalosporins	Ceftazidime (CAZ)	+++	+++	R

Symbols: R resistant (full growth); **+**Sensitive (Halo 10 - 17 mm); **++**Moderately sensitive (Halo 18 - 27mm); **+++**Highly sensitive (Halo of 28 - 37 mm).

In this sense, some studies have reported that the protective response of *L. infantum* procyclic promastigotes associated with the generation of glycoconjugates (proteophosphoglycans, acid phosphatase, lipophosphoglycans, metalloproteins) [29], is not sufficient for protection against enzymes or highly pathogenic bacterial peptides expressed by *E. cloacae*. According to the literature, in this state lifecycle (24 - 48 hrs), procyclical promastigotes of *L. infantum* present a lower degree of specialization and adaptation with respect to the metacyclic promastigotes (infective stage), which produce stronger enzymes such as chitinases that may even degrade the insects stomodeal valve and have a defence system resistant to mammalian complement factors and greater mobility [30]. The *in vitro* activity of *E. cloacae* on procyclic-like promastigotes of *Leishmania* is consistent and can justify their use in paratrasgenesis to express antitrypanosomal peptides, because other reports state that the bacteria also block the development of other parasites as *Plasmodium falciparum* in *Anopheles gambiae* and the sporogonic development of *P. vivax* in *An. albimanus* [27] [31].

Similar to *E. cloacae*, the symbiont *P. ananatis* showed a significant activity over the survival (70.17%) of the procyclic promastigotes of *L. infantum*. *P. ananatis* only has reported entomopathogenic activity for other insects [32] [33]. These aspects are interesting because these bacteria could be used to disrupt the life cycle of sandflies and the transmission of *Leishmania* spp, by the rapid spread and adaptation of these arthropods [32] [34], as previously described in a study in which *P. agglomerans* (family Enterobacteriaceae) was genetically modified, to express and secrete two anti-plasmodium effectors proteins (pelB, hly) in infected mosquitoes [35].

The dissemination of *P. ananatis* symbiont to organs or complex structures of insects suggests that it is a specialized bacterium [33], which is supported by its pan-genome that incorporates a large number of protein encoding genes that enable *P. ananatis* to colonize, persist and secrete a wide range of peptides [34]. This can also be related to the better activity over the survival of metacyclic promastigotes (50.01%) compared to *E. cloacae* and *O. anthropi*. *O. anthropi* had lower activity against metacyclic (32.95%) and procyclic promastigotes (62.33%). Unlike our results, the activity of other *Ochrobactrum* species (*O. intermedium*, *Ochrobactrum* sp., AK strain) presented greater impact (~90%) on the survival of *L. mexicana* promastigotes in co-infection trials with *Lu. longipalpis* and *in vitro* assays [16] [36].

The crude methanolic extracts exhibited similar antimicrobial activity patterns against target bacteria, with a difference appreciated mainly against the growth of *B. cereus* (22 mm), suggesting that the isolates *O. anthropi*, *E. cloacae* and *P. ananatis* are important sources of promising antimicrobial compounds with a wide biological activity spectrum. In this sense these compounds or secreted peptides, can provide selective advantages to these bacteria in different environmental niches (including the digestive tract of sandflies) and be important for colonization, providing virulence factors and defence systems to keep its niche or prevent invasion from other bacterial strains [11] [37].

Gram negative bacteria, such as those used in this study, currently have six types of protein secretion systems reported (T1SS to T6SS) associated with bacterial compete-

tion [38] [39]. Among these systems, T6SS has a role in cytotoxicity, biofilm formation, antimicrobial peptide transport and interaction with host cells. This system has recently been described for *P. ananatis*, being responsible for their potential virulence and antimicrobial activity [38].

Some members of the *Enterobacteriaceae* family are known to produce bacteriocins (3% to 26%) such as enterocins, colicins and antimicrobial lipopeptides produced by different species of *Enterobacter*, with great biopharmaceutical potential [40] [41] suggesting that bacteriocins are produced by these bacteria as part of their defense mechanism to survive complex environments such as the digestive tract of different kinds of insect vectors (*Lutzomyia*, *Phlebotomus*, *Anopheles*, *Aedes*) where *E. cloacae* is a dominant taxonomic unit [32].

Additionally, *O. anthropi*, which also exhibits antimicrobial activity against Gram positive and Gram negative bacteria, is of great interest for bioremediation and for their ability to degrade organophosphates [42]. In this sense, knowledge on antimicrobial peptides secreted by *O. anthropi* is interesting because this bacterium can transfer pesticide resistance factors to sandflies or simply remove pesticides by degradation [42] [43]. *O. anthropi* secretes detoxification enzymes, reactive oxygen species and nucleosides of great interest for their anti-tumoral, antiviral, antibiotic and antiparasitic activity [44] [45].

Few reports inform about the susceptibility of antibacterial compounds from *O. anthropi*. In our study, this isolate was resistant to most cephalosporins and penicillins, but susceptible to rifampicin, chloramphenicol, some cephalosporins (cefepime, cefoperazone), tetracycline and gentamicin. The latter two antibiotics were the most active on *O. anthropi*. Our results are consistent with other studies reporting multi-resistance patterns present in *O. anthropi* [46] [47]. However some strains of *O. anthropi* exhibit resistance patterns to cefepime [48] and only in few cases they are susceptibility to cefoperazone [49].

Unlike *O. Anthropi*, the *E. cloacae* and *P. ananatis* isolates exhibited fewer resistance patterns to the antibiotics tested, and agreed in their response to penicillin and rifampicin, while *P. ananatis* was also resistant to cephazolin and cefoxitin. Both bacteria are reported as multiresistant for its environmental ubiquity and invasion of different hosts including soils, plants, animals and insects [50]. The greatest susceptibility pattern of these two isolated correspond to cephalosporins, although some reports indicate their resistance to cefuroxime [50]. Although bacterial resitencia is analyzed in vitro in this study, the result may indicate competitive factors and/or growth of bacteria in the gut, which may favour the development or block *Leishmania* promastigotes.

The antibiotic susceptibility tests of the intestinal microbiota of insect vectors are important for co-infection based assays with parasites or viruses, in order to evaluate drugs, vaccines or to determine the autonomous vector competence of the insect. In this sense, to remove or modulate the resident intestinal microbiota depends on the resistance state to certain antibiotics, and allows to access the functional relationships between gut microbiota and their hosts.

The ability of *E. cloacae* and *P. ananatis* to inhibit the growth of procyclic-like promastigotes of *L infantum* in co-culture and the similar susceptibility patterns shown by *O. anthropic*, suggest that these isolates are promising for future control strategies aimed at evaluating the parasite load in *Lutzomyia* species when exposed to *E. cloacae* and *P. ananatis*, in order to provide new ways to reduce the transmission of leishmaniasis.

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Authors' Contributions

CXMH, GECR and SUS: Designed the study, analyzed the data and contributed to write the manuscript. RJV, SR: Designed the study, performed the experiments, analyzed the data and contributed to write the manuscript. VO: Performed the experiments and analyzed the data.

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Conflict of Interest

There is no conflict of interest from other co-others in the publication of this manuscript in this journal. All the co-others have contributed in the preparation of the manuscript up to the submission stage.

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