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PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF *BDELLOVIBRIO ISOLATES* SPP. IN MEXICO

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Abstract: *Bdellovibrio* spp. is a Deltaproteobacteria, ubiquitous, Gram-negative, uniflagellate, highly motile, preys on Gram-negative bacteria, recently reported predation on Gram-positive bacteria. It belongs to the BALOs group (*Bdellovibrio* and- like - organisms). *bdellovibrio exovores* it moves quickly using its flagellum, adhering to the outer membrane, secreting enzymes and mechanical movements consumes the prey. *b. bacteriovorus* invades the periplasm forming a *bdelloplast,* where it replicates by multiple fission. The factors involved in predation are the flagellum, mobilization, lytic enzymes, interaction genes "hit locus" bd0108 and bd0109, prey range, etc. The objective was to characterize phenotypic and molecular isolates of *Bdellovibrio* spp. in Mexico. Pathogenic bacteria of clinical interest were used as prey, and samples of water, soil, and animal feces, to isolate predatory bacteria, were confronted in order to observe lytic activity. Sequences of the 16S rRNA gene were used to amplify by PCR for the genus *Bdellovibrio,* sequences from the family Bdellovibrionaceae. Eighty-six positive isolates for BALOs were obtained, cell lysis of the prey bacteria was observed, a prey range was performed, measuring the lytic activity (prey-predator).
Keywords: Characterization, molecular, isolated, *Bdellovibrio,* pathogens.

INTRODUCTION

Gram-negative bacteria are important, because some are pathogenic to humans. Its pathogenicity lies in the composition of its cell wall composed of a lipid bilayer (lipids A), which, when an infection occurs, induce an immune response. In the Gram-negative Group: *Neisseria* are found *gonorrhoeae, N. meningitis* and some bacilli that cause respiratory infections: *Haemophilus influenzae, Klebsiella pneumoniae, Pseudomonas aeuroginosa ;* in urinary infections: *E. coli* and *Enterobacter cloacae ;* and in gastrointestinal infections, have been reported to: *Helicobacter pylori and Salmonella typhi,* among others (Gupta, 2011). In the Gram-positive Group, they contain other components in their cell wall such as peptidoglycan, teichoic and lipoteichoic acids, which are responsible for their pathogenicity. Some bacteria like *Streptococcus pneumoniae, Listeria monocytogenes, Staphylococcus aureus, S. epidermidis, S. haemolyticus, S. hominis novobisepticus,* cause serious infections in humans (Serruto *et al.*, 2010). According to the CDC EU, *Salmonella* and *Campylobacter* infections amount to 410,000 people a year. Antimicrobial-resistant infections produced per year amount to around 2 million people and 23,000 deaths (CDC, 2013). In Mexico, intestinal infections produced by other microorganisms and poorly defined have been reported, which amounted to around 6 million cases, classified as the second leading cause of disease in the country, streptococcal pharyngitis and tonsillitis left 229,000 cases, finally there were 92,000 cases by salmonellosis (SSA, 2017). In epidemiological week 52, 5 federal entities presented an increase in the number of ADD cases compared to the previous week, being the 5 main ones: Nuevo León (66.7%), Coahuila (50%), Colima (16.7%), Querétaro (16.7%) and Morelos (14.3%) (SSA, 2022). On the other hand, the increase in resistance to antibiotics has produced infections that are difficult to control, which is why emphasis has been placed on research and the search for new alternatives other than drugs. Likewise, the World Health Organization (WHO) in 2017 published a list of priority pathogens for R&D according to their resistance to antibiotics (WHO, 2017), and given the increase in resistance to antimicrobials. In 2020, the WHO classified it as a public health problem, within the list of urgent health problems of a global dimension (WHO, 2020). *Bdellovibrio* is a Gram-negative

bacterium, an obligate predator of other Gram-negative bacteria and some Gram-positive bacteria. *bdellovibrio* it moves quickly through its flagellum and joins the prey cell, attacking the outer membrane and invading the periplasm, it secretes proteolytic enzymes, giving rise to the formation of the bdelloplast, taking advantage of the nutrients of the prey bacterium until its replication by multiple *fission*, eventually the prey cell is lysed and the progeny of the predatory bacteria are released. There are different factors involved in the predation process, such as the flagellum, its mobilization, some lytic enzymes, the *bd0108* and bd0109 genes, important in coding for the retraction and extrusion of the pili involved in the detection and binding system to the prey. Therefore, the importance of this study to characterize phenotypic and molecular isolates of *Bdellovibrio* spp. of organic and environmental samples from different states of the Mexican Republic through PCR amplification of the 16S rRNA gene using specific primers for the species, as well as determining the prey range of the isolates, which provides information to determine their possible applications.

METHODOLOGY

Soil, water, and animal feces samples were obtained in falcon-type tubes and transported to the Genomic Biotechnology Laboratory of the National Polytechnic Institute, Reynosa, Tamaulipas, Mexico. *Salmonella enterica* and *Klebsiella* were inoculated *pneumoniae* individually (dams) in 20 ml of Luria Bertani (LB) broth were incubated at 37 °C/18 hours at 180 rpm, centrifuged at 3500 rpm/20 min, 4 °C, discarding the supernatant and obtaining cell pellets. On the other hand, 10 grams of soil or feces were incubated in 100 ml of milli -Q water at 180 rpm at 30 °C/1 hour, then centrifuged at 3500 rpm/20 min, 4 °C, keeping the liquid part. and discarding the solids. The dams were immediately resuspended with the liquid samples of soil and feces (co-culture), and they were incubated from 24 hours to 7 days at 180 rpm at 30 °C, until cell lysis was observed. For liquid samples, organic matter was removed with filter paper and mixed directly with the prey. The co-culture was repeated at least twice in order to obtain a larger population of the predatory bacteria.

DNA extraction was performed by heat lysis and from this the presumptive PCR to determine the presence of possible *Bdellovibrio.* spp., or BALOs, upon confirming positive by means of PCR, these were selected to obtain DNA using the commercial Wizard ® Genomic DNA Purification Kit, Ref. A1120, Promega.

PCR identification of *Bdellovibrio isolates* spp. It was carried out according to (Van Essche *et al.*, 2009). The resulting products were analyzed by 2% agarose gel electrophoresis in 1X TAE buffer solution, for 1 hour at 80 volts. Finally, the gel was visualized in a Kodak® photo documenter with a Gel Logic 112 camera using the Kodak® ds 1D bioinformatics program. In the Sequencing of the specific gene for *Bdellovibrio* spp. ExoSAP -IT™ was used to purify the PCR products, the sequencing reaction was performed using the commercial kit BigDye ™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, # 4337455). The resulting products were subjected to purification with the commercial BigDye ® X- Terminator ™ kit from Applied Biosystems. To confirm the amplified products from the isolates of predatory bacteria, the sequencing reaction was carried out using the capillary electrophoresis sequencing technique, in the Services Laboratory of the Genomic Biotechnology Center of the National Polytechnic Institute, using the ABI® 3130 equipment. genetics Applied Analyzer _ Biosystems. Sequencing analysis was performed using computer files in.ab1 format

was used to create a consensus sequence from the sequences from isolates from predatory bacteria with the direct primer. The sequences were cleaned using FinchTV 1.4. 0 and the search for homologous sequences was carried out using NCBI BLAST. Finally, they were aligned using reference sequences from the Deltaproteobacteria class. proper genera *Bdellovibrio* sp., *Peredibacter* sp. *and Bacteriovorax* sp., from the Bdellovibrionaceae and Bacteriovoracaceae families, with the programs Gblocks 0.91b and the MEGA X program was used, the *find tool was used best DNA model* was determined using the construction of a phylogenetic tree using the Neighbor-Joining method and UPGMA for the 16S rRNA sequence, with Kimura's two-parameter model at 1000 replicates using a Gamma distribution.

For prey range determination. *Klebsiella* was inoculated *pneumoniae* 18 hours at 37 °C/180 rpm, the pellets were washed 3 times with sterile milli -Q water, homogenizing and centrifuging the sample, the pellet was resuspended with 3.5 ml of 25 mM HEPES buffer, pH 7.4, mixing until dissolved. On the other hand, the isolate of *Bdellovibrio* spp. it was grown 4 days before in co-culture with *Klebsiella pneumoniae* in 4 ml of HEPES buffer, washing the co-culture, it was centrifuged to use the supernatant, filtering 3 times with 0.45 μm, the predator was concentrated by centrifuging at 15,750 rpm for 40 min at 4 ºC and the pellet was resuspended with 8 ml of HEPES. 25 mM HEPES buffer, pH 7.4. 500 μl of predator per prey were added to obtain a nutrient clean co-culture and 7:1 ratio. Determining the decrease in the optical density of the co-cultures, confronting 3 isolates of BALOs, a reference strain and the negative control, which were confronted against 12 prey bacteria, of which 4 were Gram-positive.

RESULTS

A total of 86 samples were collected in six different states of the country, of which 32 were water, 32 soil and 22 feces, Table 1 origin of the samples obtained and distribution by State. The States sampled were: Baja California (6 environmental samples), Baja California Sur (9 environmental samples), Coahuila (13 environmental samples), Nuevo León (10 environmental samples), Tamaulipas (12 environmental samples and 22 fecal samples) and Veracruz. (12 environmental samples).

The co-culture is cloudy at the beginning, and after 48 hours and up to 7 days cell lysis is observed, cell debris forms at the bottom of the flask, or on the walls, as well as co-culture clarification, Figure 1.

The gDNA of 40 isolates of predatory bacteria was obtained using the commercial Wizard ® Genomic DNA Purification Kit, Ref. A1120, Promega, previously positive by PCR-Lysis using the primers Bbs and Bds as presumptive evidence, Figure 2.

Isolates of predatory bacteria were obtained, positive by PCR using the primers Bbs (490 bp), from gDNA, Figure 3.

Twenty-one isolates of predatory bacteria were sequenced using an ABI® 3130 Genetic sequencer. Analyzer, were aligned using NCBI's Blast. 10 isolates of *Bdellovibrio* were obtained. *bacteriovorus, 9 Bdellovibrio* isolates sp., and 3 isolates from the Bacteriovoracaceae family, a phylogenetic tree was made, observing two defined groups, in the first clade, isolates from the Bdellovibrionaceae family were found, highlighting the isolates LBGBsp014 and LBGBsp015 close to *B. exovorus* JSS and *Bdellovibrio* sp. qaytius, in another subgroup, the reference isolates, close to the isolates in the laboratory. In the second clade, the isolates LBGBsp017, LBGBsp056, and LBGBsp064 were found, which aligned with the genera *Peredibacter, Bacteriovorax, and Halobacteriovorax.* The LBGBsp007 and

sample origin	Floor	Water	Stool
lower california	6	-	-
Baja California Sur	-	9	-
Coahuila	13	-	-
New Lion	10	-	-
Tamaulipas	3	11	22
veracruz	-	12	-

Table 1. Origin and Distribution of the sampling in Mexico.

Figure 1. Co-culture with lysis at the bottom of the flask, *Salmonella enterica*.

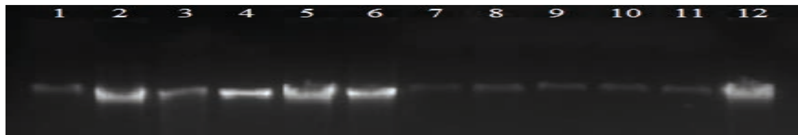

Figure 2. 2% agarose gel at 80 volts for 1 h 15 min with representative DNA with BALOs genomic DNA extracted with the purification kit (Promega, A1120). SSB218315, 2. HD100, 3. LBGBsp013, 4. LBGBsp017, 5. LBGBsp031, 6. LBGBsp034, 7. LBGBsp038, 8. LBGBsp039, 9. LBGBsp040, 10. LBGBsp041, 11. LBGBsp052.LB4.

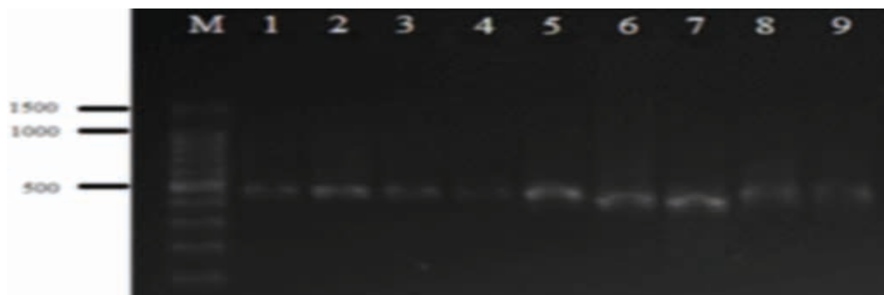

Figure 3. Representative 2% agarose gel at 80 volts for 1 h 15 min with PCR product of the 16S rRNA gene of *Bdellovibrio* spp. with an expected 490 bp fragment with Bbs primers. M. Promega G2101 marker 100 bp, 1. LBGBsp007, 2. LBGBsp013, 3. LBGBsp014, 4. LBGBsp017, 5. LBGBsp030, 6. LBGBsp031, 7. LBGBsp039, 8. LBGBsp043, 9. LBGBsp064.

LBGBsp007-1 isolates came from the same sample, with different prey, in the phylogenetic analysis we observed differences, Figure 4.

In the prey range, the quantification of the decrease in the optical density of the co-cultures was obtained, confronting 3 isolates of BALOs, a reference strain and the negative control, which were confronted against 12 prey bacteria, of which, 4 were Gram-positive, which can be observed in Table 2, where the daily results of the co-cultures are graphically represented. It was observed that the BALO isolates had different affinity against the prey bacteria. For example, the LBGBsp031 isolate showed greater predation, even greater than the control strain, the LBGBsp055 isolate had predation only against Gram-negative bacteria, on the other hand, the LBGBsp017 isolate does not prey on enterobacter prey *aerogenes* or *Esquerichia coli,* but yes to *Staphylococcus haemolyticus.* It must be noted that pathogenic bacteria were used as prey of importance for the WHO, as well as epidemiological importance for the country.

DISCUSSION

Bdellovibrio bacteriovorus, is a ubiquitous predatory bacterium mainly in aquatic environments, however, the LBGBsp007 isolate is unique, because it comes from a completely dry environment (25°38'30.3"N 100°02'51.7"W), there were few reports of isolation from a similar biome, in addition, two different isolates LBGBsp007 and LBGBsp007-1 were obtained from said sample; which had a high percentage of identity and coverage with the *Bdellovibrio strain* sp. DM11A reported to treat phytopathogens isolated from the rhizosphere (Feng *et al.*, 2016; McNeely *et al.*, 2017; Hang *et al.*, 2020) was isolated from activated sludge at the Ulu Pandan Water Reclamation Plant, Singapore. 16S rDNA gene sequencing analysis revealed that this isolate was 99% identical to 'Bdellovibrio bacteriovorus strain Tiberius' and hence is designated as 'Bdellovibrio bacteriovorus UP'. Using a novel approach based on fluorescence in situ hybridization (FISH. Also, aquatic samples were obtained and isolates such as LBGBsp009, and from LBGBsp013 to LBGBsp017, and from LBGBsp030 to LBGBsp035, as well as from LBGBsp050 to LBGBsp058 were obtained, as reported by some authors such as Feng et al. 2016 where they treated flocculated sludge isolating the *Bdellovibrio strain* sp. UP (Feng *et al.*, 2016; Feng *et al.*, 2017; Mookherjee, and Jurkevitch, 2022), recently, samples from lakes or seas have also been obtained, as in the case of Paix et al. in 2019, where they took samples from Perialpine lakes (Annecy, Bourget and Geneva lakes), and show the diversity of bacteria, observing within them members of the Peredibacteraceae families and Bacteriovoracaceae (Lambert *et al.*, 2006; Paix *et al.,* 2019). From the LBGBsp060 isolates, they were fecal samples from exotic animals in captivity, from which we were able to find several bacteria of the genus *Bdellovibrio.* sp., and one specifically from the genus *Bacteriovorax* of *ursus feces americanus.*

There are reports of bacteria isolated from the intestine of animals, such as in 2001, where six BALOs were obtained using *Proteus mirabilis.* and *Citrobacter freundii* like prey. In 2011, poultry were treated with *Bdellovibrio* sp. showing a decrease in the contamination of the environment where they developed, the microbiota of the animals barely two days old was modified, but *Bdellovibrio* did not cause a negative effect on larger chickens. Another study was done in 2017 in *Sprague-Dawley rats,* where they were inoculated with *B. bacteriovorus.* 109J, in order to observe the behavior of the immune system, showing that the bacteria was harmless (Atterbury *et al.*, 2011; Schwudke *et al.*, 2001; Shatzkes *et al.*, 2017). In the case of the isolate LBGBsp015,

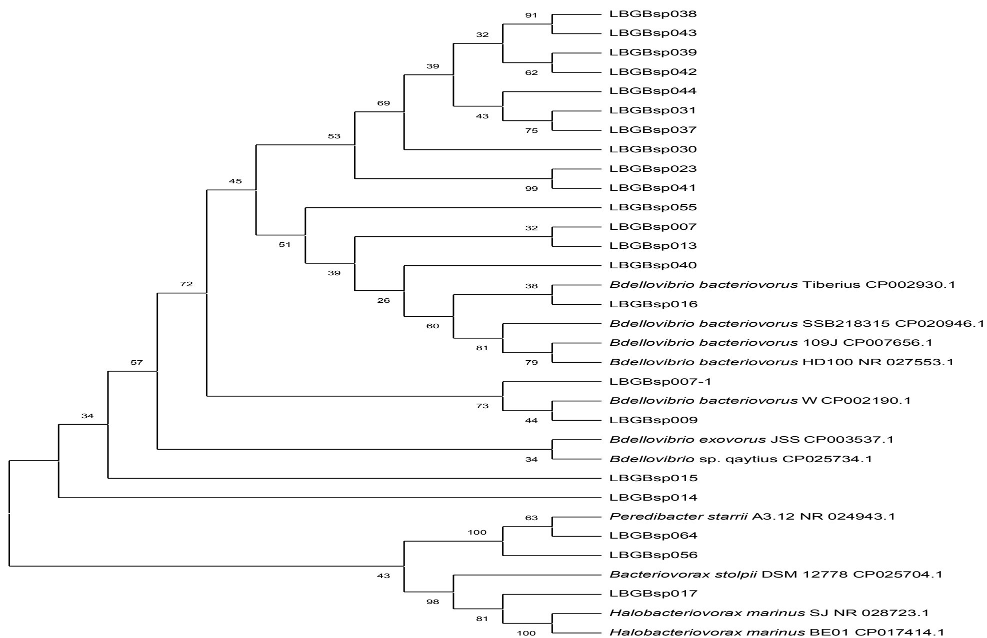

Figure 4. Phylogenetic tree of the fragment of interest of the Bds gene (490 bp).

Prey	LBGBsp017	LBGB sp 031	LBGB sp 055	HD100
***staphylococcus aureus* TO**	-	*	-	*
***staphylococcus epidermidis* B.**	-	*	-	*
***staphylococcus haemolyticus* TO**	*	*	-	*
Staphylococcus hominis novobisepticus	-	*	-	-
Enterobacter aerogenes	-	-	*	*
E. coli	-	**	-	-
Klebsiella pneumoniae	*	*	*	*
Pseudomonas aeuroginosa	*	*	*	*
Pseudomonas fluorescens	*	*	***	-
Pseudomonas putida	*	***	**	*
enteric salmonella	*	**	*	**
Vibrio cholerae	*	**	**	*

*, **, *** = predation by significance, - = There was no predation.

Table 2. Prey range assay of *Bdellovibrio* isolates. spp.

it was obtained from a water sample from the municipal trace of the city of Orizaba, Veracruz (18°50'31.9"N+97°05'01.0"W), likewise, *B. exovorus* JSS, was found in wastewater from Ontario, Canada, to date it is the only bacterium of this species taken as a reference, and it aligns with our obtained bacterium, which could give us an indication of the origin of this species. On the other hand, the isolate LBGBsp017 was obtained from a water sample in the Ojo de Agua Lagoon in Orizaba, Veracruz (18°51'49.1"N+97°04'36.0"W) near marine algae, in a depth no greater than 15 cm, which is supported by the work of Paix and collaborators in 2019 where they talk about the distribution of BALOs in samples in the Perialpina (Koval *et al.,* 2013; Paix *et al*., 2019; Mookherjee, and Jurkevitch, 2022). LBGBsp056, is an isolate characterized as *Bacteriovorax* sp., obtained from a water sample surrounding Cabo San Lucas, Baja California Sur, Mexico (22°52'45.1"N+109°54'15.3"W), as reported for Bacteriovorax *samples marinus* and *Bacteriovorax litoralis,* likewise, the isolate LBGBsp064, which was obtained from *Ursus americanus,* was also characterized as *Bacteriovorax* sp., in 2013, it was possible to isolate a strain of this species from the intestine of the fish *Ophiocephalus argus,* with which they analyzed its predation against *Aeromonas veronica* (Cao *et al.,* 2014; Koval *et al.,* 2015) .

Some studies mention plaque growth, however, only two isolates with plaque growth were obtained, among them isolate LBGBsp044, however, it was not possible to replicate the assay, various modifications were made following the recommendations of some authors., but this technique could not be standardized (Jurkevitch, 2012; Enos *et al.,* 2017; Hobley *et al*., 2012).

In Mexico, in addition to the strain SSB218315 full genome and SKB1291214 (partial sequence), other strains apparently belonging to the *Bdellovibrio genus* have been isolated, such as the strains from Cancun; however, when doing the phylogeny, we realized that the *Bdellovibrio sample* sp. Cancun7 (no data were presented, since it was sought to refer only to complete strains), had a greater similarity with a *Bacteriovorax marinus,* which belongs to a different family, in the same way it happened with the isolates of LBGBsp017, LBGBsp056, and LBGBsp064, during the PCR we worked with reference primers for the *Bdellovibrionaceae family,* however, in the alignment of the sequences, we observed that it was a different species (Jurkevitch, 2012; Snyder *et al.,* 2002).

In 2013, during a study using samples from patients with cystic fibrosis, *Bdellovibrio isolates were obtained bacteriovorus,* finding that they had activity against *Pseudomonas aeuroginosa,* however, in this same study it was found that *Staphylococcus aureus* was also responsible for the condition, for this reason they tested *Bdellovibrio* against this dam, thus finding the first indication that *Bdellovibrio bacteriovorus* not only attacks Gram-negative bacteria, but also appears to have activity against Gram-positives, which supports our prey range results, however, it has recently been shown that the HD100 strain does not prey on bacteria, but it does inhibit the formation of biofilms and the invasion of human epithelial cells, which leaves a window of possibilities open. Thus, isolates such as LBGBsp031 and LBGBsp065 are bacteria that have a wide range of prey; furthermore, the LBGBsp038 isolate seems to have very good specific bacteriolytic activity against Gram-positive bacteria (Iebba *et al. al.*, 2013; Im, *et al.,* 2018; Monnappa *et al.,* 2014; Pantanella *et al.*, 2018). Among the various attack strategies of *Bdellovibrio* is the interaction locus, which encodes the *bd0108* and *bd0109 genes,* in 2013 it was observed that the modification of the *bd0108 gene* could influence the extrusion

and retraction of type IV pili, in the same way, it has been expressed that the gene is putative of *Bdellovibrio,* however, the gene is expressed by at least 2 ORFs, in the analysis we performed, the primers used aligned better in ORF1, therefore, we cannot know if our isolates had this gene or not, and whether or not it influenced the prey range, then, when comparing the isolates LBGBsp030 and LBGBsp015, which did amplify the region of the gene, expressed different prey range activity, the same step with isolates in which the gene was not amplified, in addition, by amplifying said region in the LBGBsp017 isolate, we were able to rule out that it was putative of *Bdellovibrio* as previously mentioned (Capeness *et al.*, 2013). For the prey range, the growth kinetics strategy using optical density is a technique that has been used on other occasions, with variations in the reading conditions, since, in 2000, they worked with isolated rhizosphere samples to control phytopathogens., and the growth time was every 10 hours using a wavelength of 570 nm and in 2016, the readings were made every 20 hours at a wavelength of 600 nm, in our case, the wavelength used was 595 nm, and the readings were made every 24 hours (Jurkevitch *et al.,* 2000; Oyedara *et al.,* 2016; Ajao *et al.*, 2022).

For cases of *Staphylococcus aureus and Pseudomonas aeuroginosa,* in 2014, an assay was carried out to observe the attack of biofilms in plates, an assay similar to ours, using the *B. bacteriovorus strain as a predatory bacterium.* HD100, similar results were observed in predation, however, the negative control increased due to the constant presence of the host on the plate allowing the supply of nutrients to the medium, different from this test where washings were performed to find that the negative control neither increased nor decreased (Iebba *et al.*, 2014). In the case of the other Gram-positive bacteria, apparently there are no previous studies with the HD100 strain, however, in 2016, a prey range was carried out where work was done with the SKB1291214 and SSB218315 strains, however there was no data. positive of predation as with the isolates used in this work (Oyedara *et al.*, 2016).

In this same 2016 study, we worked with *Enterobacter aerogenes,* having positive results for predation, as well as in most of our results, similarly, in 2010, a similar work was carried out with positive results for predation, using the Bdellovibrio strain. *bacteriovorus* 109J.

In the case of *Esquerichia coli,* it was variable predation, as our results, the above due to the strain, or predator used, since in 2000 and 2010, the 109J strain was positive to prey on *Esquerichia coli,* however, other strains did not have predation, demonstrating that there is a preference for the host (Dashiff *et al.,* 2011; Jurkevitch *et al.*, 2000; Mookherjee, and Jurkevitch, 2022).

In the studies of 2011 and 2016 they worked with *Klebsiella pneumoniae* and *Salmonella enterica,* using them as prey, which have been normally used to isolate predatory bacteria, the predation results with the reference strains SSB218315 and 109J support our results, in the case of *Pseudomonas fluorescens,* the prey ranges in these studies were also positive, which means that it is likely that predatory bacteria can be used for treatments against these bacteria. Not so for *Pseudomonas putida,* which has shown resistance to being preyed on, as evidenced by other studies where all predatory strains have had no effect on this prey (Dashiff *et al.,* 2011; Jurkevitch *et al.,* 2000; McNeely *et al.,* 2017). In the case of *Vibrio cholerae,* in our study we observed predation, the same has been corroborated by other studies, which is important due to the worldwide economic interest to control this pathogen, used Bdellovibrio, *Bacteriovorax,* 109J and SSB218315 with the ability to prey

on *Vibrio cholerae,* but SKB1291214 did not prove to do so, so, again, this is a bacterium that will depend on other factors, because it has presented resistance to being preyed on or there is a preference of the predatory bacterium for a prey (Cao et al., 2014 ; *Dashiff* et *al.,* 2011; Oyedara *et al.,* 2016; Ajao *et al.*, 2022).

CONCLUSIONS

It was possible to create a bank of around 86 isolates that were positive as possible BALOs through their phenotypic characterization in co-culture, observing cell lysates from bacterial prey.

It was possible to characterize molecularly through the amplification and sequencing of the PCR fragments from the 16S rRNA gene, with specific primers for predatory bacteria, obtaining 21 BALOs, of which 19 of these isolates are aligned with *Bdellovibrio reference strains. bacteriovorus,* or, *Bdellovibrio* sp., likewise, after sequencing, it was discovered that 3 isolates of predatory bacteria from the *Bacteriovoracaceae family were obtained,* since the primers used are not specific for the *Bdellovibrionaceae family.*

On the other hand, the prey range of 20 predators was obtained, obtaining important results, since we observed that different isolates of *Bdellovibrio* had variable activity, some very specific towards some prey bacteria, and others with a wide range of prey, in addition, some isolates together with *Bacteriovorax,* decreased the CFU of Gram-positive bacteria, which is of importance for public health (according to the WHO) and agriculture, with which it is concluded that the optical density reduction assay is reliable to determine the prey range of an isolate, and that *Bdellovibrio could* be used as a biological control in the near future.

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INTEREST CONFLICT

The authors declare that there is no conflict of interest

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