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SHORT COMMUNICATION

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Cultivable autochthonous bacteria of the intestinal mucosa of *Arapaima gigas* (Pisces: Arapaimidae) with probiotic potential

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The autochthonous microbiota is closely related to the mucosa of the gastrointestinal tract, while the allochthonous microbiota is considered as only the transient bacteria (He et al., 2016; Sedláček et al., 2016). Autochthonous bacteria play an important role in fish nutrition and other biological processes (Brenner & Farmer, 2015; La Patra et al., 2014; Merrifield & Rodiles, 2015; Ramirez & Romero, 2017a; Solovyev et al., 2019).

Bacterial communities of fish are complex, and the most frequent taxa in the gastrointestinal tract are species of the phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria and Tenericutes (Merrifield & Rodiles, 2015; Pereira et al., 2017; Ramirez & Romero, 2017b; Salas-Leiva et al., 2017; Solovyev et al., 2019). Proteobacteria is a dominant taxon (Ramirez & Romero, 2017b; Salas-Leiva et al., 2017; Solovyev et al., 2019), and Enterobacteriaceae species have shown good performance in the metabolism of cofactors, vitamins, amino acids, carbohydrates and proteins, thus improving fish nutrition and helping to prevent the colonization of pathogens. Enterobacteriaceae inhibit pathogens through competition for nutrients and adhesion sites in the mucosa, which aids the fish immune system (Brenner & Farmer, 2015; La Patra et al., 2014; Ramírez & Romero, 2017a, 2017b). Therefore, such benefits indicate that species of autochthonous Enterobacteriaceae in fish intestinal mucosa may have probiotic potential for supplementation in their diet.

Little is known about the diversity and structure of autochthonous bacterial communities in the intestine of wild fish when compared to those in culture conditions. No study has investigated the probiotic potential of autochthonous Enterobacteriaceae species in *Arapaima gigas* intestine for use in dietary supplementation. Thus, the present study analysed the diversity, characterized and identified species of autochthonous enterobacteria of the intestinal mucosa of wild and farmed *A. gigas*, aiming to isolate species of Enterobacteriaceae with probiotic potential for supplementation in the diet of this giant fish species from Amazon.

Twenty specimens of *A. gigas* that showed good health and no signs of disease were collected to obtain samples of intestinal tissue, of which 10 fish specimens (54.0 ± 18.4 cm and 1187.0 ± 750.1 g) were from two commercial fish farms located in the city of Macapá and 10 fish (71.1 ± 9.1 cm and 1854.3 ± 209.9 g) were obtained from rivers or lakes in the state of Amapá, Brazil (Figure 1). Farmed fish were fed with commercial feed for carnivorous fish, but with no additional additives such as probiotics, prebiotics and immunostimulants. All fish were cryo-euthanized immediately after capture, individually packed in sterile plastic bags and transported on ice in isothermal coolers to the Laboratory of Applied Microbiology at the Federal University of Amapá (UNIFAP), Macapá, state of Amapá.

The fish were initially washed in sterile sodium chloride solution (0.85%) and, subsequently, were sprayed with asepsis with ethyl alcohol (70%) and subjected to three washes with sterile distilled water. After laparotomy, a ventral incision was made from the urogenital pore to the operculum to remove the viscera and collect the intestine. The abdominal cavity was rinsing with sterile distilled

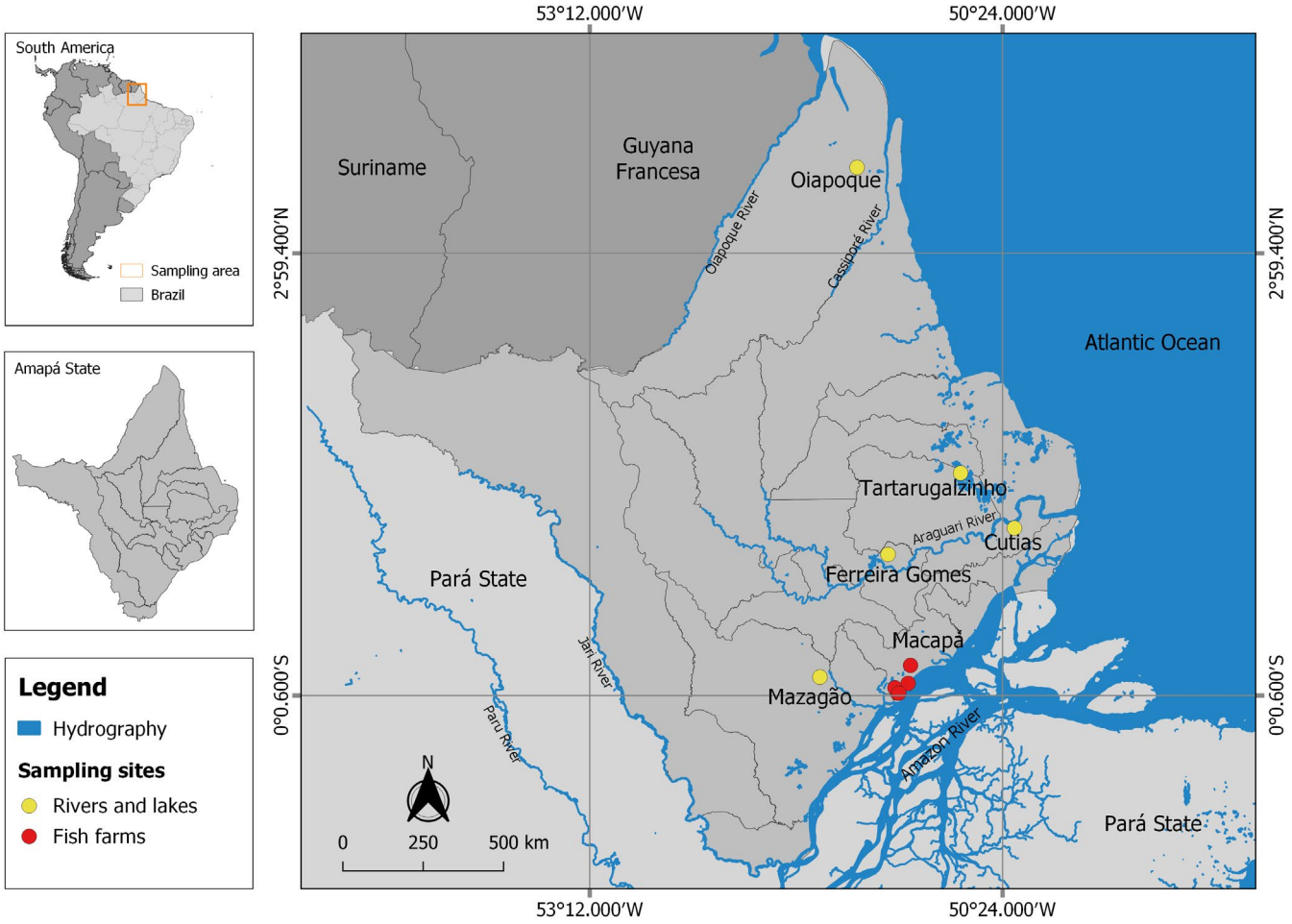

FIGURE 1 Sampling sites of *Arapaima gigas* in fish farms, and rivers and lakes from the state of Amapá, in eastern Amazon (Brazil) [Colour figure can be viewed at wileyonlinelibrary.com]

water. Then, an excision was made on the distal portion and ligation of the intestinal loops was performed with sterile sutures, washing with sterile sodium chloride solution (0.85%) and spraying with asepsis with 70% alcohol, followed by three washes with sterile distilled water. The following steps were carried out inside a Class II B2 biological safety cabinet (Trox®Technik, Brazil).

After removing the sutures from the intestinal ligations, all intestinal contents were removed, and the lumen was washed 3 times using a disposable syringe containing 20 ml of sterile sodium chloride solution (0.85%). A longitudinal incision was made in the intestine wall to collect a 10 cm^2 area sample to analyse the microbiota attached to the intestinal surface of the fish. Each intestine sample was then transferred to a sterile Falcon tube and was completed to 10 ml with tryptic soy broth/TSB (BD Bacto™, USA) and homogenized in an orbital shaker (Phoenix Luferco-AP59, Brazil) for 60 s, and serial dilutions were made in TSB to a concentration of 10^{-4}.

Each dilution was transplanted by surface streaking in triplicates with 100 µl of suspensions in concentrations of 10^{-3} and 10^{-4}, using 90 mm Petri dishes containing Chromocult® Coliform Agar/ CCA (Merck, Germany), incubated in a bacteriological incubator (REVCO ELITE II®-Kendro Laboratory Products™, USA) and maintained at 35°C for 24 hr. Plates showing growth between 30 and 300 colony-forming units (CFU) were quantified in a colony counter (Phoenix Luferco CP600-Plus, Brazil). The isolates were grouped by behaviour in a chromogenic medium, and by the morphological (Gram stain) and metabolic characteristics and were subsequently re-isolated to verify purity.

Identification of bacterial isolates and susceptibility tests to antimicrobials (Ceftazidime, Ceftriaxone, Cefepime, Ertapenem, Imipenem, Meropenem, Amikacin, Gentamicin, Ciprofloxacin, Tigecycline and Colistine) were performed in the VITEK 2 system (BioM 'Etoile, France), and the isolates were then preserved in a triptych soy broth medium with glycerol (TSB 15% glycerol) and stored in skim milk in a freezer at −70°C. Autochthonous bacterial isolates from the intestine of *A. gigas* were inoculated using streaking on surface and depth on blood agar medium (Sigma-Aldrich, Germany) with 5% sheep blood, and incubated at 35°C for 24 and 48 hr.

The preserved isolates, after reactivation in TSB at 37°C for 24 hr, were individually inoculated in tryptic soy agar/ TSA (BD Bacto™, USA) and incubated again at 37°C. After 24 hr, two colonies of each culture were transferred to a microplate (96 MSP, Bruker - Billerica, USA). The bacterial biomass was covered by a cell lysis solution (70% formic acid, Sigma-Aldrich). Then, an aliquot of 1 µL of matrix solution (alpha-cyano-4-hydroxy-cinnamic acid diluted in

50% acetonitrile and 2.5% trifluoroacetic acid, Sigma-Aldrich) was added to each bacterial biomass. The spectra of each sample were generated in Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF LT Microflex, Bruker) equipped with a 337 nm nitrogen laser in a linear path, controlled by the FlexControl 3.3 program (Bruker). The spectra were collected in a mass range between 2000–20,000 m/s, and then analysed using the MALDI Biotyper 3.1 (Bruker) program, using the standard configuration for bacteria identification, whereby the sample spectrum is compared with the references in the database. For each plate, a bacterial test standard was included to calibrate the instrument and validate the run. The identification scoring criteria were performed as recommended by Bruker Daltonics who assessed the following: a score of 2000 indicated identification at the species level; a score of 1700–1999 indicated gender-level identification; and a score of 1700 was interpreted as an absence of identification (Alatoom et al., 2011).

Tolerance test of the bacteria to low levels of pH was adapted from Vinderola and Reinheimer (2003) as described below. The stored bacterial isolates were reactivated in TSB medium, re-isolated on CCA agar after 24 hr of incubation at 35°C. Colonies were suspended and washed twice in buffer solution (0.05 M dibasic potassium phosphate and potassium chloride, pH 6.5) and centrifuged at 75 *g*/5°C/20 min. The pellets were resuspended in the same buffer and the concentrations were adjusted in a densitometer (DensichekTM, BioMerieux, France) according to the 0.5 standard of the McFarland scale. One mL of this suspension was added in a solution of pepsin (0.3% w/v) and sodium chloride (0.5% w/v) and adjusted to pH 3.5, 4.0 and 4.5, and the mixtures were in triplicates and were incubated for 3 hr. For each assay, 100 µl of suspension was plated by streaking, incubated for 24 hr at 35°C, and colonies were counted after incubation.

The bile of each *A. gigas* specimen (farmed and wild) was collected aseptically with the aid of disposable syringes and frozen at −20°C, after sterilization using a 0.22 µm Filtermax vacuum filtration system (Techno Plastic Products AG, Switzerland). Subsequently, the bile samples were thawed for 12 hr at 4°C and then at room temperature in a Biological Security Cabinet. From the bacterial isolates activated in TSB medium and re-isolated in CCA medium, bacterial suspensions were prepared in sterile PBS pH 7.2 buffer and centrifuged at 90 *g*/4°C/10 min. After two washes, the pellets were resuspended in sterile PBS pH 7.2 buffer and the concentrations were adjusted in a densitometer (DensichekTM, BioMerieux, France) according to the 0.5 standard of the McFarland scale. Then, serial dilutions of fish bile were prepared in sterile PBS buffer for final concentrations of 0.5% and 1.0% (v/v). Each tube (9 ml final volume) was inoculated with 1 ml of the bacterial suspension and incubated for 90 min at 35°C. The samples were then diluted in a series of sterile PBS buffer and 100 µl aliquots were seeded by streaking in CCA medium and incubated for 24 hr at 35°C (Mukherjee & Ghosh, 2014; Nikoskelainen et al., 2001), and colonies were counted after incubation.

The probiotic candidate bacteria were reactivated in TSB medium 24 hr in advance at 35°C. After propagation, the concentrations were adjusted in a densitometer (DensichekTM, BioMerieux, France) according to the standard 3 of the McFarland scale, for use of 100 µl to be inoculated in the wells. For this test, a standard strain of *Aeromonas hydrophila* (American Type Culture Collection/ ATCC-7966) provided by Fundação Oswaldo Cruz (CENT/LABENT, Rio Janeiro, RJ, Brazil) was used, in addition to *A. hydrophila* and *Aeromonas jandaei* previously isolated from *A. gigas* with bacteriosis (Proietti-Junior et al., 2017). The excavated well technique was used, in which cylinders of 8 mm in diameter were cut using sterilized cannulas and removed from Petri dishes with AST medium previously inoculated with suspension of each *Aeromonas*, in the 0.5 standard of the McFarland scale, not exceeding 30 min. The plates were incubated for 24 hr at 35°C and were subsequently analysed using an analog pachymeter (Etalon, Switzerland) to determine the inhibition rings around the wells containing suspensions of candidate bacteria for probiotics and their secondary metabolites (Balouiri et al., 2016).

The virulence genes *eaeA*, *lt*, *st*, *stx1*, *stx2*, *ial* and *eagg* were investigated using the Multiplex Polymerase Chain Reaction (PCR multiplex) technique. The purified bacterial DNA was extracted using a commercial PureLin® kit (Invitrogen Life Technologies, Canada). Each PCR reaction was prepared in a total volume of 20 and 2.5 µl of 10x PCR buffer (New England BioLabs, Ipswich, MA, USA), 5 pmol of each primer (MWG-Biotech AG, Germany), 'deoxynucleoside triphosphates' (dNTP) in a concentration of 200 µM (Promega), 1U Taq DNA polymerase (New England BioLabs) and 50 ng of genomic DNA. The activation temperature was 95°C in 15 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 68°C for 2 min and final extension step at 72°C for 5 min. The amplicons were subjected to electrophoresis in a 2% agarose gel (Sigma-Aldrich, USA) and stained with ethidium bromide (Omar & Barnard, 2014) and visualized on a transilluminator (ImageQuant™ 300, GE, USA). The detection of the *qnrA*, *qnrB*, *qnrS* and *rrs* genes was also performed using multiplex PCR. The reactions were performed using the previously extracted DNA in a Verit2 thermocycler (Applied Biossystems, USA), with a total volume of 20 and with 2.5 µl of 10× PCR buffer (New England BioLabs, USA), 5 pmol of each primer (MWG- Biotech AG, Germany), dNTP at a concentration of 200 µM (Promega), 1U Taq DNA polymerase (New England BioLabs) and 50 ng of genomic DNA.

The isolates were evaluated for genetic polymorphism using the Pulsed Field Electrophoresis (PFGE) technique according to the protocol of the Disease Control and Prevention Center (https://www.cdc.gov/ pulsenet/index.html). The fragments generated by the enzymatic restriction were visualized in UV light using a transilluminator (ImageQuantTM 300, GE, USA), and photo documentation was performed. PFGE standards were analysed using the BioNumerics® software, version 7.6 (Applied Maths, SintMartens-Lantem, Belgium).

The ecological terms prevalence, intensity and abundance were used as recommended by Bush et al. (1997). The Brillouin index (*HB*), evenness (*E*) associated with *HB*, Berger–Parker dominance index (d), species richness (Magurran, 2004) and frequency of dominance, which is the percentage of infracommunities for each species of bacteria is numerically dominant (Rohde et al., 1995), were calculated

to evaluate the component community of enterobacteria using the software Diversity (Pisces Conservation Ltda, UK). All data were tested for normality and homoscedasticity using the Shapiro–Wilk and Barlett test respectively. The *t* test was used to compare data on diversity parameters (*HB*, *E*, *d* and species richness) between farmed and wild fish. One-way ANOVA was used to compare the size of the inhibition rings of the isolates of the candidate bacteria to probiotics in the in vitro assays of antagonism against the three pathogens, followed by the Tukey test (Zar, 2010).

The growth in chromogenic medium allowed the isolation of 17 species (84 strains) and the results of the identification of the bacterial isolates were identical when comparing those obtained by the MALDI-TOF MS system to those of the VITEK 2 system. The prevalence, mean intensity, mean abundance and frequency of dominance are shown in Table 1. High prevalence and mean intensity were shown for *Enterobacter cloacae* and *Proteus mirabilis* in wild fish when compared to the farmed fish, whereas for *Escherichia coli*, there was a high prevalence in wild fish and greater intensity in captive fish. *Edwardsiella tarda*, *Citrobacter braakii* and *Plesiomonas*

TABLE 1 Prevalence (P), mean intensity (MI), mean abundance (MA) and frequency of dominance (FD) of autochthonous enterobacteria (CFU/10 cm^2) of the intestinal mucus of *Arapaima gigas* sampled in two different environments in eastern Amazon (Brazil)

	Phenospecies	P (%)	MI	MA	FD (%)
Environments	*Enterobacter cloacae*	20.0	17,375	3475	0.8
	Citrobacter freundii	30.0	127,778	38,333	8.6
	Citrobacter werkmanii	20.0	198,333	39,667	8.9
	Photorhabdus luminescens	20.0	34,167	6833	1.5
	Morganella morganii	30.0	174,444	52,333	11.7
	Kluyvera intermedia	30.0	160,926	48,278	10.8
	Proteus vulgaris	50.0	110,533	55,267	12.3
	Proteus mirabilis	10.0	73,333	7333	1.6
Farmed	*Cedecea neteri*	10.0	86,667	8667	1.9
	Cedecea davisae	30.0	61,667	18,500	4.1
	Edwardsiella tarda	0	0	0	-
	Escherichia coli	30.0	197,556	59,267	13.2
	Aeromonas veronii	30.0	158,889	47,667	10.6
	Aeromonas sobria	20.0	96,667	19,333	4.3
	Citrobacter braakii	0.0	0	0	-
	Klebsiella pneumoniae	40.0	107,292	42,917	9.6
	Plesiomonas shigelloides	0	0	0	-
	Enterobacter cloacae	90.0	161,926	145,733	20.8
	Citrobacter freundii	40.0	131,667	52,667	7.5
	Citrobacter werkmanii	10.0	216,667	21,667	3.1
	Photorhabdus luminescens	0	0	0	-
	Morganella morganii	20.0	95,000	19,000	2.7
	Kluyvera intermedia	0	0	0	-
	Proteus mirabilis	80.0	145,000	116,000	16.6
	Proteus vulgaris	0	0	0	-
	Cedecea neteri	0.0	0	0	-
Wild	*Cedecea davisae*	0.0	0	0	-
	Edwardsiella tarda	60.0	242,889	145,733	20.8
	Escherichia coli	60.0	122,222	73,333	10.5
	Aeromonas veronii	0	0	0	-
	Aeromonas sobria	0	0	0	-
	Citrobacter braakii	20.0	153,333	30,667	4.4
	Klebsiella pneumoniae	50.0	112,667	56,333	8.1
	Plesiomonas shigelloides	40.0	95,000	38,000	5.4

shigelloides occurred only in the intestinal mucosa of wild fish, while *Photorhabdus luminescens*, *Kluyvera intermedia*, *Cedecea neteri*, *Cedecea davisae*, *Aeromonas veronii*, *Aeromonas sobria* and *Proteus vulgaris* occurred only in farmed fish. There was a frequency of dominance for several species in farmed fish that were absent in wild fish, which showed dominance of *E. cloacae*, *P. mirabilis* and *E. tarda*. In addition, 11 isolates of *E. cloacae* showed probiotic potential.

In wild *A. gigas*, the Brillouin diversity, evenness and richness of the bacteria species were greater ($p < 0.05$) than in farmed *A. gigas*, but the Berger–Parker dominance was greater in farmed fish (Figure 2). On the other hand, farmed *A. gigas* which showed a greater value for Berger–Parker dominance index due the predominance of several species. In *Danio rerio*, no difference was reported in the structure of the composition and diversity of allochthonous bacteria between wild and captive fish (Roeselers et al., 2011). Studies of the bacterial microbiota of the mucosa and intestinal content of fingerlings and adults of *A. gigas* showed that most of the identified isolates were members of Proteobacteria, Fusobacteria and Firmicutes, and that this microbiota had more richness in fingerlings (Pereira et al., 2017). Although the structure of the intestinal bacterial communities of fish can be influenced by biotic (e.g. developmental stage, intestinal structure, diet, species, age and trophic level of the host) and abiotic (e.g. habitat and characteristics of the surrounding environment) factors, including the cultivation conditions (Ramirez & Romero, 2017a; Roeselers et al., 2011; Salas-Leiva et al., 2017), the differences observed in the present study may be due to the differences in the environment and feeding of the fish.

Isolates of *A. veronii*, *E. tarda*, *A. sobria* and *Plesiomonas shigelloides* of *A. gigas* (Table 2) were excluded as bacteria with probiotic potential due to their pathogenicity for fish (Castañeda-Monsalve et al., 2019; Shama et al., 2000) and humans. In the present study, 47% of the species of autochthonous enterobacteria showed the ability to colonize the intestinal mucosa surface of *A. gigas*. Autochthonous *Klebsiella pneumoniae* of *A. gigas* showed resistance to the antimicrobial drugs tested here and has also shown the presence of resistance genes in isolates obtained from some marine fish species (Singh et al., 2017).

The isolates of *Citrobacter freundii*, *Photorhabdus luminescens*, *Morganella morganii*, *Kluyvera intermedia* and *P. mirabilis* of *A. gigas* were excluded as bacteria with probiotic potential due to the haemolytic activity presented, while the other species were negative (Table 2). *Citrobacter freundii* also showed haemolytic activity and pathogenicity to *A. gigas* (Pereira et al., 2017) and *Pseudoplatystoma reticulatum*, and resistance to antimicrobial drugs (Pádua et al., 2014). Isolates of *P. luminescens*, *M. morganii* and *K. intermedia* from the intestinal mucosa of *A. gigas* in the present study also showed haemolytic activity. Thus, these isolates were discarded as a probiotic potential for *A. gigas*, due to its potential pathogenic in case of a disequilibrium in pathogen–host relationship. Isolates of *E. coli* in *A. gigas* were more prevalent in the intestinal mucosa of wild fish than in farmed fish, which had a greater abundance. In addition, the isolates showed multidrug resistance to antimicrobials. Bollache et al. (2018) reported that *E. coli* are producers of broad-spectrum beta-lactamases in several fish species. Therefore, *E. coli isolates*

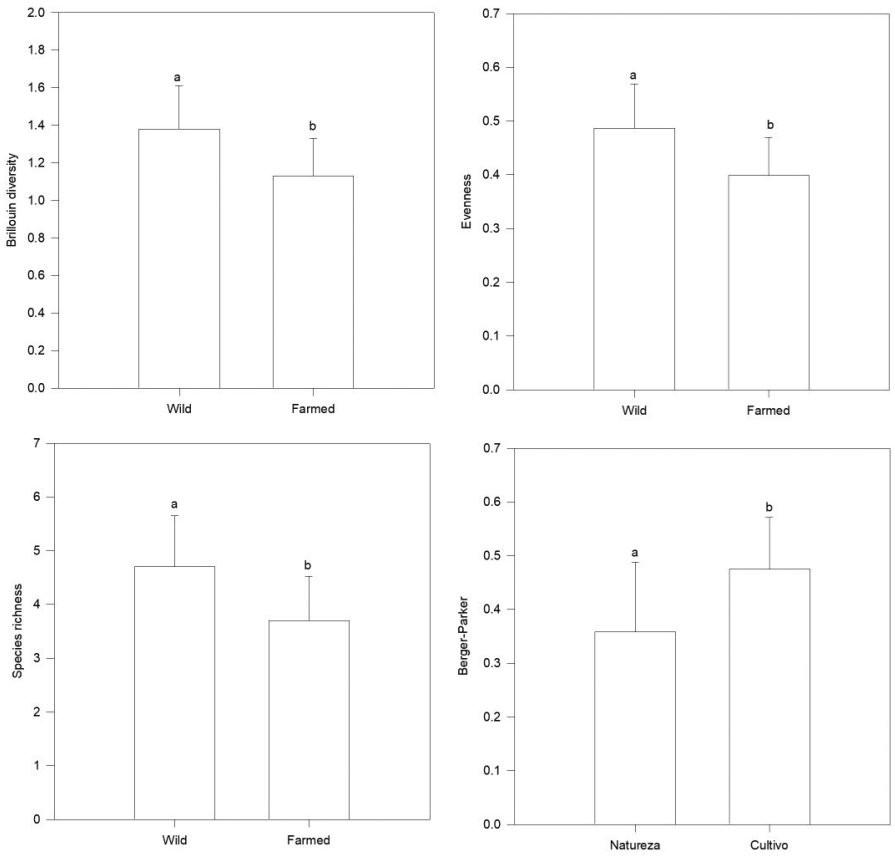

FIGURE 2 Diversity parameters of autochthonous enterobacteria in the intestinal mucosa of *Arapaima gigas* from fish farm, and rivers and lakes of eastern Amazon (Brazil). Similar letters in the same column indicate no significant differences ($p > 0.05$) according to the *t* test

TABLE 2 Results of the analyses used in the selection of enterobacteria species with probiotic potential for *Arapaima gigas*

Species	P	H	R	pH	B
Edwardsiella tarda	+	NA	NA	NA	NA
Aeromonas veronii	+	NA	NA	NA	NA
Aeromonas sobria	+	NA	NA	NA	NA
Plesiomonas shigelloides	+	NA	NA	NA	NA
Citrobacter freundii	ND	+	NA	NA	NA
Photorhabdus luminescens	ND	+	NA	NA	NA
Morganella morganii	ND	+	NA	NA	NA
Kluyvera intermedia	ND	+	NA	NA	NA
Cedecea davisae	ND	ND	+	NA	NA
Citrobacter werkmanii	ND	ND	+	NA	NA
Cedecea neteri	ND	ND	+	NA	NA
Escherichia coli	ND	ND	+	NA	NA
Klebsiella pneumoniae	ND	ND	+	NA	NA
Proteus mirabilis	ND	ND	+	NA	NA
Citrobacter braakii	ND	ND	ND	NT	NA
Proteus vulgaris	ND	ND	ND	ND	NT
Enterobacter cloacae	ND	ND	ND	ND	ND

Abbreviations: +, Positive; B, Absence of growth in any concentration of bile fluid; H, Haemolysis activity; NA, not analysed; ND, no detection; NT, no tolerance; P, Pathogenicity; pH, Absence of growth in the analysed ranges of pH; R, Resistance to at least three classes of antimicrobial drugs.

TABLE 3 Reduction of the counting of viable cells (UFC/mL) of *Enterobacter cloacae* exposed to simulated gastric fluid, after 3 hr in three levels of pH acid

Fish/isolates	pH 3.5	pH 4.0	pH 4.5
1	3.3 ± 0.5	2.4 ± 0.8	2.3 ± 0.5
4	4.2 ± 0.2	3.3 ± 0.5	3.8 ± 0.5
11	2.4 ± 0.4	1.9 ± 0.3	1.6 ± 0.6
12	2.8 ± 0.8	2.1 ± 1.2	1.8 ± 0.5
13	2.9 ± 0.6	2.4 ± 0.8	1.9 ± 0.3
14	2.6 ± 0.6	2.2 ± 1.0	1.8 ± 1.2
16	3.7 ± 0.8	2.8 ± 0.5	1.7 ± 0.8
17	2.9 ± 1.0	2.6 ± 1.3	1.9 ± 1.5
18	2.7 ± 0.4	2.3 ± 0.5	1.8 ± 0.8
19	2.5 ± 0.3	2.2 ± 0.9	1.9 ± 0.6
20	2.8 ± 1.2	2.3 ± 0.8	1.8 ± 1.2

were excluded as a probiotic potential for *A. gigas*, although this species of Gammaproteobacteria has been used as a probiotic for other animal species by one century (Wassenaar, 2016).

The isolates of *Cedecea davisae*, *Citrobacter werkmanii*, *Cedecea neteri*, *Escherichia coli*, *Klebsiella pneumoniae* and *P. mirabilis* of *A. gigas* were excluded as bacteria with probiotic potential because they are resistant to three or more classes of antimicrobial. However, further studies on these isolates are needed carried out. In contrast, the results of antimicrobial susceptibility tests for *E. cloacae* isolates are compatible with characteristics of commensal bacterial microbiota since such autochthonous bacteria were sensitive to all tested antimicrobial drugs (Table 2). *Proteus vulgaris* isolates of *A. gigas* showed a reduction in viable cell count after 3 hr of exposure to simulated gastric fluid in different pH ranges. However, all isolates of autochthonous *E. cloacae* showed resistance to simulated gastric fluid at the three pH levels (Table 3). The autochthonous *E. cloacae* isolates of *A. gigas* with probiotic potential showed tolerance to *A. gigas* bile fluid (Table 4). A reduced change in the growth of these autochthonous isolates was observed after 24 hr of exposure to bile fluid (data not shown). Isolates 11 and 16 of *A. gigas* showed greater inhibition ring against *Aeromonas hydrophila* and *A. jandaei* from *A. gigas*. In addition, the inhibition rings of all isolates against *A. hydrophila* and *Aeromonas jandaei* were greater when compared to *A. hydrophila* ATCC-7966 (Table 5). None of the *E. cloacae* isolates of *A. gigas* showed the virulence genes *eaeA*, *lt*, *st*, *stx1*, *stx*, *eaeA*, *ial* and *eagg*. In addition, none of the *E. cloacae* isolates showed the resistance genes *qnrA*, *qnrB*, *qnrS* and *rrs*. The isolates used in the genotyping allowed the detection of two distinct species of *E. cloacae*, with a gene similarity of approximately 85% (Figure 3).

Studies have shown the contribution of autochthonous intestinal bacterial microbiota to fish health (Pereira et al., 2017; Sedláček et al., 2016), of which efficacy is due to the specificity in the strain–host interaction (Salas-Leiva et al., 2017). Studies on autochthonous bacteria in the intestine of *A. gigas* with probiotic potential have focused only on lactic acid species (Fujimoto et al., 2014; Pereira et al., 2017). However, the use of the probiotic *Enterobacter* spp. from the gastrointestinal tract of *Oncorhynchus mykiss* demonstrated the ability to inhibit the in vitro growth of the pathogen *Flavobacterium psychrophilum* and reduced fish mortality after being challenged, due to the protection of the fish immune system (La Patra et al., 2014). In vitro and in vivo studies with the

TABLE 4 Growth (%) of autochthonous *Enterobacter cloacae* after 90 min of exposure to concentrations of bile fluid of *Arapaima gigas*

	Concentrations of bile fluid	
Fish/isolates	0.5%	1.0%
1	63.8 ± 2.7	59.9 ± 1.8
4	67.6 ± 3.2	41.0 ± 2.7
11	83.4 ± 4.0	77.5 ± 2.9
12	73.8 ± 2.7	69.9 ± 1.5
13	69.5 ± 1.9	63.8 ± 2.3
14	71.6 ± 4.1	65.5 ± 3.4
16	74.5 ± 1.3	68.6 ± 1.9
17	62.8 ± 3.7	57.9 ± 3.2
18	66.4 ± 2.5	62.8 ± 2.1
19	68.8 ± 3.3	65.9 ± 2.2
20	71.7 ± 3.5	68.4 ± 2.6

Note: Values expressed as mean ± standard deviation.

TABLE 5 Measures of the ring of inhibition (mm) of the isolates of *Enterobacter clocae* by in vitro inhibition of *Aeromonas* species of *Arapima gigas*

Bacteria	*Aeromonas hydrophila* (ATCC–7966)	*Aeromonas hydrophila*	*Aeromonas jandaei*	
Fish/isolates	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	*p*-value
1	11.3 ± 0.1[a]	12.5 ± 0.1[b]	12.5 ± 0.3[b]	<0.001
4	11.7 ± 0.3[a]	12.4 ± 0.2[b]	12.3 ± 0.1[b]	0.016
11	14.5 ± 0.2[a]	15.5 ± 0.2[a]	15.9 ± 0.2[b]	<0.001
12	13.2 ± 0.3[a]	14.1 ± 0.2[b]	14.7 ± 0.1[c]	<0.001
13	13.5 ± 0.1[a]	14.4 ± 0.3[b]	14.8 ± 0.3[b]	<0.001
14	13.3 ± 0.4[a]	14.3 ± 0.4[b]	15.2 ± 0.1[c]	<0.001
16	14.1 ± 0.1[a]	15.3 ± 0.3[b]	15.7 ± 0.1[b]	<0.001
17	13.6 ± 0.1[a]	14.3 ± 0.3[b]	15.5 ± 0.2[c]	<0.001
18	13.3 ± 0.1[a]	14.3 ± 0.2[b]	15.4 ± 0.2[c]	<0.001
19	13.6 ± 0.1[a]	14.5 ± 0.1[b]	15.9 ± 0.1[c]	<0.001
20	11.6 ± 0.1[a]	12.9 ± 0.3[b]	13.6 ± 0.2[c]	<0.001

Note: Values expressed as mean ± standard deviation. Similar letters in the same line indicate no significant differences according to the Tukey test.

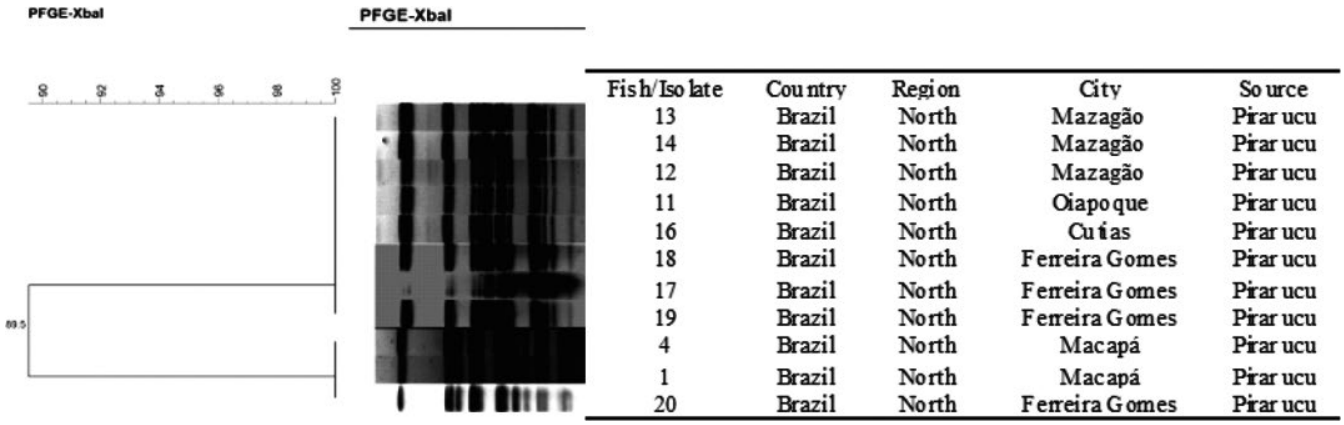

FIGURE 3 Distribution of the phylogenetic groups of autochthonous *Enterobacter cloacae* of the intestinal mucosa of *Arapaima gigas* in Brazil

probiotic *E. cloacae* showed that this bacterium had a probiotic effect, inhibiting the in vitro growth of the pathogen *P. shigelloides*, increasing the survival of challenged *Maylandia lombardoi*, and improving the intestinal structure after being added to the diet (Girijakumari et al., 2018). This study investigated the autochthonous Gammaproteobacteria communities of the intestinal mucosa of *A. gigas*, aiming to obtain isolates of Enterobacteriaceae species with probiotic potential. We selected 11 isolates of *E. cloacae* in *A. gigas* with potential for supplementation in the diet of this important Amazonian aquaculture species.

Bacteria with probiotic potential for use in aquaculture are suggested to have the following characteristics: (a) not be pathogenic for the host or consumer species; (b) be free of resistance to antibiotics encoded by extrinsic genes; (c) be resistant to bile fluid and low pH; (d) be able to colonize the epithelial surface of the host gastrointestinal tract; (e) present physiological properties compatible with its propagation in the host; (f) exhibit antagonistic activities against host pathogens; (g) be autochthonous to the host and have low impact on the cultivation environment; and (h) remain viable under storage conditions and after industrial processing (Merrifield et al., 2010; Yamashita et al., 2020). However, recent studies have used only some of these criteria for the selection of bacteria with probiotic potential for different fish species (Duarte et al., 2014; Fujimoto et al., 2014; Girijakumari et al., 2018; La Patra et al., 2014; Pereira et al., 2017; Wanka et al., 2018). The present study obtained 11 isolates of autochthonous *E. cloacae* from the intestinal mucosa of *A. gigas*, which showed potential as probiotics for supplementation in the diet of this fish, since they showed no resistance to antimicrobial drugs, were resistant to bile fluid and low pH, showed no in vitro haemolytic activity and no virulence genes (*eaeA*, *lt*, *st*, *stx1*, *stx*, *eaeA*, *ial* and *eagg*) and resistance genes (*qnrA*, *qnrB*, *qnrS* and *rrs*) were observed. In addition, the isolates showed antagonistic activity against two pathogens (*A. hydrophila* and *A. jandaei*) from *A. gigas* (Proietti-Junior et al., 2017), indicating that such bacteria are commensal to this fish species. Therefore, in vivo studies are needed to determine whether the *E. cloacae* isolates have probiotic activity

in *A. gigas*, since aquaculture of this fish requires supplementation of the diet to increase its production and reduce mortality of fry and fingerlings.

In conclusion, this is the first study regarding the diversity of cultivable autochthonous enterobacteria of the intestinal mucosa of *A. gigas*, and 84 isolates of the bacterial taxon were characterized, of which 17 species were identified. Only *E. cloacae* isolates demonstrated probiotic potential. However, further research is needed to validate the probiotic effects of these isolates, especially isolates 11 and 16, on the growth performance and immunity of *A. gigas*, and to determine the best concentration for supplementation in the diet. Studies with the same isolates are also needed for the development of biotechnological products for use in the farming of this fish.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

ETHICAL APPROVAL

The present study was carried in accordance with the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethics Committee on the Use of Animals (CEUA/UFAC: No 23107.009564/2014-29) of the Federal University of Acre and National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN N° A7EC29D) and of the Biodiversity Authorization and Information System (SISBIO No 62153-2), according to the regulations of research of the Ministry of the Environment (MMA).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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