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Original article

Effect on survival and growth (weight and length) of *Ambystoma mexicanum* (Shaw Y Nodder, 1798) of three probiotics obtained from their gastrointestinal tract

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ABSTRACT

The effect of probiotic strains (*Lactococcus lactis*; *Lactobacillus sp* and *Bacillus subtilis*), isolated from the intestinal tract of *Ambystoma mexicanum* on survival and growth of this amphibian was evaluated. 120 larvae with a weight of 3.0 ± 0.26 g and length of 1.5 ± 0.62 cm, were fed with probiotic strains (10^7 CFU mL-1) bioencapsulated in *Artemia franciscana* metanauplii. Diets were handled in triplicates (10 organisms per beaker) during 60 days. Every 15 days were measured and weighed to obtain the intrinsic growth rate (IGR) and absolute growth rate (AGR). Analysis of variance and Condition Factor was performed. Organisms that obtained a higher weight and length were fed with *B. subtilis* (5.86 ±0.42 g; 4.56 ±0.27 cm). Diets with *L. lactis* and *B. subtilis* presented the best Condition Factor (KM). Variance analysis showed significate differences (P<0.001) between all treatments. Axolotls fed with probiotics presented survival <85%, while control was 60%.

1. Introduction

Mexico is included among the richest biological countries in the world, within this diversity; amphibians are an important group being in fifth place worldwide with 55% endemism. Nevertheless; amphibian populations have dramatically decrease in the last 20 years, being Ambystomidae family one of the most affected, in particularly the specie *Ambystoma mexicanum* (Stuart *et al*., 2004). This specie has been under various environmental pressures, such as habitat destruction (reduction of Xochimilco Lake), overexploitation for food purposes, their use in traditional medicine and the insertion of other exotic species that is why, this organism is included in list of amphibian species under special protection, according to Official Mexican Standard CONABIO and PROFEPA (NOM-ECOL-059-94). In recent years, there have been efforts to recover Ambystoma species through culture in laboratory, but poor water quality, inadequate diet administration and handling have cause bacteria, virus and fungus diseases, such *Aeromonas hydrophila* (red-legged disease) and Batrachochytrium dendrobatidis (causing Chitridiomycosis) (Culp *et al*., 2007). Because of this, it is necessary to use nutritional strategies that improve nutrient assimilation, survival and disease resistance.

An alternative that has shown positive results in aquaculture industry was the use of probiotic-enriched diets. These bacteria improve the host nutrition by supplying essential nutrients, increasing the digestion, eliminate part of dissolved organic matter and produce substances that inhibit growth of opportunistic pathogens (Rengpipat *et al*., 2000; Rodríguez and Le Moullac *et al*., 2000); also constitute an alternative for replacement of chemotherapeutic agents that are misused in aquaculture. Therefore, the goal of this investigation is evaluate the growth and survival of A. mexicanum fed with probiotic strains (*Lactococcus lactis*, *Lactobacillus sp* and *Bacillus subtilis*), as a strategy for specie conservation.

2. Materials and methods

2.1. Ambystoma mexicanum collecting

Axolotls used in this investigation were donated by Centro de Investigaciones Biológicas y Acuícolas de Cuemanco (CIBAC) that belongs to Autónoma Metropolitana - Unidad Xochimilco University.

2.2. Isolated bacteria load from posterior digestive tract section of ambystoma mexicanum

Thirty juvenile stage organisms of *Ambystoma mexicanum* specie were placed in a culture beaker previously equipped in laboratory conditions to acclimatization period (15 days). After that, five randomly selected organisms were fasted during 24 hours and then, during five minutes anesthetized with clove essential oil (0.1 g L^{-1}) to make a dissection to remove the upper digestive tract. This portion of intestine was washed three times with distilled water to remove feces and food residues. Then, with aid of a sterile swab a sample of intestinal mucous was taken and inoculated in 90 mL of sterile water making dilutions 1:10. On agar plates MSR (Man- Rogosa- Sharpe), BHI (Brain- Heart infusion) and TCBS (Thiosulfate-Citrate-Bile-Salts) 0.1 mL of each dilution was inoculated three times each. Strains were purified by successive plantings. Purified strains were Gram stained to observe the cellular morphology using an optical microscope Olympus™ Cx31.

2.3. Molecular identification

2.3.1. DNA isolation

The genomic DNA from isolated strains were extracted using “Wizard Genomic DNA Purification kit” (PROMEGA™) following manufacturer's instructions. Samples were subjected to electrophoresis on agarose gel at 1% to determine the integrity and purity of DNA.

2.3.2. Protein chain reaction (PCR)

Bacterial rDNA 16S fragments were subsequently amplified by PCR using primers 8 forward 5’ AGACTTTGATCMTGGCTCAG 3’, and 1492 reverse 5’ TACGGYTACCTTGTTACGACTT 3’. A thermocycler was used for amplification reaction under the next conditions: preincubation at 95°C during 10 minutes, 30 cycles of denaturation 124 at 95°C during 30 seconds, hybridization at 55°C during 30 seconds and elongation at 72°C during 1 minute, and refrigeration at 4°C. PCR products were purified with purification QIAquick PCR (Qiagen™) kit,

following manufacturer's instructions. The PCR purified products were sequenced to MACROGEN™ Korea. Finally, the genetic sequence of each strain was analyzed with BLAST tool in GenBank database.

2.4. Tests to characterize a microorganism as a probiotic

2.4.1. Resistance to acid pH

To proof the resistance of isolated bacteria to acid pH, a gastric barrier was simulated by placing the isolated microorganisms in acid growth media with a pH of 1,5, 2,5 and 3,0. Strains that did not survive these stressful conditions were discarded.

2.4.2. Resistance to bile salts

For growth evaluation of microorganisms in bile salts, Erlenmeyer flasks were used with 100 mL of MRS broth plus 0.1%, 0.5% or 1.0% of fresh bile by triplicate. Flasks were inoculated with 1mL^{-1} of microorganism strains that survive to acid conditions, and were incubated at 37°C during three hours.

2.4.3. Inhibition test of aeromonas hydrophila (in vitro)

In vitro inhibition tests were realized with strains that obtained positive results in previous tests, for this, strain from the collection *Aeromonas hydrophila* ATCC356554A was cultured three times in BHI agar *plates*, which were incubated 24 h at 30°C. Subsequently, 70 µL of isolated strains suspension with absorbance at 620nm corresponding to a bacterial population of 10^7 CFU mL^{-1} and solutions up to 10^5 CFU mL^{-1} were added with the well diffusion method. Agar *plates* were incubated during 24 hours at 30°C, after this, formation of inhibition halos was observed. Strains that presented halos above 2 mm diameter were considered as positive.

2.5. Feeding tests

Isolated strains from *Ambystoma* intestinal, which manage high survival to acid pH and bile salts and were able to inhibit *A. hydrophila in vitro*, three of them were selected (*Lactococcus lactis*, *Bacillus subtilis* and *Lactobacillus sp.*) to perform feeding trials in following manner:

One hundred *Ambystoma mexicanum* larvae stage were acquired from Centro de Investigación Acuícola y Biológica de Cuemanco (CIBAC) with mean length of 1.50 ± 0.61cm and weight of 0.30 ± 0.25 g (Fig. 1). The organisms were distributed in 20 L plastic beakers (10 organisms per beaker) as follows: a) control diet (A. franciscana metanauplii without probiotic); b) Diet A. franciscana metanauplii with *Lactococcus lactis* (2x10^7 CFU mL^{-1}); c) *A. franciscana* metanauplii enriched with *Bacillus subtilis* (2 x10^7 CFU mL^{-1}); and d) *A. franciscana* metanauplii enriched with Lactobacillus sp (2 x10^7 CFU mL^{-1}). Each treatment *by triplicate*, having an experimental duration of 60 days. Food and feces residues were removed daily from the tanks to maintain water quality. Physicochemical parameters (temperature, pH, dissolved oxygen, nitrite and nitrate) were monitored using HACH™ Model DR/850 colorimeter 2000 equipment.

2.6.Biometry

Each 15 days the organisms were measured and weigthed with a Vernier caliper and a OHAUS™ scale ADVEnturer Pro. These data were used to determinated the Absolute Growth Rate (AGR), Instantaneous Growth Rate (IGR) and Condition Factor (KM) were calculated by using the next formulas:

$$\text{Absolute Growth Rate (AGR)} = \frac{\text{Finnal weigth or length} - \text{Innitial weigth or length}}{\text{Total experimental days}}$$

$$\text{Instantaneous Growth Rate (IGR)} = \frac{\text{LN Finnal weigth or length} - \text{LN Innitial weigth or length}}{\text{Total experimental days}} \text{x } 100$$

$$\text{Condition factor (KM)} = \frac{\text{Weight}}{\text{Length}^3} \text{x } 10^5$$

2.7. Data analysis

A database was generated in Excel 2010 (Microsoft Co., California, U.S.A) with obtained data, for the obtainment of mean values (±SD and SE). The existence of significate differences (P<0.05) was determined by a analysis of variance (ANOVA). An analysis of multiple means by Tukey technique using the program SYSTAT 13.0 was made when significant differences (P<0.05) were found.

3. Results

3.1. Molecular identification of *a. Mexicanum* intestinal microbiota

A. mexicanum digestive tract was colonized by 56 bacteria strains. Established by molecular identification, dominance corresponds to *Bacillus subtilis*, *Bacillus* sp, *Lactococcus lactis*, *Lactobacillus* sp., *Lactobacillus crispatus*, Aeromonas sobria and *Pseudomonas pscicida*. Nevertheless, just only 25 strains were resistant to acid pH, bile salts and better epithelial tissue colonization (Table 1).

Table 1
Bacteria found in the gastrointestinal tract of Ambystoma mexicanum and their probiotic capacity.

Strain	Bile salts			Acid pH			Pathogen inhibition	Molecular identification (ADN16Rs)
	0.1%	0.5%	1.0%	1.5	2.5	3.0		
Am1	+	+	+	+	+	+	+	*Lactobacillus crispatus*
Am1b	+	+	+	+	+	+	-	*Aeromonas sobria*
Am1c	+	+	+	+	+	+	+	*Bacillus subtilis*
Am2	+	+	+	+	+	+	+	*Bacillus sp*
Am2b	+	+	+	+	+	+	+	*Lactobacillus sp*
Am3	+	+	+	+	+	+	+	*Lactobacillus crispatus*
Am4	+	+	+	+	+	+	+	*Bacillus sp*
Am4b	+	+	+	+	+	+	+	*Bacillus subtilis*
Am4c	+	+	+	+	+	+	-	*Aeromonas sobria*
Am5	+	+	+	+	+	+	+	*Bacillus sp*
Am5b	+	+	+	+	+	+	-	*Lactobacillus sp*
Am5c	+	+	+	+	+	+	+	*Lactobacillus crispatus*
At1	+	+	+	+	+	+	-	*Aeromonas sobria*
Atc	+	+	+	+	+	+	-	*Pseudomonas aeuroginosa*
Atc2	+	+	+	+	+	+	+	*Bacillus sp*
At2	+	+	+	+	+	+	-	*Pseudomonas aeuroginosa*
At2b	+	+	+	+	+	+	+	*Bacillus subtilis*
At2c	+	+	+	+	+	+	+	*Bacillus sp*
At2c2	+	+	+	+	+	+	+	*Lactococcus lactis*
At3	+	+	+	+	+	+	-	*Aerominsa sobria*
At3b	+	+	+	+	+	+	-	*Pseudomonas aeuroginosa*
At3c	+	+	+	+	+	+	+	*Lactobacillus crispatus*
At4	+	+	+	+	+	+	-	*Aeromonas sobria*
At4b	+	+	+	+	+	+	+	*Lactobacillus crispatus*

Of all isolated strains from Axolotl gastrointestinal tract (GIT), five were those who managed to inhibit *Aeromona hydrophila in vitro*, but *Lactococcus lactis*, *Lactobacillus* sp. and *Bacillus subtilis* obtained higher halos diameters between 50 and 54 mm (Fig.1).

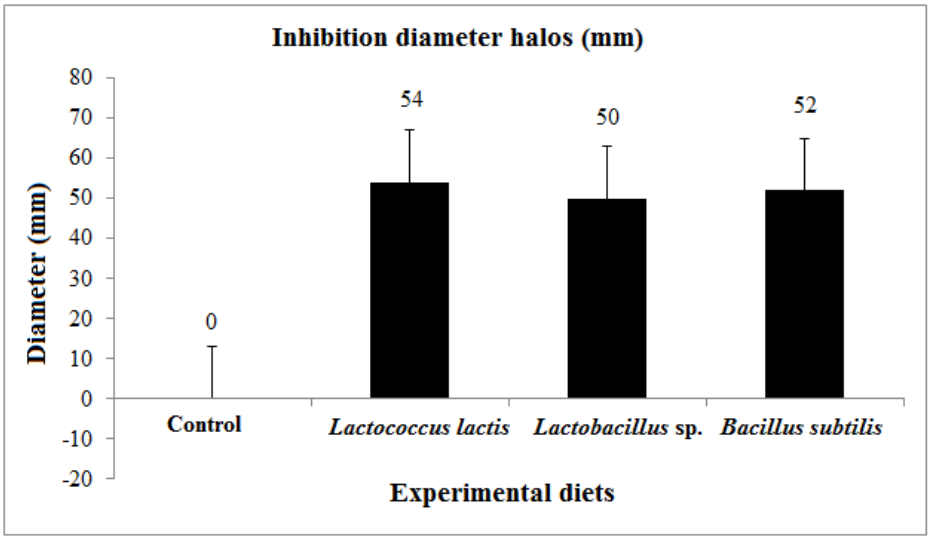

Fig. 1. Mean values of inhibition halos produced by probiotic strains against *Aeromonas hydrophila*.

3.2. Survival

Survival values of organisms fed with experimental diets are shown at Fig. 2. Organisms fed with enriched *Lactobacillus sp*. diet presented 100% survival, even though there are no significate differences (P<0.05) with *Bacillus subtilis* that presented 95% survival at the end of the experiment. Control diet presented the lowest value with 60%. ANOVA pointed out significant differences (P<0.001) between the other three diets (Control, Lactococcus lactis and Lactobacillus sp.).

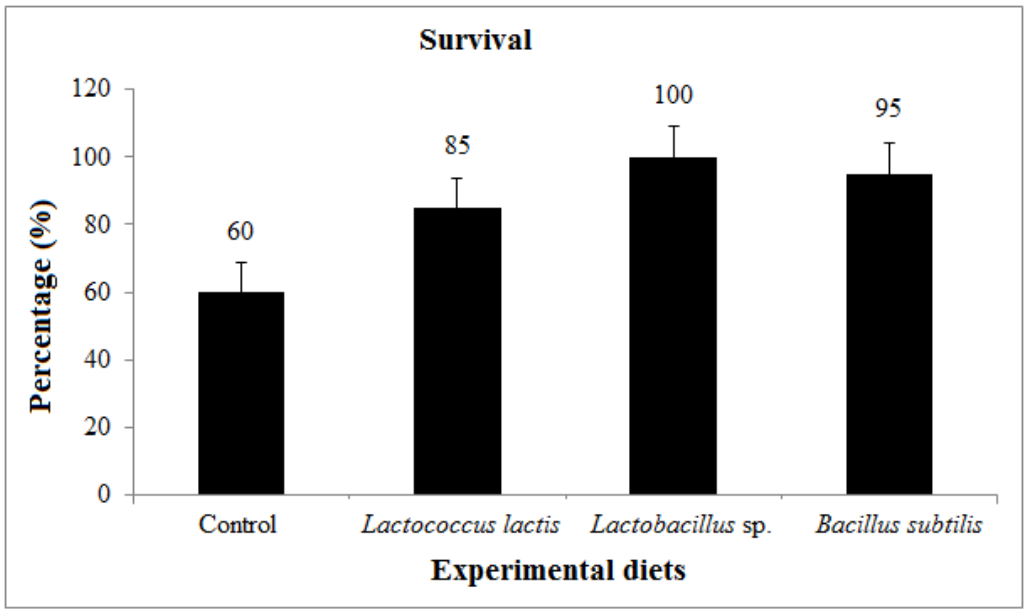

Fig. 2. Mean values of Ambystoma mexicanum survival, fed with experimental diets.

3.3. Growth evaluation

3.3.1. Gain of weight and length

Mean values (± SD.) of initial and final weight and length are shown at Table 2, and the gain of the same variables is shown in Fig. 3a,b. At Table 2 it is observed that control diet obtained the lowest values (weight 2.58 ±0.32 g; length 2.01 ±0.25 cm), with a weight gain of 2.28 g and length gain of 0.51 cm). Bacillus subtilis enriched

diet obtained the highest values (weight 5.86 ±0.42 g; length 4.56 ±0.27 cm) and a weight gain of 5.56 g and length gain of 3.06 cm.

Table 2
Mean values (± DS) of *Ambystoma mexicanum* initial weight and length fed with experimental diets.

Diet	Weight (g)		Length (cm)	
	Initial	Final	Initial	Final
Control	0.30	2.58	1.50	2.01
	±0.25	±0.32	±0.64	±0.25
Lactococcus lactis	0.30	4.26	1.50	4.03
	±0.28	±0.42	±0.61	±0.34
Lactobacillus sp.	0.30	5.05	1.50	3.22
	±0.24	±0.25	±0.62	±0.24
Bacillus subtilis	0.30	5.86	1.50	4.56
	±0.25	±0.42	±0.62	±0.27

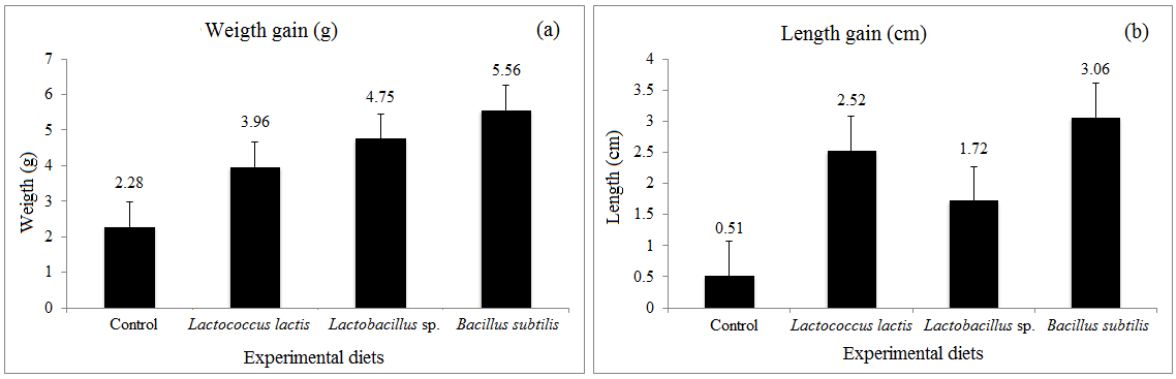

Fig. 3. Gain weight (a) and length (b) values of *Ambystoma mexicanum* fed with experimental diets.

ANOVA pointed out significant differences (P<0.001) in weight, length and gain values between all diets used in this investigation.

3.3.2. Absolute growth rate and instantaneous growth rate

At Fig. 4 (a, b) AGR and IGR values are presented respectively. It's observed that *Bacillus subtilis* diet presented the highest values in AGR with 0.093 g day-1 and 0.051 cm day-1. Control diet presented an AGR of 0.038 g day-1 and 0.009 cm day-1. ANOVA indicated significant differences (P<0.001) between all experimental diets. At IGR, the highest values was found at Bacillus subtilis diet with a daily weight increase of 4.95% and a daily length increase of 1.85%. Control diet presented the lowest values with a daily weight increase of 3.59% and a daily length increase of 0.49%.

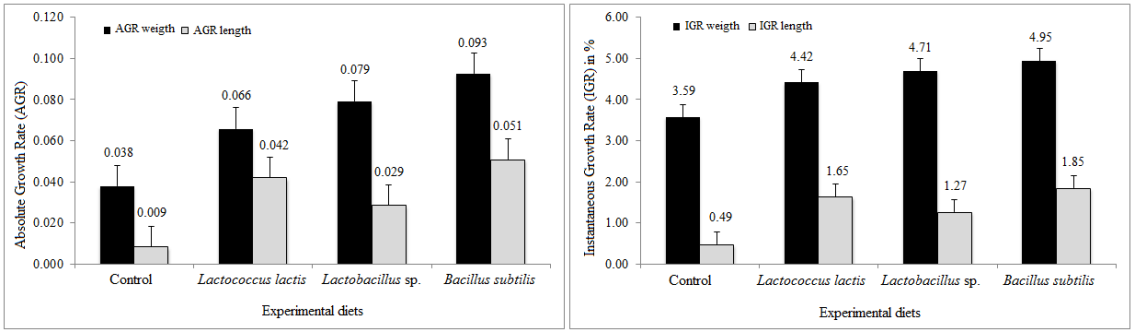

Fig. 4. AGR and IGR weight and length values in Ambystoma mexicanum fed with experimental diets.

Regarding to Condition Factor, *Lactococcus lactis* and *Bacillus subtilis* diets proportionate to axolotls a better weight and length relation (Fig. 5).

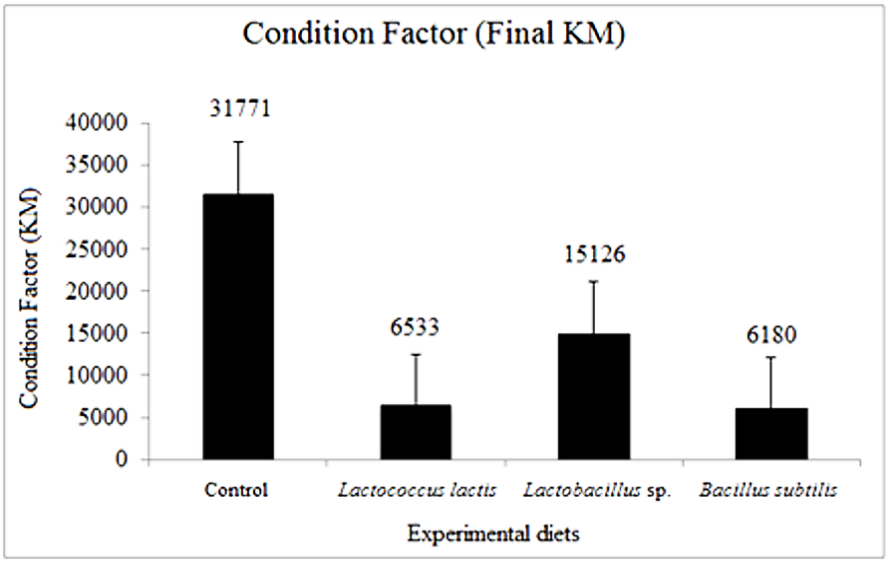

Fig. 5. Condition Factor (KM) values in Ambystoma mexicanum fed with the experimental probiotic diets.

4. Discussion

According to obtained results, it can establish that *Ambystoma mexicanum* posterior digestive tract is colonizing by 56 bacteria strains. Molecular identification determines that dominant species are *Bacillus subtilis*, Bacillus sp, *Lactococcus lactis*, *Lactobacillus* spp, *Lactobacillus crispatus*, *Aeromonas sobria* and *Pseudomonas pscicida*. It is important to point out, that identified species in this study had not been report previously as part of the intestinal microbiota of this amphibian. One of the newest was Okelley *et al.* (2010), which make an analysis and classification of intestinal microbiota of *Plethodon glutinous* salamandra, observing that 74% of recovered sequences appeared to be closely related to gender *Clostridium*, Eubacterium and Ruminococcus. Also only 10% of recovered sequences belong to enteric bacteria of the genus *Escherichia* and *Serratia*. Pryor (2008) show that Rana catesbeiana gastrointestinal tract, were dominated by bacteria *Edwardsiella tarda* and *Clostriduim*. It also mentioned that bacteria that colonize intestinal tract of amphibians are poorly studied. It is a regrettable situation, because normal microbiota has a deep significance in the history of live, ecology and evolution process in amphibians.

An important aspect of isolated bacteria strains of *Ambystoma* from their gastrointestinal tract (*Bacillus subtilis, Bacillus* sp, *Lactococcus lactis*, *Lactobacillus* sp. and *Lactobacillus crispatus*) were unable to inhibit *in vitro Aeromonas hydrophila*. This bacteria cause red-legged disease in amphibians and hence their removal indicates competitive exclusion capacity from *Ambystoma* isolated bacteria. This capacity is a probiotic characteristic of these bacteria (Balcazar *et al.,* 2006; Giri *et al.,* 2013).

Considering the organisms survival in this study, *Lactobacillus* sp. diet reach 100% survival with no significant differences ($P<0.05$) with respect *Bacillus subtilis* diet with 95% survival. Control diet presented the lowest values with 60% of survival. This results, regarding by Gatesoupe (1994); Gullian *et al*. (2004) and Bagheri *et al.* (2008), where they point out that there is always positive effects in the survival of diverse aquatic organisms when they are fed with probiotic enriched diets, because those bacteria increase the immune response that confers higher resistance to ambient variations and diseases (Bidhan *et al*., 2014).

In relation to axolotls growth, it was observed that probiotic enriched diets improve *A. mexicanum* growth, compared to control diet. Nevertheless, *Bacillus subtilis* enriched diet obtained better results in weight and length in the IGR and AGR during 60 days of experimentation. No scientific studies about *Ambystoma* growth fed with

probiotic strains has been reported. Only, it could be mentioned the work, Otto (1998), said that *Ambystoma* in captivity fed with worms, obtains better growth rates than commercial diets. According these study obtained results, it can be concluded that probiotic bacteria obtained from the same tested organism gastrointestinal tract, provide better benefit effect, because they attach and remain better in the gastrointestinal epithelium of *Ambystoma*, enhancing nutrient assimilation and exclusion of pathogenic bacteria. This benefit was reflected in better growth and increasing survival of this amphibian in culture.

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