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Growth evaluation of *Ambystoma mexicanum* and *Ocimum basilicum* with application of *Bacillus subtilis* probiotic in aquaponic system

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Abstract: The mean goal of this study was to evaluate the effect of *Bacillus subtilis* probiotic, obtained from intestinal tract of *Ambystoma mexicanum* in the growth of axolotl and basil *Ocimum basilicum* in an aquaponic system during 10 culture weeks. For this study, two experimental treatments under controlled conditions were designed and made per triplicate. Axolotl organisms final mean weight was 22.85±0.31 g (probiotic treatment) and 22.91±0.55 g (control treatment); length was 126.62±1.34 mm (probiotic treatment) and 131.94±0.55 mm (control treatment). Basil plant total length was 202.85±1.52 mm (probiotic) and 199.28±0.71 mm (control). Both experiments showed significant differences ($p<0.05$). Survival was of 100% for axolotl and basil. This study shows that *Ambystoma mexicanum* and *Ocimum basilicum* were adapted successfully in aquaponic systems without need to apply some inorganic or organic fertilizer.

Keywords: Aquaculture, axolotl, basil, functional foods

Introduction

Aquaponic is a technology that combined aquatic culture organisms (aquaculture), and plant culture without soil, called hydroponic. This technique was developed more frequently due their multiple economic and ecology benefits (Balcom, 2015), because it allows to produce several commercial cultures with reduced use of water and chemical products (Adler *et al*., 2000). Three groups were involved in optimum efficiency of aquaponic systems: plants, fishes, and nitrifying bacteria. The last one, play a key role in biofiltration, transforming ammonia toxic waste, generated by fishes, to nitrate which was the elemental mineral of plants. Therefore, it is generated a value product through a sub product, with the advantage of water recycling and reused constantly (Rakocy *et al*., 2006). Because of this, hydroponic systems have significant impact in terms of food safety, because they do not need fertilizers or chemical products. This system, works in terms of two interest production points: cost effectiveness and waste treatment (Rakocy, 1999).

Although in recent years it was developed several studies about aquaponic culture system, it still required to make some researches about commercial plant and fish production for human consumption, but also for their exploitation for those organisms which were enlisted in national or international risk category like *Ambystoma mexicanum* (UINC, 2014; NOM-059-ECOL/2010 DOF 2010).

Several studies mentioned that proper handling of microbial communities can make more efficiently the general metabolism of these production systems (Monroy *et al*., 2015). In this study, one of the most used technology to modify the microbiota in aquaculture is the use of probiotics (live microorganisms, which applied in adequate quantities, confer a benefit to host and cultured water) defined by FAO (2012). Several probiotics, like *Bacillus pumilus*, *Bacillus clausii*, *Bacillus subtilis*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* were widely used, in recent years, to improve growth and survival of fishes and crustaceans (Pérez *et al*., 2014). However, recently, it is unknow the effect of probiotic applied in aquaponic production system.

Due that, the principal goal of this research was to evaluate the growth and survival of axolotl *A. mexicanum* and culinary or medicinal herb *Ocimum basilicum* (bail), with the application of *B. subtilis* probiotic in aquaponic system.

Material and methods

Organisms procurement to experimental tests

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The axolotl organisms were obtained from one same hatch produced in Limnobiology and Aquaculture Laboratory. Bail seeds were obtained from mature bail plant from domestic greenhouse. Probiotic *B. subtilis* strain was obtained from bacterial strain collection at Laboratorio de Análisis Químico. This strain was isolated from digestive tract of adult amphibian of *A. mexicanum* (Monroy *et al.,* 2015). Both laboratories belong to Departamento El Hombre y su Ambiente of Universidad Autónoma Metropolitana Xochimilco. Previous inoculation, the bacteria strain was reactivated in MRS broth and incubated for 24 hours, at 27°C to obtain a 1x10 CFU mL^{-1} concentration.

Experimental design

Aquaponic system was designed using six fiber glass beakers of 200 L capacity. At each beaker, it was build a laminar flow system (NFT) type with two PVC tubes (1 m length and three inches diameter). Each tube had five holes (5 cm diameter), separated every 15 cm. Each PVC tube was separated from the other by 30 cm. Water flow was produced using a submergible pump (1,000 L per $hour^{-1}$) to elevate water 1 m high. Temperature was maintained constant (22°±2 °C) with a Minisplit equipment (84,000 BTU). Also, LED lights was added to bail plants of 850 lux intensity capacity (Fig. 1).

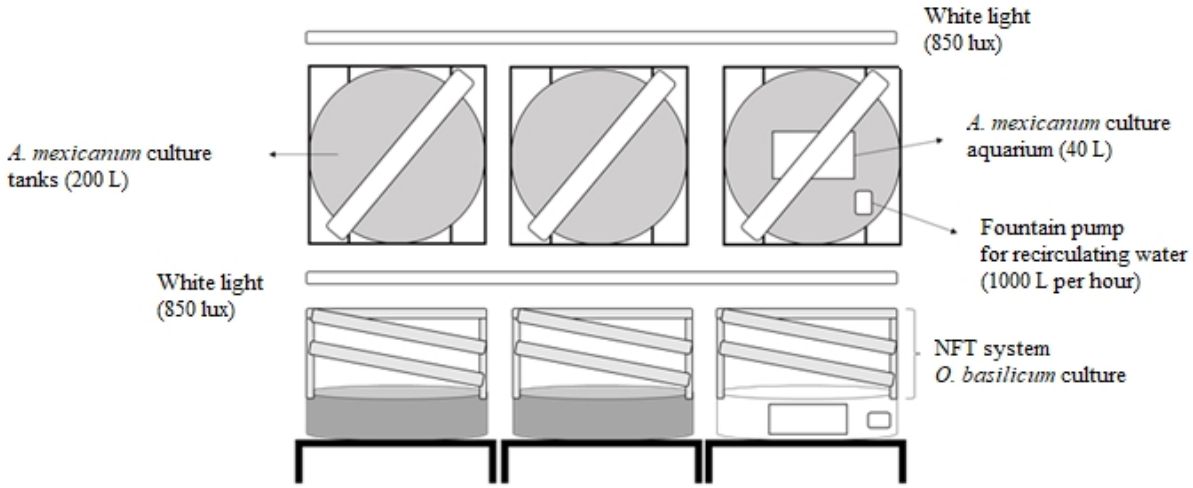

Fig. 1: Aquaponic system for *A. mexicanum* and *O. basilicum* culture.

Two experimental treatments were done: a) probiotic test group, and b) non-probiotic test group. Each treatment has three experimental units (EU) with five axolotls and 10 bail plants (For each experimental treatment 30 bail plants and 15 axolotls. Relation 2:1). Experimental test was done in two development stages of *A. mexicanum*: a) larval stages: 50 L aquarium was adapted to 200 L beaker for first six experimental weeks; the aquariums were filled with 40 L of freshwater to avoid the axolotls to find their food easily, and b) juvenile stage (week 7-10): with 70 L to avoid space competition. The experimental test was made for 70 days. Bail seed (*O. basilicum*), were introduced in plastic beakers of 7 cm high and 5 cm diameter and placed in each hole made in PVC tube (NFT) with a mix of 30 g of argillite and vemolite substrata to germinate 30 days before aquaponic experiment begin.

Food supply

The axolotls were fed with two experimental diets: a) control diet: three beakers with alternate diet of rotifers, *Artemia franciscana*, and cladocerans in 10% proportion of total biomass of organisms, and b) probiotic diet: three beakers with same diet but also *B. subtilis* strain applied directly in water culture medium with a concentration of 1×10^{-7} FCU mL^{-1}, only one day every week.

Culture medium maintenance

Each 24 h not consumed food, and feces waste was extracted from culture medium. The same water extracted was replenished.

Biometry and survival register

Each week, to *A. mexicanum*, total length was register in all organisms with Digital Vernier (Chicago Brand, Pocket Caliper, precision of 0.001 cm). Also, were weight with Digital Balance Ohaus (precision of 0.001 g).

To *O. basilicum* plants, each 15-days was estimated: total length or high (from the protruding stem of the substrate to germinal apex), distance

between leaf apices or interapical distance, and the survival. At the end of experiment test, plants were harvest and were placed in aluminum plates to dehydrated in an oven Thermolyne 9000 at 70 °C for 48 h. After that, plants were weight in a Digital Balance Ohaus (precision 0.001 g). Dry leaf's and stems were minced with a Osterizer blender and final product was weight in a Digital Balance Ohaus (precision 0.001 g).

To determine the survival for both organisms tested, the following formula was used:

Survival (%) = (Initial organisms quantity/ Final organisms quantity) X 100

Water quality

Once a week, physical-chemical parameters were registered: a) temperature and pH with a potentiometer (Hanna Instruments, Edge); b) dissolved oxygen with an Oximeter (Hanna Instruments, HI9146); c) Nitrates (NO_3), Nitrites (NO_2), and Ammonium (NH_4) with multiparametric photometer Hanna Instruments (HI83203).

Processing data

Axolotls and bail plants biometric data were registered in Excel 2013 database to obtain descriptive analysis from each experimental treatment, tendency curves were determined and, absolute growth rate (AGR) and instantaneous growth rate (IGR), proposed by Busacker (1990):

AGR = (Final length or weight – Initial length or weight)/Days of experimentation

IGR = {[NL(final length or weight)-NL(initial length or weight)]/days of experimentation} X 100

also, was determine the length and weight gain of organisms with following formula:

Gain = Final length or weight – Initial length or weight

Statistical analysis

One-way variance analysis (ANOVA) was made to determine significant differences ($p<0.05$) between treatments.

Results

Growth and survival of *A. mexicanum*

Table 1 show mean values (±S.D.) obtained per week of length and weight of axolotl *A. mexicanum* in both experimental treatments.

Final weight was 22.85 ±0.31 g for organisms in probiotic treatment and 22.91 ±0.55 g for non-probiotic treatment. ANOVA analysis showed no significant differences ($p>0.05$). With respect to total length with probiotic treatment was 126.62 ±1.34 mm and for non-probiotic treatment was 131.94 ±0.55 mm. ANOVA analysis showed significant differences ($p<0.05$) between experimental test.

Tab. 1: Mean values (±S.D.) of weight and total length per week sample of *A. mexicanum* exposed to *B. subtilis* probiotic treatment and non- probiotic treatment.

Week	Weight		Total length	
	Probiotic treatment	Non-probiotic treatment	Probiotic treatment	Non-probiotic treatment
0	0.30 (±0.05)	0.31 (±0.06)	27.87 (±2.55)	29.50 (±0.06)
1	0.59 (±0.02)	0.73 (±0.10)	37.80 (±1.33)	41.36 (±0.10)
2	1.29 (±0.07)	1.39 (±0.11)	49.77 (±2.34)	52.06 (±0.11)
3	1.59 (±0.19)	1.63 (±0.11)	55.77 (±2.17)	57.54 (±0.11)
4	2.54 (±0.25)	2.65 (±0.14)	65.01 (±1.98)	66.77 (±0.14)
5	4.83 (±0.44)	5.03 (±0.57)	76.60 (±1.97)	78.00 (±0.57)
6	7.56 (±0.55)	7.66 (±0.33)	89.74 (±2.20)	91.23 (±0.33)
7	10.03 (±0.44)	10.30 (±0.30)	99.34 (±0.69)	101.15 (± 0.30)
8	14.77 (±0.46)	15.05 (±0.39)	109.50 (±0.82)	111.71 (±0.39)
9	18.04 (±0.58)	18.16 (±1.35)	119.63 (±0.58)	124.05 (±0.58)
10	22.85 (±0.31)	22.91 (±0.55)	126.62 (±1.34)	131.94 (±0.55)

The AGR, in both treatments showed the same value (0.251 g per day). With respect total length, the AGR value was 1.09 mm dia^{-1} with probiotic treatment and 1.13 mm $día^{-1}$ at non-probiotic treatment. The IGR for weight was 1.68% and 1.66% per day respectively and for length 1.66% and 1.68% per day respectively. ANOVA analysis did not show significant differences ($p>0.05$).

The weight gain was 28.55 g (with probiotic) and 22.60 g (without probiotic). The length gain was 98.75 mm (with probiotic) and 102.44 mm (without probiotic). Figure 2 show tendency curves of weight and length for *A. mexicanum.* In both treatments showed a polynomic grade two curves, with a correlation (R^2) up to 0.90.

Growth and survival of *O. basilicum*

In Table 2 it is shown mean length values (±S.D.)

obtained each 15 days of *O. basilicum* in both experimental treatments.

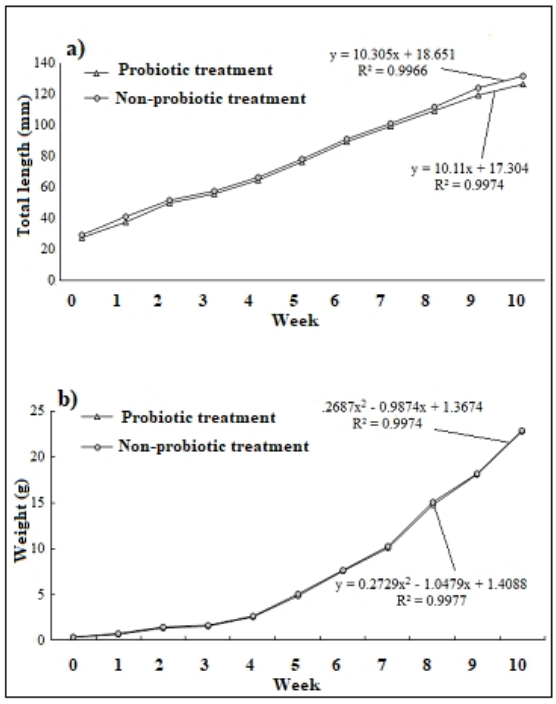

Fig. 2: Growth tendency curves of total length and weight of *A. mexicanum* in aquaponic system. a) total length; b) weight.

Tab. 2: Mean values (±S.D.) of total length (height) of *O. basilicum*, obtained every 15 days in experimental treatments.

Sample Days	Experimental treatment	
	Probiotic	Non-probiotic
15	37.06 (±0.43)	37.01 (±0.23)
30	71.42 (±0.94)	71.37 (±0.32)
45	101.93 (±1.54)	101.17 (±1.09)
60	157.63 (±0.89)	156.55 (±0.48)
75	202.82 (±1.52)	199.28 (±0.71)
90	263.45 (±1.77)	260.39 (±1.74)

Final length of bail plants for probiotic treatment was 263.45 ±1.77 mm, and for non-probiotic treatment was 260.39 ±1.74 mm. ANOVA analysis did not show significant differences ($p>0.05$) in between treatments. The AGR was of 2.51 mm per day for probiotic treatment, and 2.48 mm per day to non-probiotic test. The values of IGR were 2.17% and 2.16% increasing per day respectively. The ANOVA analysis did not show significant differences ($p>0.05$).

In probiotic treatment, the length gain was 226.39 mm and for non-probiotic treatment was 223.38 mm. The maximum values of interapical distance were 244.20±37.74 mm in probiotic treatment and 235.47±1.84 mm in non-probiotic treatment. The bail plant survival was 100%. Figure 3 show tendency curves about interapical growth of *O. basilicum* in both treatments. It shows a polynomic grade two curves, with a correlation (R^2) up to 0.90 value. The bail fresh weight was 214.31 g and dry weight of 16.47 g for probiotic treatment, and for non-probiotic treatment was 205.64 g fresh weight, and for non-probiotic treatment was 15.81 g.

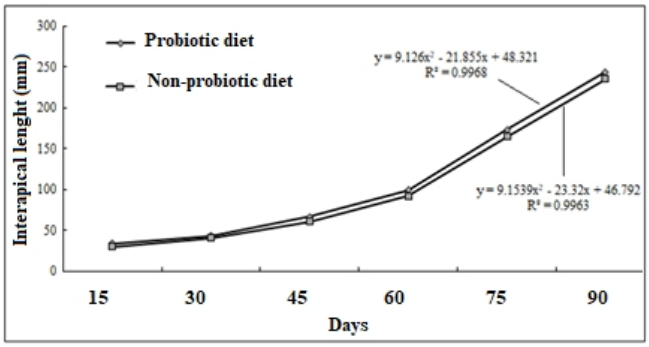

Fig. 3: Interapical length tendency curves of *O. basilicum* plants at two experimental treatments.

Physical-chemical and nitrogen compounds

The mean value of temperature was 22° ±1.52 °C, pH value was 8.42 ±0.24, and dissolved oxygen was 5.14 ±0.92 mg L^{-1}. With respect to nitrogen compounds in culture medium, are shown in Table 3.

Tab. 3: Mean values (±S.D.) of nitrogen compounds concentration showed in water culture medium at experimental treatments.

Nutrient	Treatment	
	Probiotic	Non-probiotic
Nitrites (NO_2)	0.12 (±0.02)	0.11 (±0.02)
Nitrates (NO_3)	3.78 (±0.91)	3.71 (±0.21)
Ammonium (NH_4)	0.09 (±0.04)	0.08 (±0.01)

Non-significance values: NO_2 P=0.530; NO_3 P=0.644 y NH_4 P=0.798.

Discussion

In recent years, one of technology most used to increase growth, survival, and aquatic culture organism's health, was probiotic microorganisms, which were demonstrate several benefits, without negative effects of hormones, chemicals and antibiotics application (Priyadarshini *et al*., 2013).

However, results obtained by this research did not obtained significant differences in *A. mexicanum* growth cultivated in aquaponic system with application of *B. subtilis*, unlike previous studies like Monroy *et al*. (2015), which mentioned a significant increase in survival and larval growth of *A. mexicanum* cultured in conventional aquatic system and supply of probiotic strains like: *Lactococcus lactis*, *Lactobacillus* sp. and *Bacillus subtilis.* These authors mentioned that organisms which shown bigger weight and length values were those who were fed with *B. subtilis.* Results differences with this study, may be due, because probiotic strain only was added once time a week, in comparison with other studies where probiotic strains were added for 32 continuous days period as demonstrate by Chiu *et al*. (2010), and Ferguson (2010), which obtained better growths with *Oreochromis niloticus* and *Epinephelus coioides* with probiotic *Pediococcus acidilactici* and *L. plantarum* applied.

Similarly, it should be noted that weight and length gain difference between treatments was due to continuous recirculating water variable, because bacteria strains did not stay at intestinal tract of axolotls, because were captured by plant substratum in NFT system or were maintained in water culture medium roots.

With respect to aquaponic vegetable component in two treatments, the bail plant shows high increase of length and frondiness. Seed germination occurred only in two days and at first sample, plants shows 40 mm length. Briseño *et al.* (2013), mentioned that life cycle (sowing to harvest) is approximately 90 to 110 days in bail plant. Although, do not exist previous studies about aquaponic systems with axolotls and *O. basilicum*, can be make a comparison with this aromatic herb like made by Ronzón-Ortega *et al*. (2012), which reported a growth in seedlings until 11 cm in 50 experimental days. Unlike this study, but the difference observed in Ronzón-Ortega *et al.* (2012) study, was that they installed two months early and allowed well development microbiota community in the aquaponic system. In our study, the culture medium was complete clean of microbiota, but we can conclude that bail plants were made efficient exploitation of nitrogen compounds which were in water culture medium, because when variation in this compound concentration were observed, always were maintained under permissible range to axolotls and bail plants culture. Rakocy (2006), mentioned that adequate proportion between plants and animals in aquaponic systems was the important key to obtain better integral nutrient recycling. At this way, the system works as biological filter that maintains adequate culture water quality.

It is important to mention, that there are not previous studies about axolotl's culture in aquaponic system, and despite that several authors indicated that their culture was difficult, because there are strictly carnivorous and need to be maintained between 13 to 20°C, and protected by directly light to avoid organisms stress. In this study, axolotls and bail plants were maintained at 22 ±2 °C and with light intensity of 850 lux (24 hours), but mortality or emergence of disease outbreaks in animals and plants were not observed.

It can mention that diet supply in axolotls cover the energetic source to do their basic metabolic activities, and can resist better to environmental variation and diseases, obtaining better well-being degree that translate to better growth in axolotls and bail plants, as was mentioned by Ortega- Santana (1998).

It is important to point out, that not having an external inorganic nutrient input to begin aquaponic system, the cultured organisms can be considered as 100% organic product (Cortés-Ortega, 2012).

Bernstein (2011), mentioned that in aquatic system need to be considered an equilibrium point that guarantee the nutrient availability and absorption for plants, which is achieved through the number of animals cultured balance. This author mentioned that equilibrium relation between plants and animals was 2:1, but can vary in function of specific specie cultured characteristics, use and doses from different probiotics sources. Being the first production experience of *A. mexicanum* and *O. basilicum* in aquaponic system, and did not have previous studies with these two organisms, we consider that the plant:animal relationship was adequate.

Conclusions

This study made the first steps to a new research aquaponic technique, which can be perfectible, with respect probiotic administration period and doses, which allow their use as little scale urban aquaculture system, to improve country people income and for research studies to conservation strategy in Xochimilco, Mexico habitat.

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