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Growth and survival of the axolotl (*Ambystoma mexicanum*), fed with the probiotic PROBION-forte©, under laboratory conditions.

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Abstract
For the present study, the growth and survival of 30 juvenile axolotls of the *Ambystoma mexicanum* species fed with a probiotic were evaluated. At the beginning of the investigation, the specimens had an average size and weight of 4 ± 0.5 (cm) and 2 ± 0.3 (g) respectively. Throughout the study the organisms were kept in six aquariums with 40 L of fresh water, at a temperature of 15 ± 1 °C, with a natural photoperiod and with 5 axolotls each. The axolotls were fed for 24 weeks with the *Tubifex tubifex* slimeworm inoculated with 1 X 10^7 CFU/g with the probiotic PROBION-Forte© (*Bacillus subtilis*, *Bacillus coagulans* and *Clostridium butyricum*). To verify the effect of the probiotic on the organisms, survival, growth (height and weight) as well as their degree of well-being were determined. The results reveal that the specimens fed the enriched diet present a survival of 100% compared to those fed the unenriched diet was 75%. On the other hand, the growth showed significant differences between the two treatments ($p < 0.006$) since the height at the end of the experiment was 18.4 and 11 (cm) with and without the incorporation of the probiotic in the food, respectively. In addition to this, the axolotls fed without the probiotic presented a weight of only 32.52 g; while those fed the probiotic was 57.20 g. This knowledge generated is very recent, but it has been observed that incorporating a probiotic in their diet of organisms has beneficial effects, in turn these results are important in Mexico, since it has great ecological, cultural and commercial value for the country specimens and these investigations will allow us to reduce the pressure that this organism has in its natural habitat, giving way to an increase in aquaculture production.

Keywords: *A. mexicanum*, PROBION-forte © probiotic, endemic, survival, growth.

1. Introduction
The axolotl *Ambystoma mexicanum* commonly known as axolotl or water monster, is an endemic organism of the Basin of Mexico, it has a great ecological, cultural and commercial value both local, regional, national and international, the latter thanks to the aquarium hobby and aquaculture. At present, this specimen lives in Lake Xochimilco in Mexico City, however, in recent years, its environment has been deteriorating due to urban growth, introduction of exotic species, solid waste, pesticides and fertilizers. Additionally, since pre-Hispanic times, the axolotl was used and included as food in their varied diet of the natives of the Valley of Mexico, in addition to this, this animal was considered with curative qualities for respiratory diseases such as anemia, asthma and bronchitis [1, 2, 3, 4]. Unfortunately, over time its natural populations have been disappearing, placing the axolotl in danger of extinction [5, 6] and as a species subject to special protection within the NOM-059-SEMARNAT-2010 [7]. The activity of aquaculture has been the great ally to carry out the cultivation of the axolotl in captivity under controlled conditions from eggs, young, juveniles, adults and reproduction in order not to extract the specimens from their natural environment and not affect their exploitation and extinction [8]. Given the importance of this species due to its peculiar characteristics, various educational, private and governmental institutions at the national and international level have been interested in carrying out different investigations with the axolotl covering a wide range of studies that speak of (temperature, reproductive season, quantity and quality of live and processed food in different life stages, stress conditions, photoperiod and tissue regeneration) [9-19]. Despite the fact that there is endless research, one of the great challenges that have been.

Faced throughout the different life phases of *A. mexicanum* is feeding, since the diet of the axolotl in wild life is extensive, particularly It feeds on small fish, tadpoles, aquatic insects, earthworms, crustaceans, and freshwater mollusks. When they are larvae, axolotls feed on zooplankton, made up of microscopic crustaceans such as copepods, cladocerans (*Daphnia magna, Molnama crocopa, Simocephalis vetelus, Macrothrixx triserialis*) and rotifers of different species of *Brachionus* sp. [19,20,17]. Regarding feeding in captivity, there is currently only one commercial balanced food formulated specifically for this specimen in the juvenile-adult stage (Axolotl Sticks), however, its high demand for this product by aquarium hobbyists makes that is exhausted and very difficult to obtain, so it is generally offered food from other species (*Oncorhynchus mykiss* and *Oreochromis niloticus*) that contain more than 40% crude protein, in parallel to these specimens small fresh fish fillets are offered (tilapia, carp, trout and salmon), mosquito larvae (*Culex* sp.), crustaceans (*Artemia franciscana*), decapods (*Cambarellus* sp.), cladocerans (*Daphnia magna*), mud worm (*Tubifex tubifex*) and baby fish (*Chirostoma* sp. and *Poecilia reticulata*) [19,20]. Today, in the world of aquaculture, various studies have been carried out on the implementation of live food enriched with the use of prebiotics, probiotics and symbiotics, with the purpose of eradicating pathogenic microorganisms within crops, in addition to considerably improving their survival and growth of the organisms to be studied. On the other hand, there is still little information about the use of commercial probiotics and their benefit in aquatic amphibians, since most of the research focuses on fish, molluscs and crustaceans and information on the use of probiotic strains in the amphibian culture is scarce. Therefore, the objective of this research was to evaluate the effect of live food enriched with the commercial probiotic PROBION-forte© (*Bacillus subtilis, Bacillus coagulans* and *Clostridium butyricum*) on the survival and growth of *Ambystoma mexicanum* juveniles.

2. Materials and Methods

2.1 Obtaining of organisms

The present investigation was carried out with a colony of juvenile axolotls of the *Ambystoma mexicanum* species, which were kept in captivity under controlled conditions in a property located in the Xochimilco, Mexico City.

2.2 Experimental design

In the laboratory facilities, six aquariums of 50 L were prepared with 40 L of dechlorinated water with 5 juvenile axolotls of 1.5 months of age with an average size and weight of 4 ± 0.5 (cm) and 2 ± 0.3 (g) respectively. Throughout the investigation, the organisms were kept at a temperature of 15 ± 1 ° C and with a natural photoperiod. The specimens were fed daily for 24 weeks with the *Tubifex tubifex* slimeworm inoculated with $1X10^7$ CFU/g with the PROBION-forte© probiotic (*Bacillus subtilis, Bacillus coagulans* and *Clostridium butyricum*). The treatments were distributed as follows: aquarium 1 was assigned as a control, in this, the organisms were fed with the mud worm *T. tubifex* without enrichment; Aquarium 2 was given *T. tubifex* enriched with 0.5 g of PROBION-forte© probiotic. It is worth mentioning that each treatment had its three corresponding replicas. In all cases, the feeding rate supplied daily represented 10% of the total biomass of the colony.

2.3 Record of morphometric data and survival

In order to evaluate the effect of the probiotic and minimize stress in the juvenile axolotls, the record of the sizes and weights of the organisms that make up each aquarium was averaged every 15 days from the beginning of the investigation to the end of this and with this, the proportion of food that was offered daily was adjusted.

The morphometric parameters taken from each specimen were: total length (L_T), with a modified plastic ichthyometer of 30 cm ± 0.01 precisions and the weight in (g) using an Ohaus® brand digital scale Scout pro model with a capacity of 400 g with an accuracy of ± 0.01 g, removing the extra weight of water by gently drying the body with a clean cloth.

2.4 Maintenance conditions

Food remains and feces were removed from the aquariums on a daily basis in order to maintain the quality of the water. The filling was 10% dechlorinated water so as not to stress the axolotls. On the other hand, the physicochemical parameters (temperature, pH, nitrite and ammonium) were monitored using the API water quality tests.

2.5 Data Analysis

For each treatment, the specific growth rate was calculated using the formula proposed by Ricker (1979) [21].

$$TEC\ (\%/day) = \frac{(\ln Y2 - lnY1)x^2}{T} x100$$

Where:
TEC= Specific rate of growth
ln Y1= Natural logarithm of weight and total length at the beginning of the experimental period
ln Y2= Natural logarithm of weight and total length at the end of the experimental period
T= Time in days of duration of the experiment

To evaluate the effect of the probiotic on the growth of the juvenile axolotls, the multiple condition factor was calculated, that is, their degree of well-being, with the formula proposed by (Pokniak *et al*., 2004) [22].

$$FC = \left(\frac{P}{LT^3}\right) x\ 100$$

Where:
FC= Multiple condition factor
P= weight and total length at the beginning of the experimental period
LT= total length

Weight gain was determined with the formula used by (Amisah *et al*., 2009; Vinchira *et al*., 2014) [23, 24].

$$GP = \left(\frac{Pf - Pi}{T}\right)$$

Where:
GP= Weight gain
Pf= Weight in grams at the end of the period
Pi= Weight in grams at the beginning of the period
T= Time in days of the period

Finally, survival was determined for each of the treatments using the formula proposed by (Uribe and Luna, 2003) [25].

$$\%\ Survival = \frac{Number\ of\ organisms\ at\ the\ end\ of\ the\ experiment}{Number\ of\ organisms\ at\ the\ start\ of\ the\ experiment} x100$$

2.4 Statistical Analysis
To determine the average value and standard deviation of the data generated in the different variables considered, a database was made with Microsoft Excel 2020 Software (Microsoft Corp., Washington, USA).

To determine significant differences between the two study treatments, a two-way analysis of variance (ANOVA) was performed. When finding significant differences ($p < 0.05$), the multiple means comparison technique was applied by the Tukey method. For the analysis of variance test, as well as the Tukey technique, the statistical program Systat 12 (Systat Software Inc., California, USA) was used.

3. Results and Discussion
Survival
The survival obtained by each of the *A. mexicanum* treatments with respect to feeding is shown in Fig. 1. In general, it is observed that the organisms fed with the probiotic PROBION-forte© presented a survival of 100%, in contrast, the specimens fed without the probiotic obtained 75% at the end of the investigation. Several studies [26,27,28] mention that they have obtained better survivals when they provide live food enriched with probiotics extracted from the digestive tract, mucus from the scales and gills of different aquatic organisms (fish, crustaceans and amphibians) to their organisms under study. An example of this is the work carried out [29], with the species *Pangasius hypophthalmus* to which they provided an inert food based on vegetables, fruits and tilapia fillet, enriching it with a probiotic extracted from the intestine of angelfish and lobster red presenting in the latter the best results in terms of survival and weight in 180 days. On the other hand, researchers [30] evaluated the effect of *Lactobacillus* sp. and *Bacillus subtilis*, probiotic strains previously isolated from the digestive tract of the axolotl *Ambystoma mexicanum* and supplied to larvae of this same amphibian to determine their survival and growth in a period of 60 days. The results showed that axolotls that had a diet supplemented with the first probiotic had a 100% survival and organisms fed *Bacillus subtilis* had a 95% survival. This is because the use of probiotics in aquaculture with the genus *Bacillus* sp. it has been recognized for having multiple beneficial effects that they cause in the hosts, since they stimulate organisms to survive, accelerate their growth, strengthen their immune system against some pathogens and thus reduce their mortality rate [31]. Another example is the study carried out with juveniles of the *Ambystoma velasci* species, where they obtained a survival of 100% when they were given a live food enriched with the probiotics *B. subtilis* and *C. butyricum* [32]. Various authors [33, 34, 35] mention that survival is the estimation of the levels of living organisms in cultivation systems, which may be feasible for calculating the daily ration, production and cultivation status in terms of profitability. Therefore, providing it with a functional food that meets the nutritional requirements that the species needs and incorporating it with a probiotic increases the survival of the organisms and thus tends to develop optimally. On the other hand, researchers [36] point out that two important aspects should be considered to obtain survivals above 60%: 1) Number of times the organisms are fed per day and 2) Consider the growth phase in which they are finds the organism in experimentation, because the larger the organism, the greater the number of times it must be fed. The same authors point out that an intake greater than 3 rations of food a day not only causes the animals to form the necessary structures, but also that weight and height increase significantly. Regarding the statistical analysis, ANDEVA indicated that there are significant differences in survival with respect to food enriched with the probiotic and food without fortification ($p < 0.004$). With the aforementioned, it can be corroborated in this research that the best survivals in juveniles of *A. mexicanum* were obtained with food enriched with the probiotic PROBION-forte© since it is made up of (*B. subtilis* + *B. coagulans* + *C. butyricum*) and each of these probiotics have a positive impact on survival.

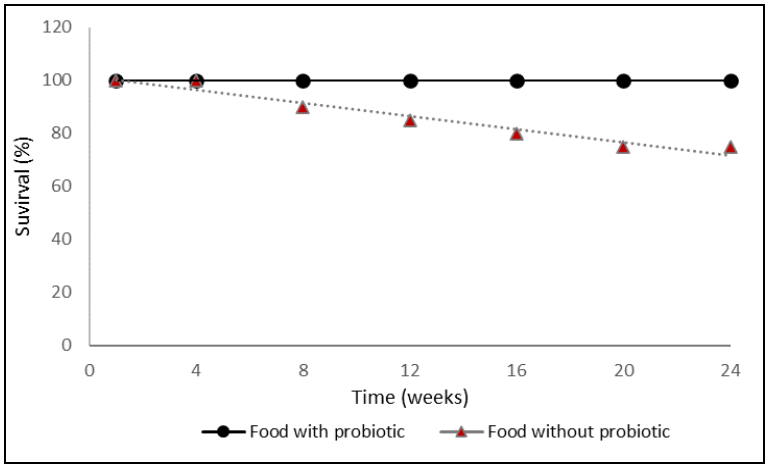

Fig 1: Survival of *Ambystoma mexicanum* with respect to time (weeks) with the food enriched with the probiotic and without enriching the food.

Growth (weight and height)
The average values (± SD) of the growth in weight and height obtained by the juveniles of *A. mexicanum* in the two treatments studied are found in Table 1 and Fig. 2. It is observed that at the end of 24 weeks the specimens fed with the probiotic had a height and weight of 18.4 (cm) and 57.20 (g) respectively, in comparison the organisms fed without the probiotic were 11 (cm) and 32.52 (g). In different investigations it has been found that probiotics are a replacement alternative to growth promoters, since when administered in adequate amounts, they reduce mortality and increase food conversion [37], improving the digestive capacity and increasing the health status of the animal, not to mention that they stimulate the growth of beneficial microorganisms and suppress pathogens by competition and production of lactic acid. In addition to this, the genus *Bacillus* has been

used in different aquatic organisms such as sole, which obtained weight gain and reached their commercial size faster, in the same way it has been used in shrimp and fish obtaining good results both in size and weight and survival [38]. Regarding the *C. butyricum* bacteria, benefits have been obtained that favor the growth of the organisms, as observed in the study [39] that by supplying this probiotic bacterium at 10^9 CFU/g in the *Miichthys miiuy* fish, the organisms showed greater results in the growth parameters, such as the total weight gain 97.3 ± 20 (g), unlike the fish that were not given the probiotic, was 48.5 ± 3.5 (g). On the other hand, an author [32] incorporated to *T. tubifex* a mixture of probiotics (*Bacillus subtilis* + *Clostridium butyricum*) in juveniles of *A. velasci*, at the end of the study the final weight was 54.47 g and 49.81 g and a height of 17.66 cm and 16.84 cm, the weight and height being greater with the enriched diet. In addition to this, researchers mention that the application of the yeast *Saccharomyces cerevisiae* does not significantly modify the weight and height of the axolotls at 120 days of the experiment ($p>0.05$); however, it was observed that there is a linear trend in weight with mean values of 46.06 ± 4.2 and 44.19 ± 1.1 g [40]. This is corroborated in the present investigation since the results obtained in weight and height were superior in comparison with the same authors. Other studies that have used these same probiotic strains, mention that their organisms develop in a significantly shorter time, have a lower incidence of diseases compared to specimens whose diet was not enriched, additionally they observed that there is a significantly increase in fertility, production and size of the offspring [41, 42, 43]. Another example is the study carried out by Menezes *et al.*, 2008 [44], with the probiotic *B. subtilis* and its effect on the growth, survival and immunological analysis of the bullfrog (*Rana catesbeiana*) in organisms 14 days before finishing their metamorphosis. The results in this study showed that there was a greater weight gain (4.25 g) at the end of the experiment with the probiotic. Regarding the immunological analysis, a positive influence is observed in the organisms that were fed with the enriched diet. However, it is always important to know the eating habits of each of the species to study, since in their different life stages they can be detritivores, herbivores, opportunists and omnivores without forgetting that their behavior can present cannibalism [45, 46, 47, 48]. For this reason, it is important to mention that axolotls prefer to consume animal protein [49], but the most important thing is to verify that adding different types of probiotics to aquatic organisms in the diet will accelerate the growth rate. According to the above, the inclusion of beneficial bacteria in diets for aquatic animals can have very varied effects with the last consequence of an improvement in the health and/or growth of the animals, although the latter may be partly a consequence of the first [50, 51]. Due to this, the proportion of *T. tubifex* as live food in this diet was adequate to observe growth. Regarding the statistical analysis, ANDEVA indicated that there are significant differences between the size of the organisms fed with the diet enriched with the probiotic and the unenriched food ($p <0.006$).

Table 1: Average values of length and weight in (cm) and (g) respectively of *Ambystoma mexicanum* with respect to time (weeks) with the food enriched with the probiotic and without enriching the food. Similar letters in rows do not show significant differences ($P <0.05$).

Week	Size		Weight	
	Food with Probiotic	Food without probiotic	Alimento con probiótico	Food with probiotic
0	4^a	4^a	2^a	2^a
D.S.	± 0.5	± 0.5	± 0.3	± 0.3
4	6.3^a	5.2^a	6.15^a	4.70^b
D.S.	± 0.4	± 0.1	± 0.5	± 0.1
8	8.1^a	6.9^b	12.72^a	8.29^b
D.S.	± 0.8	± 0.3	± 1.8	± 0.9
12	10^a	7.5^b	19.45^a	14.82^b
D.S.	± 0.5	± 0.3	± 2.1	± 0.7
16	13.4^a	8.1^b	26.90^a	19.10^b
D.S.	± 0.4	± 0.1	± 1.9	± 1
20	15.2^a	9.8^b	38.40^a	25.91^b
D.S.	± 0.3	± 0.1	± 1.2	± 1.9
24	18.4^a	11^b	57.20^a	32.52^b
D.S.	± 0.3	± 0.1	± 2.4	± 1.1

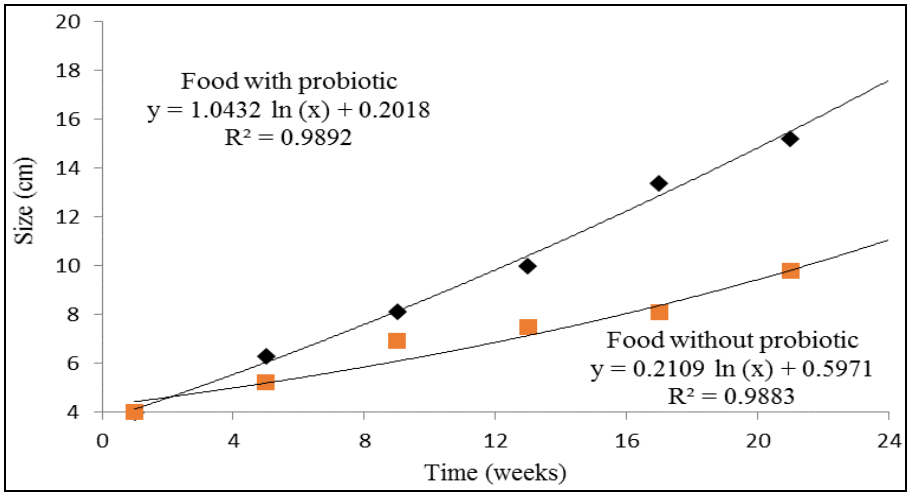

Fig 2: Values of the growth curve with respect to the height of *Ambystoma mexicanum* juveniles fed with and without the probiotic.

Regarding the condition factor (KM) and the absolute growth rate, significant differences were observed between the treatments, being the organisms fed with the probiotic the one that presented the best weight-length relationship, contrary to what happened with the unenriched feed as their KM remained less than optimal. Some authors emphasize [52, 53] that the condition factor is used to compare the degree of well-being of the organism or population, based on the fact that the specimens present a greater weight, a certain length and thus present a better condition to survive in its environment, it should be noted that its interpretation may depend on several factors such as; food availability, seasonality and growing conditions. However, when its KM is lower, this indicates that the diet supplied does not meet the necessary requirements so that the height corresponds to the weight of the animals and thus smaller and low-weight organisms are obtained [54] Fig. 3.

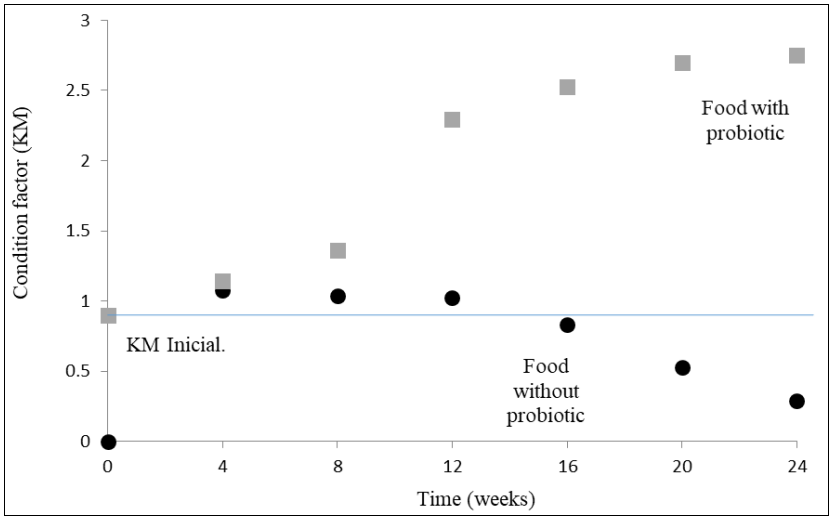

Fig 3: Values of the condition factor (KM) of *Ambystoma mexicanum* fed with the diets under study.

4. Conclusion

In the present research, it can be affirmed that by incorporating a probiotic in food (inert or alive) in aquatic organisms, it will play a very important role since its effect will be positive, improving and increasing survival and growth, in addition to their offspring they will be of better quality. This is confirmed in the present study where survival was 100% with organisms fed the probiotic in contrast to organisms that did not have a probiotic in their diet was 75% in a time of 24 weeks. On the other hand, their growth in size was 18.4 and 11 (cm) and their weight was 57.20 and 32.52 (g) with the organisms with the enriched and unenriched diet, respectively. The use of probiotics in the cultivation of *Ambystoma mexicanum* is very recent, but it has been observed that it has beneficial effects on organisms, in turn these results are important in Mexico, since it has great ecological, cultural and commercial value for the country these specimens, having said the above, these investigations will allow us to reduce the pressure that this organism has in its natural habitat, giving way to an increase in aquaculture production and, in turn, allowing the recovery of natural populations in Lake Xochimilco.

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