Administration of Three Autochthonous *Bacillus subtilis* Strains Induce Early Appearance of Gastric Glands and Vestiges of Pylorus in *Piaractus mesopotamicus* Larvae

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**Abstract**

Autochthonous microorganisms as probiotics are a novel alternative to antibiotics. The genus *Bacillus* is highly used by its ability to survive on adverse conditions. *Piaractus mesopotamicus*, a native fish, is the most produced specie in Argentinian aquaculture. The low level of knowledge, high stress conditions and demand of animals led to a deficit in the number of larvae and fry. The aim of this study was to evaluate the probiotic effect of previously selected autochthonous *Bacillus* on biometrical parameters of *P. mesopotamicus* larvae when administered together in a combined suspension in different doses and stages. Results showed that the bacterial administration did not cause significant effects on the variables evaluated. However, the dose containing 6 × 10^7 CFU L^-1 of each strain showed the highest average values of *P. mesopotamicus* biological parameters, regardless the stage of administration. Histological evaluations of animals administered with this dose did not show adverse effects and indicated the capability of this dose, administered in a particular period of time, to stimulate the early appearance of gastric glands, vestiges of what would be the pylorus and a higher development of liver. Thus, we proposed the safe use of this dose in a combined probiotic mixture to be studied in further assays.

**Keywords:** *Bacillus subtilis*; *Piaractus mesopotamicus*; Probiotics; Aquaculture; Larvae; Beneficial effects

**Introduction**

The use of native species in aquaculture of Northeastern Argentina allowed their predominance in the National fish production chart. *Piaractus mesopotamicus* is the most cultivated fish in the country since 2012, representing a 52.22% of the total production. Indigenous fishes had the advantage of resist to native environmental conditions. The demand of animals for breeding and fattening, the stress conditions in intensive culture systems and the low survival indexes of larvae and fry led to a deficit in the number of animals available. These facts brought the need of novel techniques to increase the production parameters. Antibiotics emerged as a solution, frequently applied as growing factors, anti-infectious agents and tranquilizers [1,2]. However, the European Food Safety Authority (EFSA) proposed the prohibition on their use as additives in foods [3] supported by the resistance transference [4], the toxicity of antibiotic residues [5], the appearance of allergies [6], the unbalance of intestinal microbiota [2] and environmental risks. Although expected, these regulations urged the search of more extensive production systems without the requirement of using additives, and/or the application of natural, novel and safe products.

The use of probiotics attempts to replace the use of chemotherapeutics in animal production [6-8]. Probiotics for aquaculture were defined by Verschueren et al. [6] as “a live microbial adjunct which has a beneficial effect on the host by modifying the host associated or ambient microbial community, by ensuring improved use of feed or enhancing its nutritional value, by enhancing the host response toward disease or by improving the quality of its ambient environment”. They were applied in a variety of terrestrial and aquatic hosts (www.isapp.org) with a wide range of beneficial effects: inhibitory activity against pathogens, nonspecific immune response stimulation, immunomodulatory effect, decrease of mortality and increase of growth rate and production [9-21]. Commercial probiotics are relatively ineffective in fishes, mainly because most of them include non-aquatic strains unable to survive and remain viable in the intestinal environment of fishes [22]. Then, it is essential to study cultivable autochthonous microorganisms as putative probiotics since they are more likely to survive and remain in the fish gastrointestinal tract [23].

*Bacillus* spp. has been used for several years in fermentation products or as spore-based supplements [24]. Recently they were proposed as gut commensals rather than solely soil micro-organisms [25-28]. Over other non-spore formers microorganisms, *Bacillus* have the advantage of being stable and resistant to the gastric barrier and storage conditions [24,29].

In previous *in vitro* studies our group selected three autochthonous *B. subtilis* strains as novel putative beneficial microorganisms [30]. However, the definitive manners and beneficial effects of potentially probiotic strains should be evaluated through animal models (www. isapp.org) [31]. The *in vivo* assays for the study of probiotics for aquaculture have three critical items: the stage of biological cycle, the optimal dose and the way in which microorganisms should be administered to the host. Pasteris et al. [32] suggested that the colonization of the skin (or scabs) and the gastrointestinal tract occurs together with the ontogeny and afterwards, the ingestion of...
microorganisms in larval stages could result in the establishment of a dominant intestinal microbiota that persist during the first stages of the biological cycle. Unfortunately, there is no general consensus on the other two critical terms. Bibliographic references suggest doses of administration ranging from $1 \times 10^7$ to $1 \times 10^{12}$ CFU. The last critical item is the way of administration, being widely used the balanced feed or the live alimen as a way to deliver microorganisms [16,33,34]. Therefore, the aim of this work was to evaluate the effect of the administration of the suspension containing these three autochthonous strains on the survival, mean weight, biomass and histological parameters of *P. mesopotamicus* larvae.

**Material and Methods**

**Bacillus strains**

Autochthonous *B. subtilis* strains A252, A253 and A254 were isolated from gills and medium intestine sections of *P. mesopotamicus* specimens. They were selected as potentially beneficial microorganisms based on the ability to express beneficial properties in "*in vitro*" tests. They were probed to resist the conditions of the gastrointestinal tract of fishes and to therapies used in aquaculture facilities. They were included in the Laboratorio de Sanidad Animal of the Estación Experimental Agropecuaria Rafaela belonging to the Instituto Nacional de Tecnología Agropecuaria (INTA) under Budapest treaty [35] for patent aims [30].

**Bacteria culture**

The spore forming bacteria described in the above section were grown daily in 400 mL nutrient broth (Britannia*) and incubated at 37°C under constant shaking conditions for 8 h. Bacterial cells were harvested by centrifugation at 3000 g for 10 min at 4°C, washed twice with sterile distilled water and suspended to obtain the adequate numbers.

**Live food preparation**

1 L suspension of freshwater containing 1 g of brine shrimp cysts *Artemia* sp., 15 g of NaCl and 2 g of sodium bicarbonate was submitted to incubation process under intense aeration and lightening for 24 h. Live hatched nauplii were filtered, suspended in freshwater and counted in order to obtain an approximate concentration of the solution [36].

**Fish reproduction**

Pacu larvae were obtained by controlled reproduction from *P. mesopotamicus*’s broodstock. Spawning was induced by injection of pituitary extract of *Prochilodus lineatus* according to Da Silva et al. [37]. The sexual gametes were obtained by the stripping technique, mixed immediately in a bowl, and suspended at the required concentration [38]. Aliquots of 1 mL were counted in order to obtain an approximate concentration of fedeculated eggs.

**Experimental design and sampling**

Experimental units were settled as 5 L plastic fishbowls with a constant recirculation system and approximately 300 fedeculated eggs. The three *Bacillus subtilis* strains were administered, in a combined suspension (BAC), in three different concentrations: 4 ($6 \times 10^7$ CFU L$^-1$), 7 ($6 \times 10^8$ CFU L$^-1$) and 10 ($6 \times 10^9$ CFU L$^-1$) of each strain and at different stages of the biological cycle of larvae: E (from the time of fecundation of the eggs until the beginning of the exogenous feeding 5 days), L (from the beginning of the exogenous feeding until the end of the assay in laboratory–from day 5 up to day 15) and E&L (from the fecundation of the eggs until the end of the assay in laboratory–from day 0 up to day 15). Control units (CTRL) were assayed with no addition of bacteria.

During stage E the bacterial suspension was added directly to the fish bowls four times a day with previous stop of water recirculation which was restarted one hour after. In L experimental units, bacteria were co-incubated with live food during two hours previous to administration; this procedure was performed *ad libitum* four times a day. Water recirculation was interrupted before alimentation and restored one hour after. Water quality determinations of pH, dissolved oxygen and temperature were performed daily in each experimental unit.

**Sampling**

At day 15, larvae were counted and weighed to obtain values of survival, mean weight and biomass. To evaluate the normal development, macroscopic evaluations were performed with binocular magnifier Optical Kyowa model SDZ. For histological studies, 10 larvae per treatment were collected and anaesthetized by chilling on ice, fixed in Bouin’s solution (saturated picric acid 3000 mL, formaldehyde 1000 mL, glacial acetic acid 200 mL) for 12 h, washed twice with alcohol 70° and maintained in this solution until processing. Samples were then routinely processed for histology, stained with haematoxylin and eosin and analyzed by light microscopy using a Leica DM500 microscope, a Leica ICC50 digital camera and the Leica Application Suite 3.4.1 image analysis system [39].

The procedures and experimental protocols applied to the animals in this work were in accordance with the ethical principles of animal experimentation, and approved according to protocol n. 0019/14-2011-02204 and 14-2012-03865 by the Ethics and Biosafety Committee of the School of Veterinary Sciences of the Northeast National University (UNNE) of Argentine.

**Statistical evaluation**

All the assays were performed by triplicate using a completely randomized design. Each replicate corresponds to different parents, excluding the genetic factor of the experiment. Statistical analyses were carried out using Statistica 6.0 for Microsoft Windows. Comparisons were performed, first, by a one way ANOVA including the ten experimental groups followed by a control vs. treatments comparison. Later, results were compared, excluding the control, by using a factorial two-way ANOVA with subsequent post hoc test in order to evaluate the main effects of doses and stages as well as the interactions between them. When interaction and significant effects were not detected, results were evaluated by orthogonal polynomials for trend analysis.

**Results and Discussion**

The values of survival, mean weight and produced biomass obtained after the administration of the microbial mixture BAC to eggs and/or larvae of *P. mesopotamicus* analyzed through one way ANOVA, did not indicate significant differences between treatments and control group (p>0.05) (Table 1). The two ways ANOVA analysis did not show neither interaction between doses and stages nor significant effect of dose or stage over any of the variables analyzed (p>0.05) (Figure 1). Although statistical evaluations did not demonstrate significant differences, the average values of each biometrical parameter indicated that larvae administered with the BAC suspension containing 6 $\times 10^7$ CFU L$^-1$ of each strain, regardless the stage of administration, showed the best outcome (Figure 2). These results indicate that there is not a remarkable effect of the probiotic on the evaluated variables.
### Table 1: One way ANOVA of mean weight, survival and biomass of *P. mesopotamicus* larvae administered with mixture BAC in different doses and stages.

| Variable | N  | SS  | df | MS  | F   | p-value |
|----------|----|-----|----|-----|-----|---------|
| Mean weight (mg) | 30 | 8.14 | 9  | 0.90 | 1.03 | 0.4490  |
| Survival (%)     | 30 | 552.03 | 9  | 61.34 | 0.85 | 0.5841  |
| Biomass (mg)     | 30 | 30362.53 | 9  | 3093.73 | 1.09 | 0.4117  |

N: Number of values; SS: Sum of squares due to the source; df: Degrees of freedom in the source; MS: Mean sum of squares due to the source; F: F-statistic.

However, dose 7 (6 × 10^7 CFU L^-1 of each strain) of the BAC suspension (composed by *B. subtilis* strains A252, A253 and A254), showed to be the most suitable concentration for its incorporation to a composite probiotic formula. This potentially probiotic mixture will be tested *in vivo* in future assays for *P. mesopotamicus* aquaculture. Thus, these strains were included in a type collection culture under Budapest treaty to initiate patent procedures [35]. The better effect of medium doses (quadratic effect) was described by several authors in aquaculture. Bagheri et al. [34] established that more probiotic cells in diets and host intestine does not necessarily result in a highest or improved growth, survival and protein efficiency ratio of animals, when administering two *Bacillus* strains in different concentrations to rainbow trout. Faramarzi et al. [40] obtained similar results when administering *Lactobacillus acidophilus* at different doses to rainbow trout, indicating better results on the survival percentage when administering an intermediate dose. Kapareiko et al. [41] obtained similar results over survival when different concentrations of *Vibrio* sp. was administered to oyster larvae challenged with pathogens.

With the aim of evaluate that the previously selected dose did not produce any adverse effect to the animals, they were observed macro and microscopically. Macroscopic observations did not show differences in behavior and/or observable malformations between treated and control group. The histological evaluations demonstrated no translocation or aggrupation of microorganisms in any of the treatments, basic characteristic of the strains to be considered for probiotic use [31]. Histological samples of animals administered with dose 7 in stages E (from the time of fecundation of the eggs until the beginning of the exogenous feeding) (Figure 3B) and E&L (from the fecundation of the eggs until the end of the assay in laboratory)
(Figure 3C) were consistent to those obtained in fishes of control group (Figure 3A) and those reported in literature as normal for this stage of the biological cycle [42]. On the other hand, when evaluating the histological parameters of animals administered with dose 7 in stage L (from the beginning of the exogenous feeding until the end of the assay in laboratory), the appearance of gastric glands was evidenced, together with a higher developed liver and vestiges of what would be the pylorus (Figures 4 and 5), histological characteristics of animals in a more advanced stage of their biological cycle, as described by several authors for this and other fresh water fish species [43-45]. There are no references indicating the early development of the gastrointestinal tract of fishes as those described in the present work. Instead, there are several studies based on the ability of probiotics to restore or prevent histological alterations caused by pathogens and only a few evaluate minor modifications of the normal histological structure of different organs. Standen et al. [46] published that tilapia administered with a multi-species commercial probiotic showed a significant increment in the absorptive surface of intestine. Merrifield et al. [47] proved the ability of *Pediococcus acidilactici* to increase significantly the length of microvilli of proximal intestine of rainbow trout and Rodríguez Estrada et al. [48] reported that the administration of *Enterococcus faecalis* reduces the number of lipid vacuoles of enterocytes, increasing their functionality.

**Figure 3:** Structure of stomach (*), liver (#) and esophagus (+) of control group, without the addition of microorganisms, (A) and larvae administered with suspension BAC in dose 7 and stages E (B) and E&L (C). Bar indicates 100 µm. Magnification = 40X.

**Figure 4:** Structure of stomach (*), liver (#), intestine (X), pylorus (-) and esophagus (+) of control group, without the addition of microorganisms, (A) and animals treated with BAC L7 (B, C y D). Bar indicates 300 µm. Magnification = 10X.

**Figure 5:** Structure of stomach (*) and esophagus (+) of control group, without the addition of microorganisms, (A) and animals administered with BAC suspension in dose 7 and stage L (B, C y D). Bar indicates 100 µm. Magnification = 40X.

**Conclusion**

The present work allows our research group to select the BAC suspension; containing $6 \times 10^7$ CFU L$^{-1}$ of *Bacillus* strains A252, A253 and A254, to be assayed in further assays. In addition, it supports a new research area to evaluate the methods by which this bacterial suspension, administered to larvae from day 5 to 15, induce an early development of the gastrointestinal tract.

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