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SHORT COMMUNICATION

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In vitro selection of autochthonous lactic acid bacterium from clownfish *Amphiprion ocellaris*

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Ornamental aquaculture has been growing around the world as an important economic activity (FAO, 2017). However, its increase in production has promoted the outbreak of diseases due to high stocking densities and inadequate handlings in captivity (Pavanelli, Eiras, & Takemoto, 2008). In this scenario, probiotics stand out as a sustainable alternative to antibiotics, which are commonly used for disease control. Probiotic is a live microorganism supplemented in the diet to promote benefits to the host (Sayes, Leyton, & Riquelme, 2018). In recent years, various reports have been published about the use of autochthonous probiotics in aquaculture, such as *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus acidophilus*, *Bacillus subtilis*, *Enterococcus faecium* and *Bacillus velezensis* (Jatobá et al., 2018; Nandi et al., 2017; Sayes et al., 2018).

The first step to determine the probiotic potential is through in vitro tests (Vieira et al., 2013). The ability to colonize the intestinal tract is the main characteristic of probiotic bacteria (Balcázar et al., 2006). For this reason, specific in vitro tests, such as resistance to pH, NaCl and bile salts, would aid in the selection for adequate probiotics (Vieira et al., 2013).

Nowadays, few papers have been published about the use of probiotics for clownfish (Wesseling et al., 2015), but there is no reports about the use of autochthonous bacteria as probiotics for *Amphiprion ocellaris*. Thus, the current study aimed to isolate and apply in vitro tests in autochthonous bacteria with probiotic potential to the clownfish *A. ocellaris*.

In the experiment, six fish were anesthetized (benzocaine solution 20 mg/L) (Pramod, Sajeevan, Ramachandran, Thampy, & Pai, 2010) and euthanized by medullar section according to animal ethics committee (CEUA Number 010218). Their intestinal tract was removed, macerated in sterile saline solution (NaCl 1.5%), serially diluted in a factor of 1:10 and inoculated on Petri dishes with De Man, Rogosa, and Sharpe agar (MRS agar) supplemented with NaCl 1.5% and aniline blue 1%. Each plate was incubated for 48 hr at 35°C to bacterial growth.

Colonies (coccus or bacillus) presenting gram-positive coloration, negative catalase and affinity for aniline blue were selected. To determine the growth kinetics, each selected colony was inoculated in MRS broth culture medium and incubated for 24 hr at 35°C, and growth evaluated each 2 hr aided by spectrophotometer at absorbance of 630 nm. At the same evaluation time, agar plates also inoculated and incubated to allow bacterial growth. The absorbance values were converted to colony-forming units (CFUs) through exponential regression, and the maximum growth rate and duplication time were determined (Ramirez et al., 2017).

For resistance tests, experiments were performed in a completely randomized design using different levels of NaCl (0, 1.5, 3.0 and 4.5%), pH (2.5, 5.0, 7.5 and 9.0) and salt bile (5% weight/volume) in triplicate. The reduction in percentage absorbance (630 nm) of colonies grown in MRS broth (35°C for 48 hr) was evaluated.

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Another test carried out was the antagonistic ability against pathogens such as *Aeromonas hydrophila* (CPQBA22808 DRM), *Aeromonas jandaei* (LAQUA), *Vibrio fluvialis* (LAQUA), *Vibrio parahaemolyticus* (LAQUA), *Shewanella putrefaciens* (LAQUA), *Citrobacter freundii* (LAQUA), *Staphylococcus aureus* (ATCC 29213), *Enterococcus duran* (ATCC19432) and *Escherichia coli* (D363).

For this test, each selected bacterium with probiotic potential was inoculated in MRS agar and incubated at 35°C for 48 hr. After bacterial growth, agar discs with a diameter of 0.8 cm were sectioned from these plates and allocated into other plates containing tryptone soya agar (TSA) inoculated with the pathogens. A positive control without probiotic bacteria containing an antibiotic (oxytetracycline 3 mg/L) was used for comparison. After incubation (35°C at 48 hr), the inhibition halo (mm) against pathogens was determined.

In vitro results were submitted to the Kruskal–Wallis and post hoc Dunn non-parametric test ($p < .05$). The best strain selected as a possible probiotic was identified by MALDI-TOF-MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) using the molecular weight of ribosomal proteins with laser shots at a wavelength of 260-337 nm. Scores ≥1.7 were used to identify the genus and species (Tekippe, Shuey, Winkler, Butler, & Burnham, 2013).

Eight lactic acid bacteria with different morphological aspects were isolated in MRS agar. Only five followed to in vitro tests due to gram-positive coloration, negative catalase activity and an affinity for blue aniline (ST01, ST02, ST03, ST04 and ST05). Two strains (ST01 and ST05) had the largest values ($p < .05$) of maximum growth rate (1.16 ± 0.03 and 1.16 ± 0.01a cell/hr) and total viable cells (14.11 ± 7.54 and 22.04 ± 6.12a/ml × 10^8 CFU). In the resistance test, the highest growth observed was for ST03 in the presence of bile salt (5% w/v) followed by ST05 and ST01. The ST05 presented the highest resistance ($p < .05$) to NaCl and pH alterations (Table 1), and the lowest resistance was observed to strains ST02 and ST04.

Regarding to antagonistic ability against pathogens, the ST05 showed the highest inhibition halo against *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Aeromonas hydrophila*, *Aeromonas Jandaei*, *Escherichia coli*, *Citrobacter freundii* and *Shewanella putrefaciens* (Table 2) being similar to the antibiotic. All other isolated strains (ST01, ST02, ST03 and ST04) showed similar or reduced performance when compared to the ST05. The bacterium with the best results for resistance tests and antagonistic ability was identified by MALDI-TOF-MS as *Lactobacillus plantarum* DSM 2360 (score of 2.01).

The lactic acid bacterium *Lactobacillus plantarum* has been used in recent years for aquaculture, reporting various benefits to the host (Jatobá et al., 2018; Van Doan, Doolgindachbaporn, & Suksri, 2016). Its ability to quickly colonize the intestinal tract is the main characteristic (Jatobá et al., 2018). This specific ability could be related to the growth rate, which is a fundamental factor for probiotic bacteria (Vine et al., 2004). However, despite of fast growth it has low resistance in bile salt. According to Balcázar et al. (2008), *Lactobacillus plantarum* isolated from rainbow trout *Oncorhynchus mykiss* showed low viability in bile salt. This reduction was also observed in the present study. Bile salt works as a natural emulsifier making soluble fats and some vitamins, thereby acting as a bactericidal, breaking down the cellular wall of microorganisms (Lambert, Bongers, de Vos, & Kleerebezem, 2008).

Resistance to pH is another important aspect from probiotic bacteria (Succi et al., 2005; Vieira et al., 2013). According to Son et al. (2017), *Lactobacillus plantarum* shows good growth in low pH levels. In the present study, pH resistance was observed to both acid pH (2.5) and alkaline pH (9.0) when compared to other isolated bacteria. This characteristic allows the probiotic bacteria be used in the diet of the host (Sayes et al., 2018).

Among the main water quality parameters, bacterial growth could be affected by different salinity levels (Vieira et al., 2013). For marine fish, due to the specific mechanism of osmoregulation, different salinity concentrations are observed in their intestinal tracts (Baldisserotto, Mancera Romero, & Kapoor, 2007; Whittamore, 2012). Nonetheless, some lactic acid bacteria used as probiotics show resistance to different salinity levels (Ricciardi, Parente, & Zotta, 2009; Vieira et al., 2013), for example *L. plantarum*. For these reasons, it shows resistance to factors such as stomach acidity, different salinity concentrations and bile enzyme activity that make colonization by a probiotic feasible. The bacteria colonizing the intestinal tract inhibit the pathogenic bacteria through the competition for nutrients and space (Jatobá et al., 2018; Sayes et al., 2018).

The ability to inhibit any pathogenic bacteria is the most important characteristic for a probiotic in aquaculture (Cornélio et al., 2013; Dias et al., 2018). In this scenario, lactic acid bacteria stand out due to the production of specific components with bactericidal potential (Balcázar et al., 2006). According to Vieira et al. (2013), *Lactobacillus plantarum* shows a good ability to inhibit the growth of pathogens such as *Aeromonas hydrophila*, *Escherichia coli*, *Enterococcus duran*, *Vibrio*

TABLE 1 Mean values of reduction resistance tests of different strains (in %)

Strain	Salt Bile	NaCl 0%	NaCl 3%	NaCl 4.5%	pH 2.5	pH 5.0	pH 9.0
ST01	21.07 ± 1.69abc	43.40 ± 4.37c	72.26 ± 1.33ab	66.72 ± 1.36a	65.15 ± 2.12ab	74.98 ± 2.08b	94.74 ± 2.09ab
ST02	15.74 ± 1.76bc	54.36 ± 2.52ab	55.06 ± 2.08c	49.92 ± 2.16ab	56.57 ± 7.04b	70.24 ± 3.79b	88.82 ± 8.32b
ST03	27.47 ± 3.82a	47.49 ± 0.97bc	60.54 ± 2.77abc	37.45 ± 2.30ab	41.36 ± 5.01c	92.56 ± 1.92a	91.41 ± 4.77ab
ST04	15.00 ± 2.04c	55.54 ± 2.06a	57.27 ± 1.12bc	29.97 ± 0.88b	39.30 ± 5.54c	47.68 ± 7.06c	94.74 ± 2.11ab
ST05	24.47 ± 1.73ab	52.24 ± 1.96abc	76.30 ± 2.91a	67.47 ± 2.03a	74.17 ± 5.36a	97.45 ± 1.95a	98.44 ± 1.02a
p-value	.000046	.000174	.000001	.000001	.000010	.000012	.003090

Note: Lowercase letters in the column mean statistical difference ($p < .05$).

TABLE 2 Mean values and standard deviation of inhibition halo against pathogens

	***V. fluvialis**	**V. parahem**	**A. hydrop**	**A. jandai**	**S. aureus**	**E. duran**	**E. coli**	**C. freund**	**S. putrefac**
Antibiotic	17.54 ± 0.05a	17.36 ± 0.15a	17.42 ± 0.13a	17.38 ± 0.16a	18.00 ± 0.25a	17.82 ± 0.45a	17.80 ± 0.64a	17.04 ± 0.09a	17.22 ± 0.18a
ST01	15.06 ± 0.44abc	15.02 ± 0.65ab	13.64 ± 0.59b	15.68 ± 0.28ab	14.88 ± 0.35c	11.40 ± 0.86c	15.68 ± 0.51b	15.60 ± 0.20abc	15.76 ± 0.17ab
ST02	13.6 ± 0.55c	14.56 ± 0.78ab	14.40 ± 0.89ab	14.40 ± 42b	15.58 ± 0.63c	12.06 ± 0.49c	14.76 ± 0.36bc	14.08 ± 0.23bc	14.12 ± 0.30b
ST03	16.08 ± 0.75abc	16.26 ± 0.71ab	16.04 ± 1.05ab	16.10 ± 1.24ab	17.28 ± 0.68ab	13.86 ± 0.63b	16.56 ± 0.97ab	15.62 ± 0.50abc	15.60 ± 0.51ab
ST04	12.12 ± 0.18c	11.82 ± 0.78b	12.32 ± 0.41b	13.14 ± 0.26b	14.68 ± 0.34c	11.06 ± 0.68c	13.42 ± 0.68c	13.96 ± 0.29c	13.86 ± 0.22b
ST05	16.50 ± 0.91ab	16.38 ± 1.56a	16.16 ± 0.97ab	16.62 ± 0.66ab	16.92 ± 0.80b	14.22 ± 0.72b	15.88 ± 0.82ab	16.12 ± 0.27ab	15.92 ± 0.27ab
p-value	.00034	.00040	.00010	.00022	.00007	.00001	.00020	.00010	.00011

Note: Lowercase letters in the column mean statistical difference ($p < .05$)

**Vibrio fluvialis, Vibrio parahaemolyticus, Aeromonas hydrophila, Aeromonas jandai, Staphylococcus aureus, Enterococcus duran, Escherichia coli, Citrobacter freundii and Shewanella putrefaciens.*

harveyi, *Vibrio anguillarum* and *V. alginolyticus*. For *Lactobacillus plantarum*, in this study, its inhibitory ability was mainly observed against *Vibrio parahaemolyticus*, showing the same results with the antibiotic oxytetracycline. This ability could be explained by the production of lactic acid, hydrogen peroxide and plantaricin (Hernandez, Cardell, & Zarate, 2005; Sugita, Ohta, Kuruma, & Sagesaka, 2007). According to Klaenhammer (1993), this specific component 'plantaricine' acts as powerful bactericide against gram-negative bacteria. Thus, *Lactobacillus plantarum* may be an efficient probiotic for aquaculture. This is the first report of the autochthonous probiotic for clownfish *Amphiprion ocellaris* recommending *L. plantarum* as a potential probiotic.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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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REFERENCES

Balcázar, J. L., De Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., & Muzquiz, J. L. (2006). The role of probiotics in aquaculture. *Veterinary Microbiology*, *114*(3–4), 173–186. https://doi.org/10.1016/j.vetmic.2006.01.009

Baldisserotto, B., Mancera Romero, J. M., & Kapoor, B. G. (2007). *Fish osmoregulation* (pp. 333–359). Boca Raton, FL: CRC Press, Taylor & Francis Group.

Bolívar Ramírez, N. C., Rodrigues, M. S., Guimarães, A. M., Guertler, C., Rosa, J. R., Seiffert, W. Q., … Vieira, F. D. N. (2017). Effect of dietary supplementation with butyrate and probiotic on the survival of Pacific white shrimp after challenge with Vibrio alginolyticus. *Revista Brasileira De Zootecnia*, *46*(6), 471–477. https://doi.org/10.1590/s1806-92902017000600001

Cornélio, F. H. G., Cargnin-Ferreira, E., Borba, M. R. D., Mouriño, J. L. P., Fernandes, V. A. G., & Fracalossi, D. M. (2013). Growth, digestibility and resistance to pathogen infection in Nile tilapia fed with probiotics. *Pesquisa Agropecuaria Brasileira*, *48*(8), 863–870.

Dias, J. A., Abe, H. A., Sousa, N. C., Couto, M. V., Cordeiro, C. A., Meneses, J. O., … Fujimoto, R. Y. (2018). Dietary supplementation with autochthonous *Bacillus cereus* improves growth performance and survival in tambaqui *Colossoma macropomum*. *Aquaculture Research*, *49*(9), 3063–3070.

FAO. (2017). *Food and Agriculture Organization of the United Nations*, *Aquaculture Newsletter* (pp. 38–39). Rome, Italy: FAO. Retrieved from http://www.fao.org/3/a-i7171e.pdf

Hernandez, D., Cardell, E., & Zarate, V. (2005). Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: Initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. *Journal of Applied Microbiology*, *99*(1), 77–84.

Jatobá, A., Pereira, M. O., Vieira, L. M., Bitencourt, M., Rodrigues, E., Fachini, F. A., & Moraes, A. V. (2018). Action time and feed frequency of *Lactobacillus plantarum* for Nile tilapia. *Arquivo Brasileiro De Medicina Veterinária E Zootecnia*, *70*(1), 327–332. https://doi.org/10.1590/1678-4162-9870

Lambert, J. M., Bongers, R. S., de Vos, W. M., & Kleerebezem, M. (2008). Functional analysis of four bile salt hydrolase and penicillin acylase family members in *Lactobacillus plantarum* WCFS1. *Applied and Environmental Microbiology*, *74*(15), 4719–4726.

Nandi, A., Dan, S. K., Banerjee, G., Ghosh, P., Ghosh, K., Ringø, E., & Ray, A. K. (2017). Probiotic potential of autochthonous bacteria isolated from the gastrointestinal tract of four freshwater teleosts. *Probiotics and Antimicrobial Proteins*, *9*(1), 12–21. https://doi.org/10.1007/s12602-016-9228-8

Pavanelli, G. C., Eiras, J. C., & Takemoto, R. M. (2008). *Doenças de Peixes: Profilaxia, diagnóstico e tratamento*. Maringá, Brazil: Eduem.

Pramod, P. K., Sajeevan, T. P., Ramachandran, A., Thampy, S., & Pai, S. S. (2010). Effects of two anesthetics on water quality during simulated transport of a tropical ornamental fish, the Indian tiger barb *Puntius filamentosus*. *North American Journal of Aquaculture*, *72*(4), 290–297.

Ricciardi, A., Parente, E., & Zotta, T. (2009). Modelling the growth of *Weissella cibaria* as a function of fermentation conditions. *Journal of Applied Microbiology*, *107*(5), 1528–1535.

Sayes, C., Leyton, Y., & Riquelme, C. (2018). Probiotic Bacteria as a Healthy Alternative for Fish Aquaculture. In S. Savic (Eds.), *Antibiotic use in animals*. Rijeka, Croatia: InTech.

Son, S. H., Jeon, H. L., Jeon, E. B., Lee, N. K., Park, Y. S., Kang, D. K., & Paik, H. D. (2017). Potential probiotic *Lactobacillus plantarum* Ln4 from kimchi: Evaluation of β-galactosidase and antioxidant activities. *LWT-Food Science and Technology*, *85*, 181–186. https://doi.org/10.1016/j.lwt.2017.07.018

Succi, M., Tremonte, P., Reale, A., Sorrentino, E., Grazia, L., Pacifico, S., & Coppola, R. (2005). Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from parmigiano reggiano cheese. *FEMS Microbiology Letters*, *244*(1), 129–137.

Sugita, H., Ohta, K., Kuruma, A., & Sagesaka, T. (2007). An antibacterial effect of *Lactococcus lactis* isolated from the intestinal tract of the Amur catfish, Silurus Asotus Linnaeus. *Aquaculture Research*, *38*(9), 1002–1004. https://doi.org/10.1111/j.1365-2109.2007.01765.x

Tekippe, E. M., Shuey, S., Winkler, D. W., Butler, M. A., & Burnham, C. A. D. (2013). Optimizing identification of clinically relevant gram-positive organisms using the Bruker Biotyper MALDI-TOF MS system. *Journal of Clinical Microbiology*, *51*, 1421–1427.

Van Doan, H., Doolgindachbaporn, S., & Suksri, A. (2016). Effect of *Lactobacillus plantarum* and Jerusalem artichoke (*Helianthus tuberosus*) on growth performance, immunity and disease resistance of Pangasius catfish (*Pangasius bocourti*, Sauvage 1880). *Aquaculture Nutrition*, *22*(2), 444–456.

Vieira, F. D. N., Jatobá, A., Mouriño, J. L. P., Vieira, E. A., Soares, M., Silva, B. C. D., ... Vinatea, L. A. (2013). In vitro selection of bacteria with potential for use as probiotics in marine shrimp culture. *Pesquisa Agropecuária Brasileira*, *48*(8), 998–1004. https://doi.org/10.1590/S0100-204X2013000800027

Vine, N. G., Leukes, W. D., Kaiser, H., Daya, S., Baxter, J., & Hecht, T. (2004). Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. *Journal of Fish Diseases*, *27*(6), 319–326.

Wesseling, W., Wittka, S., Kroll, S., Soltmann, C., Kegler, P., Kunzmann, A., ... Lohmeyer, M. (2015). Functionalised ceramic spawning tiles with probiotic Pseudoalteromonas biofilms designed for clownfish. *Aquaculture*, *446*, 57–66. https://doi.org/10.1016/j.aquaculture.2015.04.017

Whittamore, J. M. (2012). Osmoregulation and epithelial water transport: Lessons from the intestine of marine teleost fish. *Journal of Comparative Physiology B*, *182*(1), 1–39. https://doi.org/10.1007/s00360-011-0601-3

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