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Research article

Probiotics reduce anxiety-related behavior in zebrafish

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ABSTRACT

There is increasing evidence that gut microbiome could have effects on neurological processes and on behavior. In this study we used the novel tank test (NTT) to analyze zebrafish exploring behavior after four months' supplementation with probiotics with probed antioxidant and anti-inflammatory properties. Results showed that prolonged ingestion of *Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347 significantly alters the swimming pattern and mean swimming speed in the zebrafish model. After treatment, zebrafish strongly reduced their bottom-dwelling geotactic behavior when placed in a new tank, which could be correlated to a lower state of anxiety.

1. Introduction

The increasing use of probiotics has led broadened our knowledge of the relevant effects of the composition of gut microbiome and bacterial metabolites on neurological and behavioral processes through different regulatory routes (Foster et al., 2017, Sandhu et al. 2017). Most studies have focused on humans or other mammals. However, the use of fish model species has become more common in recent years due to the fact that they are easy to breed in in captivity and also because some of their neurological pathways are homologs to those of with that of mammalian species (Maximino et al., 2015; Oliveira, 2013).

Zebrafish (*Danio rerio*) are an example of a model species in which results from probiotic experiments can be used as a reference for human treatment as they have similar intestinal microbiota and colonization patterns (Gioacchini et al., 2014). The novel tank test (NTT) is one of the most widely-used experimental procedures designed to test anxiety-related behavior in zebrafish (Maximino et al., 2010). It is based on their natural preference for swimming close to the bottom when first placed in a novel tank. Differences in time the fish take to before they leave the bottom of the tank and start to explore other areas are generally considered to be a reflection of the anxiety they experience (Cachat et al., 2010; Egan et al., 2009; Speedie and Gerlai, 2008). Multiple research has analyzed the effect of different substances on the performance of the fish using this method, a positive correlation with anxiolytic drugs such as diazepam, buspirone (Bencan et al., 2009), and fluoxetine (Egan et al., 2009) being observed. Nevertheless, collateral effects in the behavioral response derived from the intake of probiotics have not been studied in depth, a reduced response to stress being found in some cases (Davis et al., 2016) and no direct correlation in others (Schneider et al., 2016). Therefore, the mechanism triggering this response remains unclear.

In the present study, we analyzed how exploring behavior is affected in zebrafish adults given a probiotic mixture (1:1) of *Lactobacilus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347 strains, which have already demonstrated their effect on improving spermatogenical performance in humans (Valcarce et al., 2017) and zebrafish (Valcarce et al., 2019).

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2. Materials and methods

2.1. Ethics statement

All protocols and procedures involving animals in the present experimental design were approved by the institutional Animal Care and Use Committee (authorization number PI-10-16) at the Marine Culture Plan *El Bocal* of the Spanish Institute of Oceanography in Santander (Spain). All animals were standard manipulated in accordance with European Union Council Guidelines (2010/63/EU), following Spanish regulations (RD/2013) for the use of laboratory animals.

2.2. Subjects and housing

Two groups of nine adult (8 months old) wild-type zebrafish (AB strain) raised at the Spanish Institute of Oceanography facilities in Santander (Spain) were used in this study. All fish were housed in 3 L tanks with constant water exchanged from a recirculation system equipped with mechanical, chemical and biological filters. The water was kept at a mean temperature of 26 °C, and the room on a 14/10 light/dark cycle.

In order to evaluate the effect of probiotic administration in fish behavior, different feeding regimes were supplied to each group for four months (December 2018–March 2019). A control group (CTRL) was fed twice daily on a commercial zebrafish diet (Zeigler, PA, USA) and the experimental group (PROBIO), in addition to the commercial diet, were provided with a daily probiotic dose of 10^9 CFU mixture of *Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347, as described in Valcarce et al., (2019).

One month prior to the behavioral test each fish was anesthetized in 110 mg/L buffered tricaine methane sulfonate (MS222) and individually tagged with visible implant elastomers (Northwest Marine Technology, WA, USA). One fish from CTRL died during the experimental period.

2.3. Novel tank test

A quadrangular glass aquarium (20 × 8 × 18 cm; length × width × depth) was filled to a total volume of 3.5 L and used as an evaluation arena. Each fish was placed individually at the bottom of the tank using a net and its swimming behavior video-recorded (1920 × 1080 px) for 6 min. Once the trial was over, the fish were transferred to a recovery tank, separately from the naïve fish in the different groups. The video files were analyzed with Noldus Ethovision® tracking software, generating a virtual grid dividing the tank at half depth in upper and lower areas. Time spent in each area (expressed as a percentage of total time), number of crosses between areas, mean speed, total distance moved and latency to first cross to upper area, were determined.

2.4. Data analysis

Swimming results were expressed as the mean ± s.e.m. of each group analyzed. Differences between groups were evaluated by t-Student test for impaired samples using Prism 8 statistical software (GraphPad Software, CA, USA). P-values < 0.05 were deemed as statistically significant.

3. Results and discussion

When a fish is placed in a new environment, its instincts induce protective diving behavior at the bottom of the water column, reducing the risk of being attacked by a potential predator closer to the surface (Bass and Gerlai, 2008). The novel tank test (NTT) is a commonly used test in zebrafish behavior experiments based on this response (Nathan et al., 2015; Stewart et al., 2011; Wong et al., 2010; Egan et al., 2009; Levin et al., 2007). The assay could be defined as an analog of the rodent open field paradigm. Thus, the NTT enables the study of the strength of the geotaxis escape diving instinct of a fish under specific experimental conditions since it has a conflict between “safe” diving behavior and “exploration” swimming behavior. Under normal controlled laboratory conditions, fish generally tend to spend more time at the bottom of the tank in the first minutes of monitoring, showing a higher rate of erratic movements and immobility events (Cachat et al., 2010). After the habituation period, the animals gradually explore the upper areas of the water column. Our results show that adult zebrafish fed on a mixture of antioxidant and anti-inflammatory (Valcarce et al., 2017) probiotic strains (Figure 1A) for four months, present a strongly modified behavior pattern when compared to control-fed fish. Using Ethovision software, we created two homogeneous virtual subareas (Figure 1B) with the purpose of analyzing the exploration pattern (the spatial and temporal dynamics of behavior). Our results showed statistically significant differences ($p = 0.0137$; $t = 2.790$) in preference for the swimming area (upper--lower) virtually generated in the novel tank test. Probiotic-fed animals showed an overall strong preference for the upper area when heat maps were analyzed (Figures 1C and 1D). On the other hand, standard-fed animals did not show this predilection for the surface. Indeed, the mean value for the percentage of time scored in the upper zone for the PROBIO group (80 ± 6.5%) compared to the mean value recorded for CTRL (40 ± 13.2%). In the control group, only 50% of studied fish showed a preference for the upper zone whereas 88.89% of the probiotic-fed animals preferred to be closer to the surface. In fact, 7 of 9 fish in the PROBIO group spent over 80% of the 6-minute novel tank test experiment in the upper subarea. We also evaluated speed, total distance swum by each animal (Figure 1E), number of transitions between subareas (Figure 1F) and latency to the first crossing (Figure 1G). The statistical analysis only recorded significant differences ($p = 0.0158$; $t = 2.719$) in mean speed (Figure 1E). In our experiment, CTRL showed a higher speed (6 ± 0.5 cm/s) when compared to the probiotic-fed fish siblings (4.2 ± 0.3 cm/s). Latency to enter the upper subarea in the PROBIO group was surprisingly low (none of the fish lasted more than 10 s until first exploration of the upper area) (Figure 1H). These data are of considerable interest because combined with clear spatial preference; they indicate a robust difference in the behavioral pattern between groups. Stress can modulate NTT pattern behavior in zebrafish. Some anxiolytic drugs usually prescribed for the treatment of anxiety in humans, such as buspirone and diazepam (Bencan et al., 2009) or chronic fluoxetine (Wong et al., 2010), tend to increase the time zebrafish spend at the top of the experimental device. These similarities with our results evidence that the probiotic mixture may have an effect on anxiety modulation in the model animal. Further studies could address the incorporation of anxiolytic control and a demonstration of strain colonization. Nevertheless, we consider that constant feeding during 4 months could ensure the continuous presence of bacteria and their metabolites in the intestines.

In summary, we have determined that prolonged ingestion (4 months) of *Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347 alters the swimming pattern in the zebrafish model. Our results show that adult zebrafish fed with this probiotic mixture of antioxidant and anti-inflammatory probiotic strains, approved for human consumption, strongly reduced the bottom-dwelling geotactic behavior of zebrafish when placed in a new tank. This primary behavioral response is correlated to a lower state of anxiety. Therefore, the ingestion of this mixture of probiotics could be considered as a beneficial treatment for anxiety episodes.

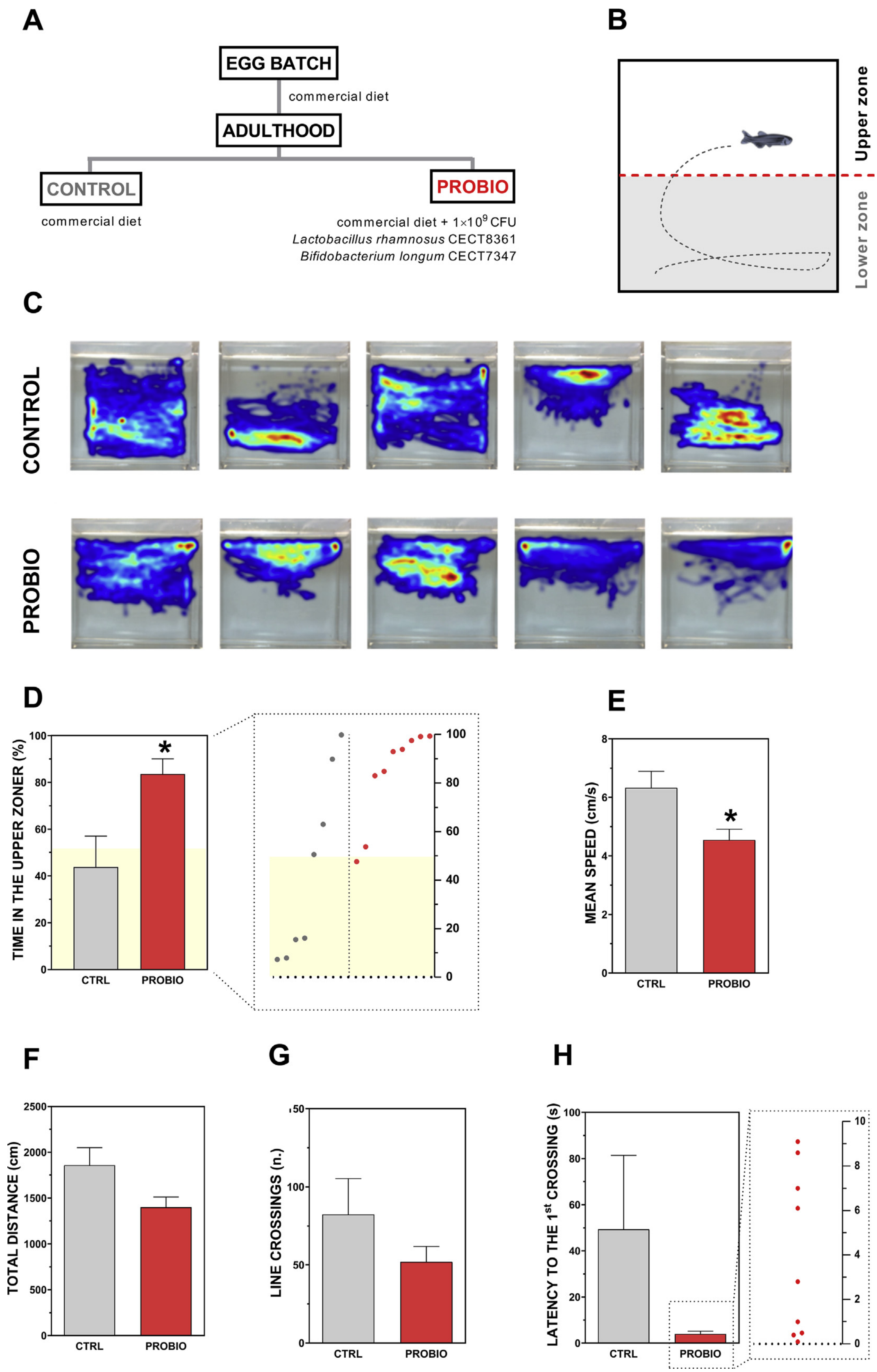

Figure 1. *Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347 ingestion modulate the behavior in zebrafish after 4 months. (**A**) Schematic representation of the experimental design. (**B**) Schematic representation of the novel tank test (NTT) and the established areas of analysis. (**C**) Heatmaps obtained after Ethovision analysis of the trajectories in both experimental groups (CONTROL and PROBIO). Column graphs and dot graphs showing: (**D**) Time spent by the animals in the upper zone (%); (**E**) Mean speed (cm/s); (**F**) Total distance (cm); (**G**) Line crossings (n.) and (**H**) Latency to the first crossing (s). Data are presented as means ± s.e.m. Asterisk shows statistically significant differences *($p < 0.050$).

Declarations

Author contribution statement

David G. Valcarce, Juan M. Martínez-Vázquez: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Marta F. Riesco: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Vanesa Robles: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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