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RESEARCH PAPER

Preliminary Safety Assessment of Potential Aquaculture Feed Additives *Lactiplantibacillus plantarum* BCCa32 and BCCa36 Using Zebrafish Larvae as a Model Organism

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Abstract

With ever-increasing food demand, aquaculture has witnessed a boom over the past century. As part of the measures to revolutionize the industry, probiotics have emerged as essential feed additives aiding in producing healthy livestock. Before their incorporation into fish feed, potential probiotic strains need to be tested for safety in a relevant model system; hence *Danio rerio* has been regarded as the model system of interest for testing aquaculture feed additives. In this study, the safety of two *Lactiplantibacillus plantarum* isolates, namely BCCa32 and BCCa36 were assessed as potential probiotics by using zebrafish larvae. The probiotic isolates did not show any mortality at lower cell densities; however, higher cell densities of BCCa36 increased larval mortality. Of interest, no gut colonization was noted under any condition. The results of this study serve as proof of the principle that zebrafish is a valuable and relevant model system for aquaculture feed and nutrition-related studies.

Introduction

The aquaculture industry is one of the fastest growing food industries with an annual 8.8% increase in fish production worldwide (FAO, 2012). With the decreased availability of fishmeal and reduced fish supply, continuous efforts have been made to diversify aquafeed ingredients (Ulloa et al., 2014). Probiotics have emerged as potential feed additives that aid in production, feed utilization, immunity, survival and nutrition (Ulloa et al., 2014; El-Saadony et al., 2021).

Probiotics can adhere and colonise the host gut, contributing to nutritional physiology, prevention and/or treatment of intestinal infection, anti-bacterial effect against antibiotic resistant bacteria and modulation of gut mucosal immunity (Hoseinifar et al., 2018). The effects of probiotics on the growth of fishes have been studied extensively (Ringø and Gatesoupe, 1998; Mohapatra et al., 2012; Tan et al., 2019). Probiotics specifically those from the genus *Lactobacillus, Lactococcus, Leuconostoc, Bacillus, Aeromonas, Vibrio* and *Shewanella* have demonstrated higher fish performance and growth by mitigation of stress (Nayak, 2010). Moreover, their use as a prophylactic and sustainable alternative to antibiotics in order to curb the antimicrobial resistance in aquaculture has also been suggested (Hoseinifar et al., 2018).

In recent years, the OMICs approach is widely exploited to screen potential probiotic candidates (Kiousi et al., 2021). In order to test and predict the

probiotic potential and safety of probiotics, several *in-vitro* models have been used. In particular, animal cell lines are the most commonly used model systems that simulate direct host-microbe cellular contact and adhesion. Although *in vitro* models give a fair idea of the microbial potential to colonize the human gut and toxicity, there is still much to be investigated using *in-vivo* models. In addition, it is necessary to comprehend the molecular pathways involved in determining the effects of these probiotic feeds on fish physiology. This is a long-term and expensive strategy. Therefore, to expedite the process, a cost-effective approach by using *Danio rerio* as a model for testing aquaculture feed and additives was suggested (Ulloa et al., 2014).

The use of zebrafish as a promising vertebrate model of aquaculture animal nutrition presents several advantages compared to other models because of their high fecundity, small size, fast development, the optical transparency of the embryos and early stages of larvae and genetic and structural similarities to mammals (Kimmel et al., 1995; Ulloa et al., 2014). Therefore, the aim of this study was to assess the safety of newly isolated indigenous probiotics *Lactiplantibacillus plantarum* BCCa32 and BCCa36 (Jobby et al., 2020) using zebrafish larvae as a model system.

Materials and methods:

Bacterial Strains and Growth Conditions

Indigenous probiotic strains *Lactiplantibacillus plantarum* strains BCCa32 and BCCa36 were previously isolated (Jobby et al., 2020) and maintained in 20% glycerol (1:1) at -80^{0}C. The bacteria were grown in 50 mL of Man de Rogosa and Sharpe (MRS) broth for 18h at 37^{0}C, under anaerobic conditions. The broth was centrifuged at 8000 rpm for 12 minutes and the supernatant was discarded. The pellet was then washed twice with sterile distilled water and adjusted to 1×10^7, 1×10^8 and 1×10^9 to colony forming units (CFU) / mL at an absorbance of 610 nm. These adjusted cells were used for further analysis.

Germ-free Embryo Generation

Adult zebrafish were maintained and cultured according to the protocol by Tungare et al. (2020). Briefly, adult fishes (2:1, male: female ratio) were maintained in a 250-litre glass aquarium at 27±1^{0}C with 14h light period. Spawning was triggered at dawn and a mesh (~5 mm diameter) was placed below to provide a preferable spawning condition of shallow region for the zebrafish. Zebrafish embryos were collected within 2h post-fertilization (hpf) and washed twice with sterile distilled water and maintained at pH 7.2-7.6 at 27±1^{0}C for further use.

Germ-free embryos were generated as mentioned by Pham and Yin (2019) with slight modifications. Briefly, embryos were washed with sterile distilled water to remove the faecal matter and any microbes attached to the outer surface of the chorion after which the embryos were washed with an antibiotic solution (200 µg/mL ampicillin and 15µg/mL kanamycin) followed by three washes using sterile distilled water. The embryos were then immersed in 0.02% polyvinylpyrolidone (PVP) solution for two minutes and washed immediately with sterile distilled water. The embryos were then immersed in 0.003% bleach solution for 30 minutes followed by a final wash with sterile distilled water. The germ-free embryos were maintained in E3 medium (0.005M NaCl, 0.00017M KCl, 0.0004M $CaCl_2$, 0.00016M $MgSO_4$) for further experimentation.

Zebrafish Larvae Acute Toxicity Assessment

In vivo toxicity was performed as per Organization for Economic Co-operation and Development (OECD) test guideline no. 236. The bacterial cell culture was prepared as mentioned above. To assess the bacterial toxicity, a zebrafish larvae acute toxicity assay was set up according to Xiong et al. (2022). In brief, 4 days post-fertilization (dpf) healthy larvae were added to 6-well plates (6 larvae per well) containing 2mL E3 medium. Thirty larvae were exposed to each test condition by immersion method (Dey and Kang, 2020) as mentioned in Table 1. The larvae were monitored for 48h at an interval of every 12h, using an inverted microscope (Radical Scientific Equipments, RTC-05) and the photographs were captured using Procam (Radical Scientific Equipments). The larvae were observed for mortality, heartbeat, coagulation and significant morphological malformation or changes in the tail, somites, eyes, and pigmentation (Tungare et al., 2022).

Gut colonization Assessment

Zebrafish larvae that survived the exposure at the highest dose were monitored up to 8dpf (i.e. 4 days of bacterial exposure) and processed as per Russo et al. (2015) with slight modifications. Briefly, after 4 days of exposure to BCCa32 and BCCa36, the surviving larvae were washed thrice with sterile distilled water followed by three washes with sterile saline (0.85% NaCl) in order to remove the bacteria loosely attached to the outer

Table 1. Exposure conditions

	Exposure Conditions		
Experimental set	LD Low Dose ($1x10^7$ CFU/mL)	MD Medium Dose ($1x10^8$ CFU/mL)	HD High Dose ($1x10^9$ CFU/mL)
L. plantarum BCCa32	BCCa32 LD	BCCa32 MD	BCCa32 HD
L. plantarum BCCa36	BCCa36 LD	BCCa36 MD	BCCa36 HD
Control	NA	NA	NA

skin of the larvae. For each experimental condition, 10 larvae were manually homogenized using a sterile mortar pestle in 1 mL of sterile saline. Finally, 100 µl of the recovered suspension was serially diluted and spread on MRS agar plates and incubated at 37^0C for 48h and the colony-forming units were noted.

Statistical Analysis

All experiments were conducted in triplicate using independent assays. Values are expressed as mean ± the standard deviation (*SD*). Statistical significance of data comparisons was calculated by ANOVA using GraphPad Prism Version 8.0. Values of $P<0.05$ were considered to be statistically significant.

Results

In vivo Acute Toxicity Assessment

The toxicity of two *Lactiplantibacillus plantarum* isolates was tested using zebrafish larvae. The developmental stages of the embryo are depicted in Figure 1. Figure 2 depicts the survival rate of probiotic-administered zebrafish larvae. No death upon exposure to 0 (i.e control), low dose (LD), medium dose (MD), high dose (HD) of BCCa32 was observed. Similarly, the larvae exposed to lower densities of BCCa36 also showed zilch mortality even after 48h post-exposure (Figure 3). In both cases, the probiotic strains were well tolerated at low and medium doses with no observed adverse

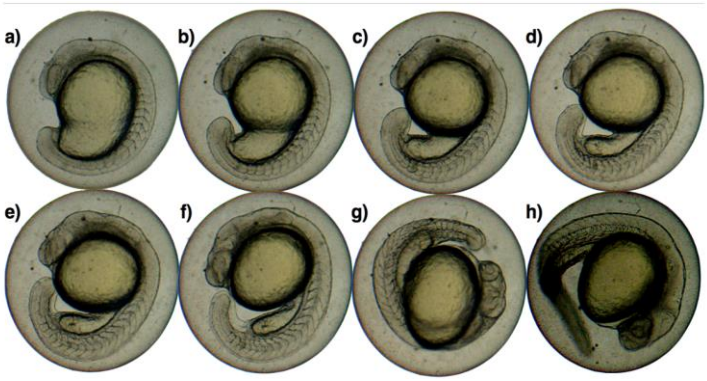

Figure 1. Developmental stages of the zebrafish embryo a,b,c,d,e,f,g,h represent development at 14,15,16,17,18,19,20,22 hours post fertilization captured using an inverted microscope. Magnification 40X.

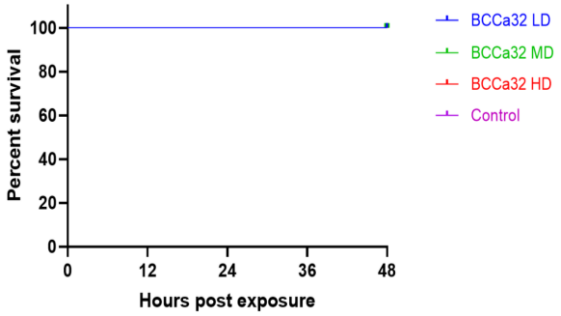

Figure 2. Kaplan-Meier survival curve comparing the percentage survival of zebrafish larvae exposed to BCCa32 at different doses

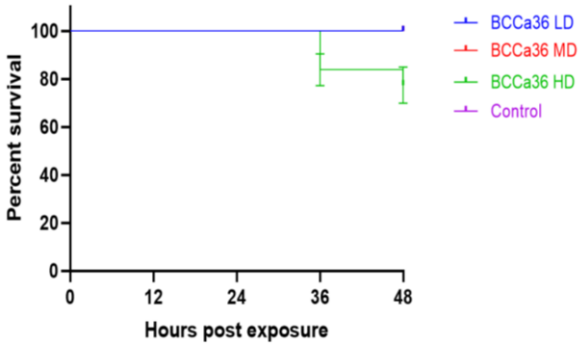

Figure 3. Kaplan-Meier survival curve comparing the percentage survival of zebrafish larvae exposed to BCCa36 at different concentrations.

effects. However, the larvae exposed to the higher dose of BCCa36 displayed 17% and 23% mortality after 36 and 48h post-exposure, respectively.

Effect of Probiotic Exposure on the Heartbeat

The toxic effects of probiotic exposure on zebrafish heartbeat were evaluated and the results at 48 hours post-exposure are depicted in Figure 4. The average heartbeat of zebrafish on exposure to BCCa32 and BCCa36 was found to be between 170-180 bpm at all three doses. There was no significant difference in the heartbeat of larvae exposed to probiotics in comparison to the control group. The highest heartbeat (176 bpm) was observed in the BCCa36 group at HD ($1x10^9$).

Effect of Probiotics on Larval Morphology

The effects on zebrafish larvae were recorded in terms of somites development, coagulation, development of eyes and pigmentation and malformation if any in the tail. There were no malformations in the control and BCCa32 exposed groups even after 48 hours post-exposure as seen in Figure 5. Mortalities were reported in the BCCa36 high dose group; the cause of death remains unclear, although the prominent morphological alterations included opacification, scoliosis and gradual disintegration of the body (Figure 5).

Gut Colonization

There was no bacterial growth observed on the MRS plates even after 48h of incubation at the requisite condition. This suggests that neither of the isolates was able to colonize the larval gut.

Discussion

Mammalian models are predominantly used in toxicity research. However, zebrafish has emerged as an advantageous model due to its higher human genome homology and structure similarity (Xiong et al., 2022). Zebrafish larvae are a robust and cost-effective system, and hence were selected for this study. Probiotics are known to produce bioactive compounds, nonetheless, their effects on eukaryotic systems are poorly understood (Imhoff et al., 2011). Therefore, an attempt was made to assess the preliminary toxicity of indigenous probiotics.

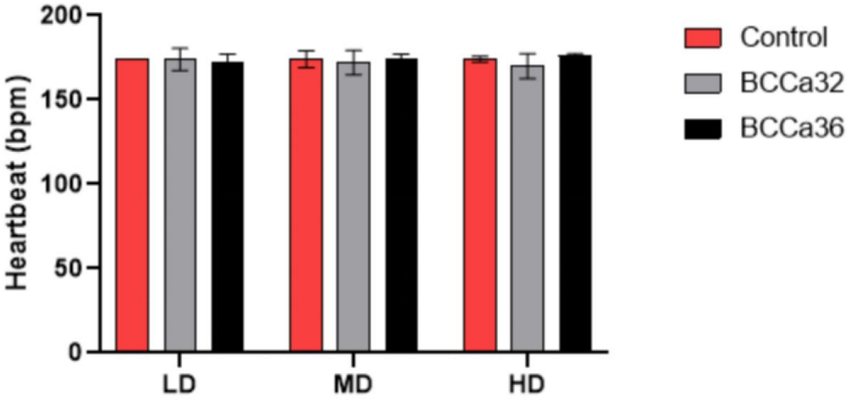

Figure 4. Effect of probiotic exposure on the heartbeat of zebrafish larvae at 48 hours post-exposure. Data expressed as mean ±SD. No significant difference was observed between and amongst groups.

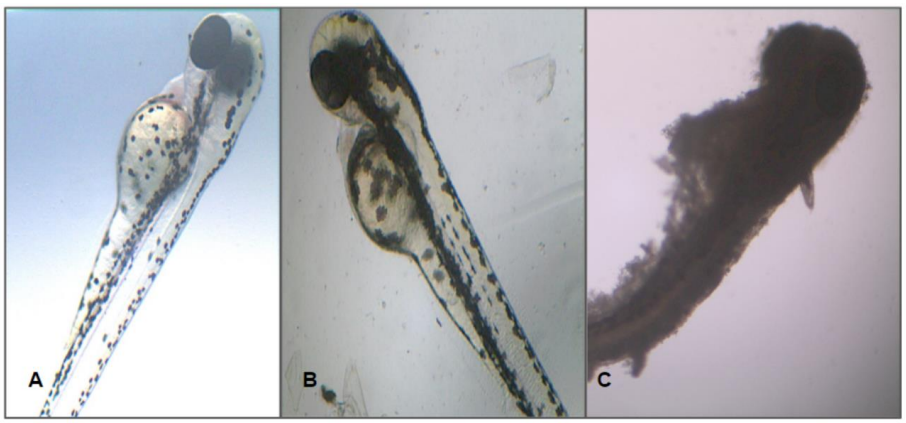

Figure 5. Representative pictograph depicting morphology of zebrafish larvae at 48h post-exposure A) Control; B) upon exposure to HD suspension of BCCa32, C) upon exposure to HD suspension of BCCa36.

Percent mortality in zebrafish larvae was reported by considering factors such as the cessation of the movement, crumpling or disruption of the larval body, the transparent larval body turning opaque, and caesurae of the heartbeat (de Luca et al., 2014). The survival rate results suggest that BCCa32 is safe for administration even at higher doses, while BCCa36 is safe at lower densities. Similar results were reported by Dey and Kang (2020) where they co-cultured *Weisella confusa* DD A7 with zebrafish larvae at a cell density of 1×10^8 CFU/mL for 12h and did not observe any significant changes in the mortality rate. Nonetheless, chronic toxicity studies need to be carried out to validate their safety. Furthermore, the higher dose of isolate BCCa36 resulted in lower survival rates of the zebrafish larvae. Strain-specific and dose-specific activities of probiotics have been reported earlier (Viega et al., 2020). Moreover, probiotic bacteria produce organic acids, which could have led to higher mortalities.

Heartbeat per minute is a critical parameter to assess cardiac function and to check for any signs of stress (Schwerte et al., 2006). The normal heartbeat of zebrafish larvae at 72 hpf is 120-180 beats per minute (bpm) (Russo et al., 2015) and is vital for its growth and development. Results from this study were in accordance with the literature, hence impressing no stress on cardiac functions.

It is speculated that the probiotics need to adhere to the host gut in order to impart the benefits (Monteagudo-mera et al., 2019), but it is not validated. Hence, the adhesion and colonization ability of the two strains were checked. No growth on the plates indicated no gut colonization, which could be attributed to the inability of the larvae to feed. As the larvae depend on the yolk sac until 8 dpf (Ulloa et al., 2014), no external feeding occurred. This suggests the need to expose higher developmental stages of the zebrafish to evaluate the colonization.

Conclusion

The current study was carried out to evaluate the possible use of zebrafish model systems to access probiotic safety. Toxicity assessment showed that both isolates did not show any mortality at lower cell densities. However, higher cell densities of BCCa36 increased larval mortality. A strain-specific difference was observed which recommends a thorough safety evaluation of bacteria for aquaculture use. These results act as proof of the principle that zebrafish can be used as a valuable model system for microbiome-related and nutritional studies. This was a preliminary assessment of toxicity using larvae; further studies will focus on the effect of probiotics on zebrafish, their effect on the gut microbiome, adhesion ability, immune system, host metabolism, and protection against pathogens. The molecular aspects of survival, persistence, and alteration of host gut microbiota by these probiotic strains will be elucidated. The toxicity of bioactive compounds and secondary metabolites will also be studied.

Ethical Statement

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The authors did not receive any funding for the work.

Author Contribution

Conceptualization, Supervision and Visualization: RJ, Data curation, formal analysis and investigation: AF and AKY, Resources and Project administration: SJ, Writing and editing: AF, AKY, KT, PJ.

Conflict of Interest

The authors declare no conflict of interest.

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