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Faculty of Science & Engineering

**MSc Sustainable Aquaculture Systems
Research Project**

**Effects of dietary probiotic (*Pediococcus acidilactici*) on breeding efficiency and reproduction
of adult zebrafish (*Danio rerio*)**

by

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1 **Effects of dietary probiotic (*Pediococcus acidilactici*) on breeding efficiency and**
2 **reproduction of adult zebrafish (*Danio rerio*)**

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12 **Abstract**

13 Probiotic administration influences many aspects in host organism such as growth per-
14 formance, immune responses and disease susceptibility. Present study demonstrates the
15 possible effects on breeding efficiency and reproduction. Adult zebrafish (*Danio rerio*)
16 specimens were supplemented with a probiotic (*P. acidilactici*) diet commercially
17 known as Bactocell for 4-weeks period. Each week ovulated eggs were collected and
18 documented in relationship with fertility and egg quality. Probiotic administration in-
19 creased number of eggs laid per spawning with a greater hatching rate. Eggs collected
20 on week 3 monitored from early developmental stages to post-embryonic stages (15
21 dpf) for survival and growth. Eggs laid on week 4 exposed to heat stress (34°C – 2h at
22 72 hpf) and stained with alcian blue (at 120 hpf). Alcian blue stained specimens were
23 examined for jaw and branchial arches development in addition to growth parameters
24 such as eye diameter and length. Probiotic treated species differentiated significantly
25 from the control group in terms of length, jaw and branchial arches developments.
26 Adult zebrafish specimens were examined for known oocyte development modulator
27 genes such as *Ihgcgr*, *cbr1l*, *paqr8*, *gdf9* and *bmp15*. The results of gene expressions
28 interpreted as a vindication of beneficial effects of probiotics as feed additive in repro-
29 ductive process.

30
31 **Keywords:** zebrafish, probiotic, bactocell, reproduction, development
32

DECLARATION

I hereby certify that this research project constitutes my own work, that where the language of others is set forth, quotation marks so indicate, and that appropriate credit is given where I have used the language, ideas, expressions or writings of another.

I declare that the research project describes original work that has not previously been presented for the award of any other degree of any institution.

Signed,

Muhammed Doğan

Özdemir

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LIST OF ABBREVIATIONS

175		
176		
177	CHMC	Distance between ceratohyal and meckel's cartilage
178	dpf	Days post fertilization
179	dph	Days post hatch
180	EFSA	European Food Safety Authority
181	EU	European Union
182	FAO	Food and Agriculture Organization of the United Nations
183	FC	Fold change
184	H₂O₂	Hydrogen peroxide
185	hpf	Hours post fertilization
186	hph	Hours post hatch
187	KOH	Potassium hydroxide
188	LCH	Length of ceratohyal
189	NH₄	Ammonium
190	NO₂	Nitrogen dioxide
191	NO₃	Nitrate
192	p	Value of significance, probability
193	SD	Standard deviation
194	SE	Standard error
195	WHO	World Health Organization
196	UN	United Nations

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1. Introduction

The scope of this research project is to examine the relationship between a dietary probiotic additive known and used in aquaculture Bactocell (*Pediococcus acidilactici*), and the fish, using zebrafish (*Danio rerio*) as a model to some extend of feeding, fish breeding, reproduction and gene expression at adult stages and survival, growth, cartilage development and response to heat shock at early life stages.

1.1 The state of aquaculture

Fishery production in 2014 was stated as a total of 167.2 million tons consisting of 93.4 million tons of capture fisheries (55.9%) and US\$160.2 billion worth 73.8 million tons of culture fisheries (44.1%, up from 42.1% in 2012 and 31.1% in 2004) (FAO, 2016). In the last 20 years total capture fisheries have fluctuated between 80 and 100 million tons and is projected to stay below 100 million tons in 2025. Given the stagnated status of wild capture fisheries in addition to the increasing demand for animal protein and the technological developments, aquaculture production for human consumption is expected to continue to grow and is projected to surpass 100 million tons by 2025 (FAO, 2016).

The current world population of more than 7.4 billion is predicted to reach 8.5 billion by 2030 and 9.7 billion by 2050 (UN, 2017). In 2013, about 17% of all animal protein and 6.7% of all protein required by the world population is provided by fish species. In 2014, the global finfish aquaculture production (362 different finfish species including hybrids) reached approximately 70% (~50 million tons of finfish with an estimated value of US\$99.2 billion) of all aquaculture production (FAO, 2016). With the increasing demand and the developments in aquaculture systems, cultured seafood products are promising for a further expansion.

1.2 Probiotics and Aquaculture

Probiotics are described by FAO and WHO in 2001 as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001) and are “able to persist in the digestive tract because of its tolerance to acid and bile salts” (Irianto & Austin, 2002). Therefore, probiotics are usually applied as feed additives (if not applied directly to the water) in aquaculture

with this method live probiotics are introduced to the host organism usually fish to form colonies and benefited from. Numerous studies have documented different types of benefits that probiotic colonies can provide to the host such as influence on growth performance (using *Streptomyces* strains and *Lactobacillus casei*) (Dharmaraj & Dhevendaran, 2010; Hernandez et al., 2010), pathogen inhibition (*Lactobacillus acidophilus*, *Lactococcus lactis*) (Al-Dohail et al., 2011; Sun et al., 2012), nutrient digestibility (*Lactobacillus acidophilus*, *Shewanella putrefaciens*) (Al-Dohail et al., 2009; Tapia-Paniagua et al., 2012), water quality (*Bacillus coagulans*) (Zhou et al., 2009), stress tolerance (*Pediococcus acidilactici*, a member of a Alteromonadaceae family strain Pdp11) (Castex et al., 2009; Varela et al., 2010) and reproduction improvement (a commercial probiotic Primalac containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium* and *Bifidobacterium thermophilum*, *Lactobacillus rhamnosus*) (Abasali & Mohamad, 2010; Gioacchini et al., 2010). A large amount of studies concerning such beneficial microorganisms is often aims to ascertain their ability to prevent or control disease, promote growth and increase stress tolerance while only a few evaluated the topic reproduction.

1.2.1 The use of *Pediococcus acidilactici*

The strains of *P. acidilactici* commercially known as Bactocell (the first probiotic authorized for use in aquaculture industry in EU) is commonly used as a feed supplement not only to fish but also to chickens and pigs as a fattening agent (EFSA, 2012). The efficiency of Bactocell is assessed in numerous studies and field trials on shrimp (*Litopenaeus stylirostris*) (Castex et al., 2008; Castex et al., 2009), rainbow trout (*Oncorhynchus mykiss*) (Merrifield et al., 2011), pollock (*Pollachius pollachius*) (Gatesoupe, 2002) and many other fish species. Such trials have demonstrated the ability of Bactocell to promote growth, increase survival and often stated effective against disease susceptibility.

In one study, Lamari and his co-workers have examined the ossification in sea bass (*Dicentrarchus labrax*) larvae fed with *Pediococcus acidilactici* MA18/5M since 5 dph (days post hatch) and have documented 60% ossification in probiotic fed group at 62 dph in comparison to the control fed group whereas the ossification was only 13% suggesting the beneficial effects of *P. acidilactici* is not only limited to growth but also can be effective in bone development (Lamari et al., 2013).

In another study conducted in a commercial sea bass hatchery in Greece with Dias Aquaculture the scientists have examined approximately 400,000 fish for 33 days starting from 64 dph in 6 tanks 3 replicates of with and without Bactocell fed specimens. The trial results showed no significant differences between the probiotic fed treatment group and the control group in terms of mortality and/or growth. Nonetheless, statistically significant differences are observed in terms of deformities (17.1%) and lordosis (4.13%) in comparison to control group (22.3% and 6.53%, respectively) (EFSA, 2012).

1.3 Zebrafish: A model fish species

Zebrafish is a small cyprinid teleost species lives in freshwater originating from South Asia (Briggs, 2002; Lawrence, 2007). Surveys conducted in India and Bangladesh elucidate the choice of habitat of a zebrafish as calm waters, somewhat alkaline ($\text{pH} \approx 8$), clear and comparably warm waters ($16.5^{\circ}\text{C} - 34^{\circ}\text{C}$) (McClure et al., 2006; Spence et al., 2006). Suggested temperature for culturing zebrafish is 28.5°C (Westerfield, 2000) and supported by other studies while acquiring relatively high growth rates (Schaefer and Ryan, 2006). Zebrafish have asynchronous ovaries and are batch spawners. When physical (such as maturation, healthy reproductive organs etc.) and chemical (such as water quality, temperature, pH etc.) conditions are optimal a continuous spawning occurs daily and the spawning is highly dependent on photoperiod which usually but not necessarily occurs a few hours before light (Selman et al., 1993; Spence and Smith, 2005). Zebrafish larvae under favourable conditions hatches after 2-3 days of fertilization and becomes sexually mature in three to four months (Figure 1.) (Kimmel et al., 1995; Westerfield, 2000; D'Costa and Shepherd, 2009).

Zebrafish has been used as a vertebrate model fish species due to a few characteristics such as its small size, ease of housing, husbandry and care and rapid development rate (Kimmel et. al., 1995; Briggs, 2002), short re-spawning time (usually on daily basis) and large amount of eggs at each time (Kimmel et. al., 1995; Briggs, 2002), ease of examination at early life stages due to external fertilization and transparent eggs (Briggs, 2002; Seth et. al., 2013), fully sequenced genomes (Woods et al., 2000) and its comparability and relationship to other fishes and humans (Howe et al., 2013). Innumerable amount of trials has conducted using

Zebrafish in different fields such as genetics and development (Fishman, 2001), human diseases (Penberthy et al., 2002), environmental studies (Scholz et al., 2008), pharmacological studies (Langheinrich, 2003), toxicology (McGrath & Li, 2008), the screening of therapeutic drugs (Sumanas and Lin, 2004).

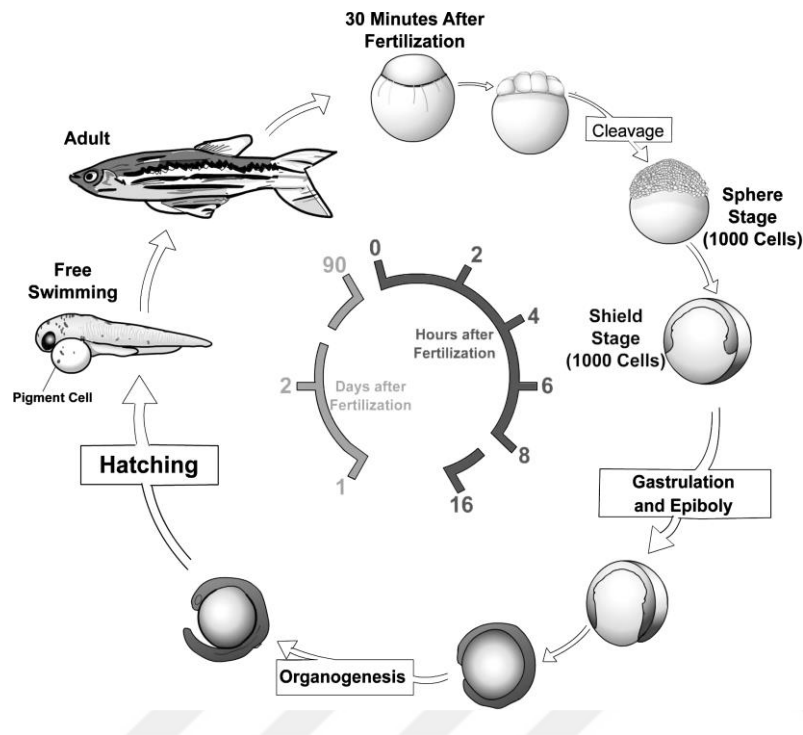


Figure 1. Lifecycle of a zebrafish (D'Costa and Shepherd, 2009).

The reproductive system of zebrafish is well documented, and it's modulated through hypothalamus–pituitary–gonadal axis (HPG) (Peter & Yu, 1997). HPG governs the process of reproduction by the transcription and release of *gnrh* mRNA (which is very sensitive to energy availability and regulates oogenesis by secreting necessary hormones) (Hill et al., 2008). Those hormones; follicle stimulating hormone (FSH) and luteinizing hormone (LH) are secreted by the pituitary gland (Patiño et al., 2001). FSH and LH controls the two stages; oocyte growth and maturation. FSH controls the production of a phospholipoglycoprotein vitellogenin (vtg, also known as yolk protein) by stimulating the estradiol production, and vtg modulates the growth stage of oocyte development (Clelland & Peng, 2009). LH triggers the oocyte maturation stage by binding to *lhcg*r. And *cbr1l* plays a key role in converting 17-alpha hydroxy progesterone to maturation-inducing hormone (MIH). MIH is activated by *paqr7b* and *paqr8* genes (Patiño et al., 2001).

Maturation stage is not only under the influence of LH and FSH, but also autocrine and paracrine factors such as *bmp15* and *gdf9* genes (Clelland & Peng, 2009).

Thus, zebrafish was chosen as a test organism in this trial due to before mentioned characteristics especially its reproduction wise capabilities but also its cost and time efficient housing requirements. And to confirm the possible findings of the study in a genomic level a few key genes (*lhcg*, *cbr1l*, *paqr8*, *bmp15* and *gdf9*) expressions were analysed due to their role in the reproductive performance of the zebrafish.

1.4 Aims of the study

The main aim of this study is to assess beneficial effects of the dietary probiotic (*P. acidilactici*) on breeding efficiency and reproduction of adult zebrafish. With the aim of doing;

- Evaluating the potential probiotic administration effects on factors playing a crucial role in reproductive productivity and fitness of adult zebrafish.
- Assessing the potential effects on the offspring of a probiotic supplemented zebrafish regarding growth and survival from early stages to post embryonic stages.
- Examining the potential effects on the offspring of a probiotic supplemented zebrafish when exposed to heat treatment.
- Investigating the potential effects on larval and cartilage development of a probiotic supplemented zebrafish offspring.
- Assessing the potential probiotic supplementation effects on genes playing a key role in reproductive biology of adult female zebrafish in terms of oocyte maturation and development.

Concomitantly, the broad objective of this study is aiming to provide a better knowledge and understanding in probiotic use such as *P. acidilactici* as a feed additive on broodstock such as female zebrafish. This way providing applicable knowledge of a known example of a teleost fish and her reproductive system which have similarities to many other globally cultured fishes is in benefit of the many

345 stakeholders in aquaculture industry (feed producers, farm owners, researchers
346 etc.).
347



2. General material and methodology

2.1 Experimental design

This experiment was designed in 3 stages (Figure 2.);

- 1- Broodstock (feeding) stage: adult zebrafish was supplemented with a probiotic diet for 4 weeks; ovulated eggs were sampled each week (reproductive fitness analysis) and ovaries were sampled at the end of this stage of the trial (gene expression).
- 2- Observation (growth and survival) stage: zebrafish larvae were monitored until day 15 pf.
- 3- Heat treatment (cartilage development) stage: zebrafish larvae were treated with heat shock (34°C) for 2 h at 3 dpf and sampled at 5 dpf analyzed for differences in growth and cartilage development.

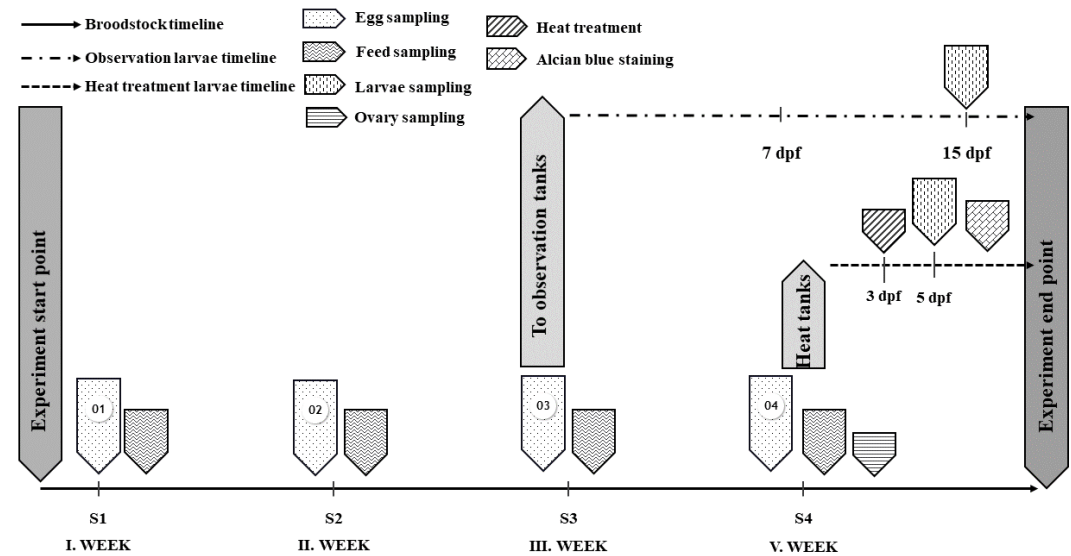


Figure 2. A demonstration of experimental setup.

2.2 Rearing Facilities

Housing have taken place at Plymouth University Zebrafish Rearing Facility, Plymouth, UK. All fish acquired from Plymouth University Zebrafish Facility with no history of probiotic diet. Adult zebrafish female and males (a total of 96

fish 1:1 sex ratio, 2 years old, weight: 0.64 ± 0.14 g length: 3.72 ± 0.23 cm) (Table 1.) have kept in a total of 6 rectangular 25L glass tanks at 12:12 light: dark photoperiod with recirculating water (with appr. 10% daily water change) and used as breeders. The trial lasted for 4 weeks and during this period water conditions for pH (7.83 ± 0.42), dissolved oxygen (7.66 ± 0.16 mg L⁻¹) and water temperature (27.21 ± 0.69 °C) were checked on daily basis along with room temperature (min 25.33 ± 0.93 °C, max 25.93 ± 0.80 °C) and water quality parameters for ammonium (NH₄⁺ 0.031 ± 0.005 ppm), nitrogen dioxide (NO₂ 0.023 ± 0.015 ppm) and nitrate (NO₃ 20.88 ± 7.31 mg L⁻¹) were checked on weekly basis. The trial setup was planned in triplicates with 3 tanks of control group fed with basal diet and 3 tanks of treatment group fed with probiotic diet.

2.3 Diet formulation and making

All diet used during the experiment was produced in Plymouth University Food and Nutrition Lab in Plymouth, UK. Two types of diets were formulated containing 38% crude protein, 8% fat, 3.65% ash, 0.55% crude fiber and 7.52% moisture. Control group was fed with a basal diet (9% SPC62, 32.5% Corn starch, 20% HiPro Soy, 6.80% sunflower oil, 13% herring meal, 13% wheat gluten, 2% premix beef, 2% gelatine, 0.6% DL methanine, 0.6% Lysine HCL and 0.5% CMC) and the treatment group was fed with a probiotic diet (25% Bactocell at the expense of Cornstarch was added to the basal diet ingredients to form the probiotic diet) (Table 2). Ingredients were carefully weighed and mixed using a Kitchenaid mixer. Mixed ingredients shaped as long sticks using P.T.M. P6 pasta extruder. Extruded feed sticks have left drying for 24h at 37°C using Memmert Universal Oven. Dried feed sticks were then grinded using a coffee grinder and to achieve the desirable size pellets the feed sieved into 4 different size of 0.2 mm, 0.5 mm, 0.85 mm and 1 mm but during the experiment only the 0.85 mm pellets were used.

Table 1. Weight and length measurements of adult zebrafish specimens.

Category	Group	Factor	Mean	Standard deviation (SD)	Standard error (SE)
Female	Control	Wet weight	0.70 g	0.14	0.03
Male	Control	Wet weight	0.52 g	0.10	0.02
Both	Control	Wet weight	0.63 g	0.15	0.02

Female	Treatment	Wet weight	0.69 g	0.14	0.03
Male	Treatment	Wet weight	0.59 g	0.09	0.02
Both	Treatment	Wet weight	0.65 g	0.13	0.02
Both	Both	Wet weight	0.64 g	0.14	0.01
Female	Control	Length	3.74 cm	0.22	0.04
Male	Control	Length	3.62 cm	0.22	0.05
Both	Control	Length	3.69 cm	0.22	0.03
Female	Treatment	Length	3.77 cm	0.27	0.05
Male	Treatment	Length	3.69 cm	0.18	0.04
Both	Treatment	Length	3.74 cm	0.24	0.04
Both	Both	Length	3.72 cm	0.23	0.02

395

396 Table 2. Feed formulations for control and treatment groups.

Components	Control Diet	Treatment Diet
Chemical composition		
Crude protein (%)	38	38
Fat (%)	8	8
Ash (%)	3.65	3.65
Crude fiber (%)	0.55	0.55
Moisture (%)	7.52	7.52
Ingredients		
SPC 62 (%)	9	9
Corn starch (%)	32.5	7.5
HiPro soy (%)	20	20
Sunflower oil (%)	6.8	6.8
Herring meal (%)	13	13
Wheat gluten (%)	13	13
Premix beef (%)	2	2
Gelatin (%)	2	2
DL Methanine (%)	0.6	0.6
Lysine HCL (%)	0.6	0.6
CMC (%)	0.5	0.5
Bactocell (%)	0	25

397

398 2.4 Feeding and sampling

399 Adult zebrafish specimens were fed twice daily, once in the morning an hour
400 after the first light at 9 a.m. and once 6 hours after that at 3 p.m. Feed intake for all
401 adult fish tanks weighed and monitored daily (control group: 0.196 ± 0.067 g/day
402 and the treatment group: 0.220 ± 0.064 g/day). Feed sampled every week during the

experiment and microbiological analyses carried out to determine bacterial count and probiotic viability. Zm-000 (Zebrafish Management Ltd; Winchester, United Kingdom) was used for larval feeding few times a day after day 5pf. No feed was given to the samples collected on 4th week and sampled at 5dpf.

Larvae sampling occurred once in each week starting on at the start of the 2nd week till the end of the trial, 4 times in total (Figure 2). For egg collection purposes tanks are netted night before to separate fish and the eggs from each other. The first two batches were only counted under a stereomicroscope to determine fertilization rate and egg count. The third batch of eggs were counted and 200 fertilized larvae from each tank (total of 1200 sample in 6 tanks of 5L) kept alive for 15 days and monitored for hatching rate, survival and growth (Figure 2). The last batch of eggs were collected and counted on week 4. Hundred specimens of each tank kept alive and divided into two groups of 6 tanks (a total of 12 tanks of 3L, 50 larvae per tank forming sub-groups for heat shock) control (28°C and 34°C – 2h) and treatment group (28°C and 34°C – 2h) for 5 days and challenged with heat shock at 34°C for 2h at day 3pf and moved back to rearing facility (Figure 2). Euthanized specimens were kept in 10% neutral buffered formalin for further examinations.

At the end of the feeding part of the trial all adult female zebrafish specimens were euthanized and dissected using a dissection microscope and a dissection kit to sample ovaries. All sampled ovaries were stored in RNAlater for overnight at 4°C and moved to -80°C freezer day after for further investigations.

An overdose of MS-222 (tricaine methane sulfonate 300 mg L⁻¹) was used to euthanize adult fish and larvae.

2.5 Sample Analyses

2.5.1 Feed sample analysis

Feed sample analysis were performed once in every week during the trial period. Bacterial cultivation and counts were conducted by preparing MRS agar plates. Sample preparation and enumeration were carried out by following the procedures described elsewhere (Vanderzant & Splittstoesser, 1995).

2.5.2 Breeding stock and egg sample analysis

All specimens from zebrafish breeding stock were euthanized using MS-222 for sampling purposes. During the experiment one female and one male zebrafish mortality recorded due to jumping off the tank. Two female zebrafish specimens from the broodstock disregarded due to bad health conditions detected after dissection. Euthanized specimens were measured for length and weight and ovaries sampled for further analysis (gene expression).

Eggs collected each week were enumerated under a stereomicroscope during the trial. A total number of 12,481 ovulated eggs were counted during the experiment. Egg samples of the 2nd batch (a total number of 117 sample) were also measured for width under a stereomicroscope with an attached colour camera using computer software imageJ (Figure 3.).

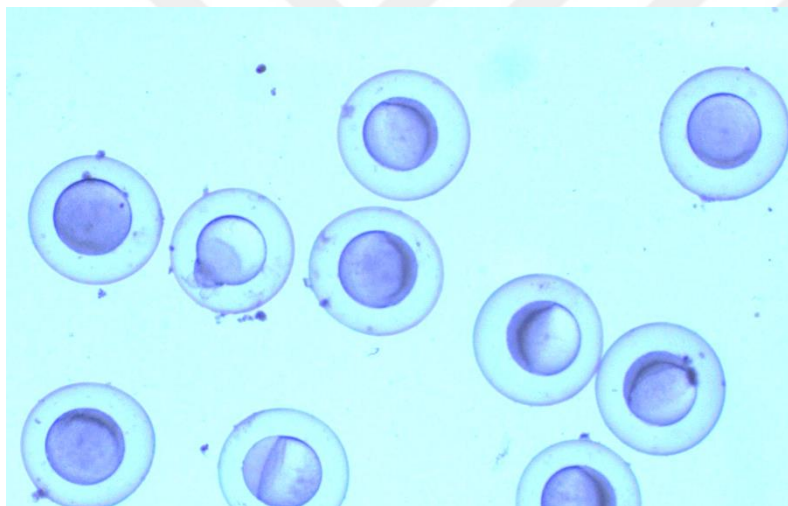


Figure 3. Representative image of zebrafish eggs under stereomicroscope (appr. 7 hpf).

2.5.3 Survival and Growth analysis

Embryos collected and counted after breeding at week 3 and kept alive for 15 days (600 specimens from each group control and treatment with 3 replicates). Survival was monitored daily. At day 15 all larvae were euthanized with a lethal dose of MS-222 and examined for total length (146 sample is analysed) using a stereomicroscope with a camera attached (Figure 4). All measurements were taken using ImageJ computer software.

2.5.4 Heat shock and cartilage development analysis

Embryos collected at week 4 were divided into two groups forming duplicates of treatment and control groups (a total of 6 replicates of each group), 3 replicates of each group exposed to heat stress (34°C) for 2 hours at 72 hpf; and moved back to rearing facility (28°C), the other 3 replicates kept at 28°C during the trial (Figure 2). All 6 replicates (600 larvae) were maintained in system water until 5dpf after Long et al., 2012. Health and mortality monitored daily along with a daily 20-25% water exchange. Larvae's from both groups were collected and stained using alcian blue staining following a protocol described earlier (Schilling et al., 1996; Helland, 2009). Euthanized larvae were fixed using 4% neutral buffered formalin overnight. Twenty mg of alcian blue dissolved in 70 ml absolute ethanol and 30 ml acetic acid and filtered. Samples were moved in to the staining solution as whole and agitated at room temperature for 2h, to prevent overstaining the process was monitored every 30 minutes through a stereoscope. To prevent calcium loss, samples were moved into a saturated sodium borate solution and agitated at room temperature for 4 hours. A bleach solution of 1 ml of 3% hydrogen peroxide (H₂O₂) and 1 ml of 2% potassium hydroxide (KOH) were added after having sodium borate removed and left at room temperature with lids open for 30 minutes, bleaching ended immediately after bubble formation was observed. Samples were cleared using a solution of 1 ml of 50% glycerol and 0.25% KOH and agitated at room temperature for 2 hours. Specimens were viewed under the same solution and measured using stereomicroscope attached with a camera using computer software ImageJ measurements for total length (462 sample), eye diameter (195 sample), distance between ceratohyal and meckel's cartilage (266 sample) and ceratohyal length (214 sample) (Figure 4). Such parameters were used before to examine the cartilage development of zebrafish (Mukhi & Patiño, 2007; Long et al., 2012).

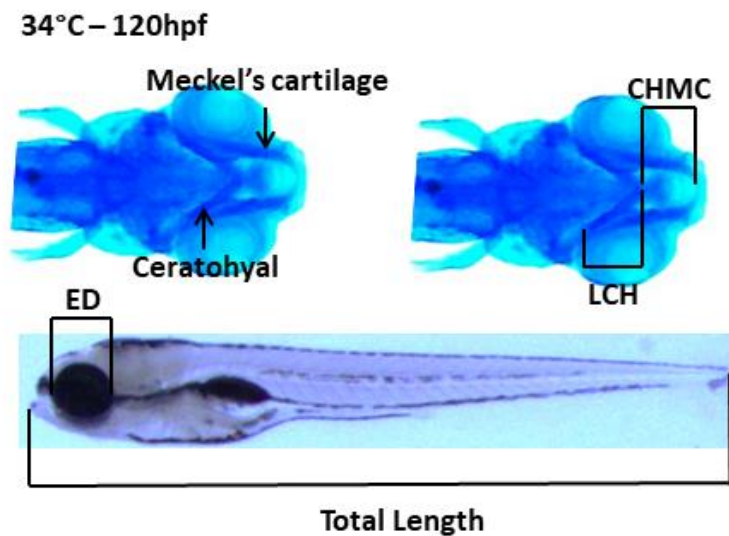


Figure 4. Representative images of zebrafish larvae at 120 hpf. Zebrafish larvae were treated with 34°C heat shock for 2 hours at 72hpf and images taken under a stereomicroscope with a camera attached. CHMC: Distance between Ceratohyal and Meckel's cartilage. LCH: Length of Ceratohyal. ED: Eye diameter.

2.5.5 Gene expression analysis

RNA extraction from adult zebrafish tissue was completed following the standard operating protocol (SOP) guidelines. Tissue removed from RNAlater homogenized with 1 ml of TRI Reagent (Sigma Aldrich T9424) after complete dissociation of nucleoprotein complexes 200 μ l of Chloroform (Sigma Aldrich C2432) was added and centrifuged for 15 minutes at 12,000g forming three phases; the aqueous phase (RNA), the interface (DNA) and the organic phase (lipids, proteins etc.). The aqueous phase was then transferred into a new tube and added 500 μ l of 2-propanol (isopropanol) (Sigma Aldrich I9516) and centrifuged for 15 minutes at 14,000g and the RNA was precipitated forming a pellet and the pellet was washed using 70% ethanol and left for 10 minutes for evaporation. 40 μ l of nuclease free water added to the washed pellet and stored at -80°C for further analysis.

RNA quantity/quality measurements were carried out using nanodrop. Before using nanodrop 1 μ l of Nuclease free water was used to clean and calibrate. Each sample was analysed, and readings were noted for evaluation purposes.

Genomic DNA elimination and Reverse-transcription reaction processes were carried out using QuantiTect® Reverse Transcription Kit, and followed the

protocol stated in QuantiTect Reverse Transcription Handbook. Samples were stored at -20°C as suggested in the handbook for further real-time PCR analysis.

Real-time PCR analysis were conducted with the SYBR green method in an iQ5 iCycler thermal cycleron. Ovary samples from adult zebrafish were targeted oocyte maturation phase inducer genes; *paqr8*, *cbr11* and *lhcg*, and preventer genes; *gdf9* and *bmp15* normalized against *gapdh* and β -actin genes after Gioacchini et al., 2010. Primers were designed using Primer3 (version 4.1.0) (Table 3). Calculations were carried out following the method known as Pfaffl method (Pfaffl, 2001).

Table 3. List of forward and reverse primers for real-time PCR analysis.

Genes	Forward Primer	Reverse Primer
Gapdh	GCGGCAAACTGGTCATTGAT	ATGTTGTGGAGTCTACTGGTGT
β -actin	ATCCACGAGACCACCTTCAA	ATGTACCCTGGCATTGCTGA
paqr8	AGCCACAGTACACGCTTCT	TGTAGCCTCTTCCAATGTCACA
cbr11	GGTGTGACTGTGCTTTCAAGAA	TCTATTGAACGCTTGCTGTCCA
Lhcgr	CCTGGAGGCTCATTTTCACTAG	TAGAGAGCCATGCCTTCAATGG
gdf9	CTCAAACCTGGACCATTTGATCA	CAATGCACACCATGGTTCAGA
bmp15	TGAACCGAGTGACCAAGAACAA	GGATACTGCATGGGAGACTGT

2.6 Statistical analyses

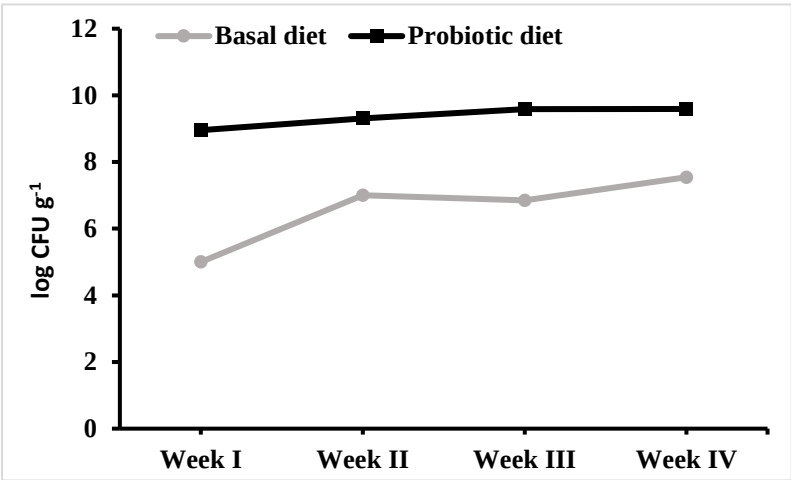
Statistical analysis conducted using Minitab version 17.3.1. Two sample t-test was used to compare normally distributed data sets and Kruskal-Wallis for the not normally distributed data sets. General linear model, a two-way ANOVA equivalent was used to analyse 2 factor conditions. Gene expression analysis were carried out using one sample and two sample t-test in logarithmic scales. All analysis were conducted within 95% confidence level.

3. Results

3.1 Feed results

During the experiment changes in viable cell counts of probiotic bacteria (*P. acidilactici*) was monitored on a weekly basis. Results shown in Table 4 confirms viability of probiotic cells during the period of feeding and storage.

Table 4. Line chart presenting MRS plate count data of the diets used, in log colony forming unit (CFU) per gram by time during the experiment.



3.2 Reproductive performance and fecundity

Reproductive performance of adult zebrafish female specimens was assessed after 7 days of basal and probiotic diet supplementation period on a weekly basis. Each week a new batch of ovulated eggs of zebrafish was collected and enumerated. Number of spawned eggs showed no statistically significant differences between groups until the third batch of eggs collected. After the 2nd batch of eggs, the number of eggs laid per female in treatment group have significantly differentiated from the control group (Figure 5) suggesting possible beneficial effects of the probiotic *P. acidilactici* to the reproductive fitness of adult zebrafish specimens.

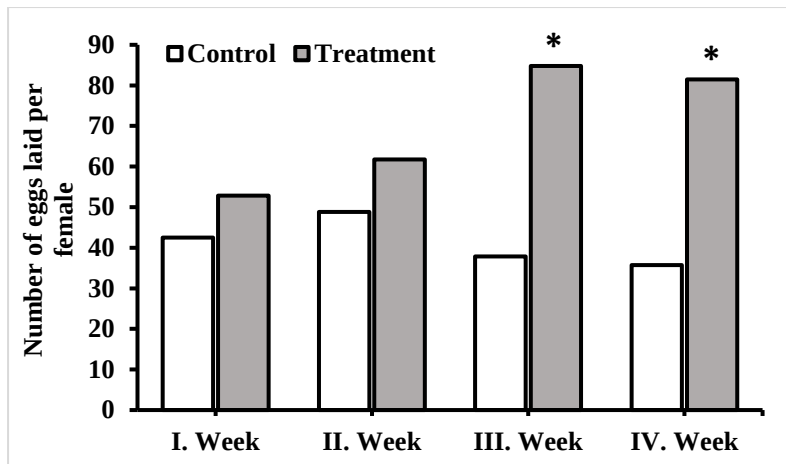


Figure 5. Number of eggs spawned per females (n=28) by control and treatment each week. Asterisks above the data bars indicates the significant difference ($P<0.05$) evaluated using t-test. Mean= 42.50, 48.82, 37.82, 35.71 (control), 52.86, 61.75, 84.82, 81.46 (treatment) from left to right, respectively.

Even though spawned number of eggs by treatment group have statistically differentiated from the control group, there was no significant differences between groups regarding fertilization success rate (Figure 6.). Nonetheless, it was observed both groups have showed good reproductive performances in terms of ovulated eggs and fertilization rate revealed by the results.

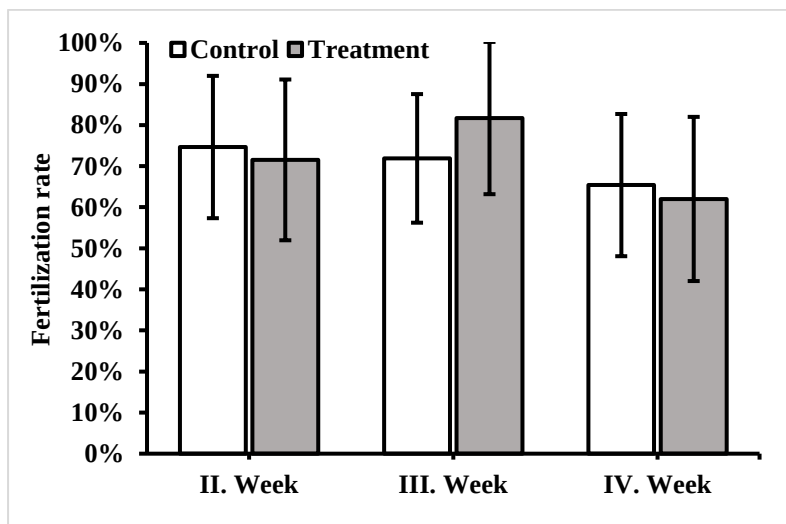


Figure 6. Bar chart demonstrates the fertilization rate of control and treatment groups in percentage.

In addition to the analysis conducted to determine reproductive fitness and fertilization rate, eggs collected from the 3rd week were measured for width. However, samples from both groups showed no significant differences regarding size

(Figure 7). In conclusion, these findings extrapolate that the probiotic administration increased the total number of eggs laid per spawning or in other terms fecundity but failed to increase fertilization rate or egg size.

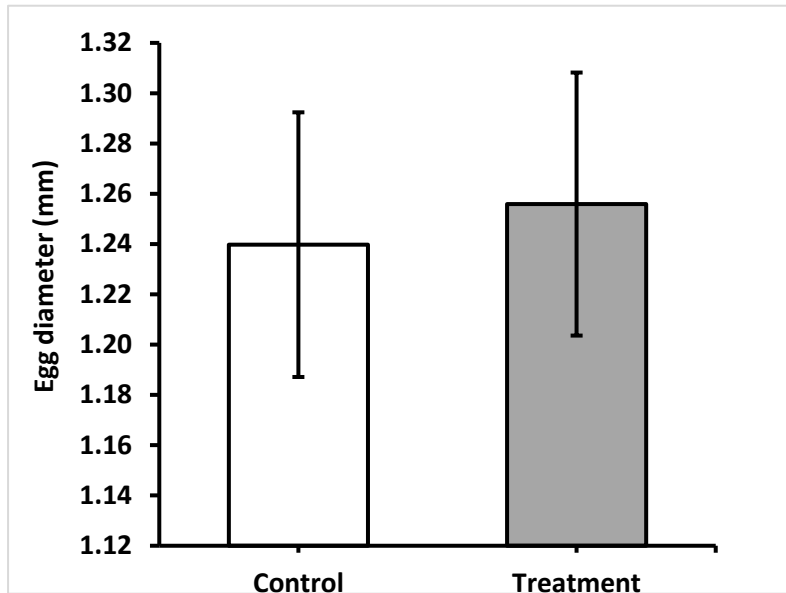


Figure 7. Bar chart demonstrate the possible effect of probiotic administration on egg diameter (appr. at 7 hpf) with error bars of standard deviations of each group (n: 58 and 59, respectively). Groups showed no statistically significant differences evaluated by t-test ($P>0.05$). Mean= 1.240 and 1.256, SD= 0.053 and 0.052, SE=0.007 and 0.007 for control and treatment groups respectively.

3.3 Growth and survival

To characterize the possible beneficial effects of probiotic supplementation to the extent of second generation, growth and survival of zebrafish larvae at early stages until day 15 pf were examined. Hatching was completed at approximately 72 hpf with a success rate of $59.5\pm5.7\%$ and $73\pm8.3\%$ for control and treatment groups, respectively. It was also observed that the hatching process started earlier on treatment groups in comparison to the control and completed with a greater hatching rate. Daily and cumulative mortalities shown in Figure 8. After hatching until day 15 pf, 72 and 44 mortalities recorded for control and treatment groups respectively. Cumulative mortalities in each group followed a similar pattern except a few peak points where the difference was significant, but statistically speaking there was not enough evidence to claim a significant difference between groups ($P>0.05$).

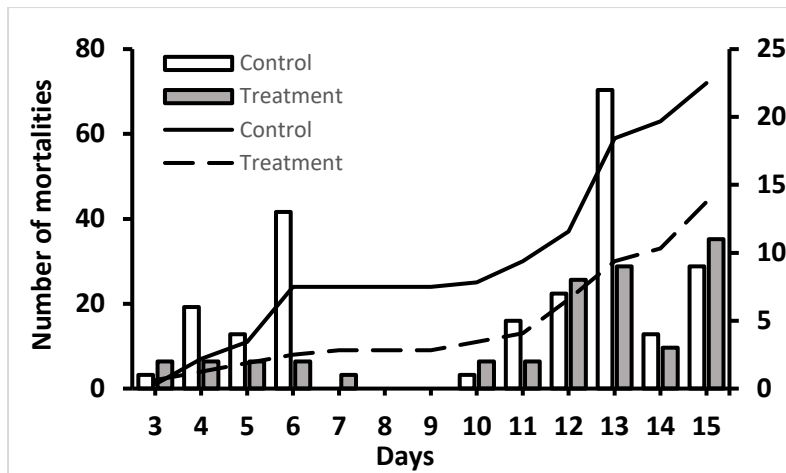


Figure 8. Combined chart of daily mortalities in control and treatment groups. Lines indicates the cumulative mortalities (primary axis) where bars indicate daily total number of mortalities (secondary axis).

On the other hand, length is accepted as one of the most important determining factors of the growth and staging the normal development of the zebrafish, especially at post embryonic development (Parichy et al., 2009). In Figure 9, measurements of length were illustrated as a bar chart. Specimens from the treatment group showed statistically significant increase in length with the analysis conducted with 68-78 specimens compared to the control group ($P<0.05$). This corroborates with the results regarding probiotic supplementation influences on reproductive fitness and fecundity.

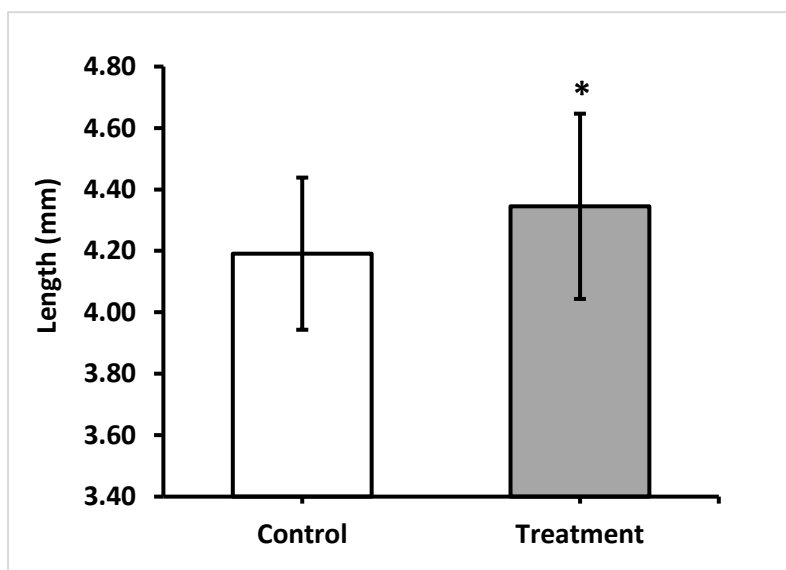


Figure 9. Bar chart illustrates the possible effects of probiotic administration on larvae length (at 15 dpf) with error bars of standard deviations of each group (n:

68 and 78, respectively). Asterisk above the error bar of the treatment group present statically significant differences determined by t-test ($P<0.05$). Mean= 4.191 and 4.345, SD= 0.248 and 0.302, SE= 0.030 and 0.034 for control and treatment groups respectively.

3.4 Cartilage development

To examine the relationship between the cartilage development at post-embryonic stage of probiotic fed zebrafish offspring and the heat exposure, larvae of both probiotic and basal diet fed zebrafish treated with 34°C for 2 hours at 3 dpf. Morphological differences such as length and eye diameter were compared between (temperature) and within (treatment) groups (28°C control, 28°C probiotic, 34°C – 2h control, 34°C – 2h probiotic).

The samples exposed to 34°C for 2h showed statistically significant increase compared to the 28°C groups regarding length ($P<0.05$). The mean values and standard deviations were given in Figure 10A. However, specimens showed unexpectedly no significant differences within groups ($P>0.05$) suggesting the differences were heat related at this point rather than the probiotic treatment. It's interesting that the differences found in comparisons of 15 dpf specimens of control and treatment groups in respect of length was significant (Figure 9). And yet, both groups development were accelerated by the heat treatment at an early post-embryonic stage. Considering the possible effect of a probiotic treatment in a longer period, the difference is likely to grow.

In terms of eye diameter (Figure 10B), results showed no significant differences neither between groups nor in-between groups ($P>0.05$).

In contrast, significant differences were observed between groups and within-groups after the alcian blue staining. As a part of the experiment to examine the jaw and branchial arch developments of zebrafish the length of ceratohyal and the distance between ceratohyal and meckel's cartilage were measured (Figure 4). Both probiotic treated groups showed significant differences ($P<0.05$) compared to the control groups (28°C treatment group compared to the 28°C control group and 34°C – 2h treatment group compared to the 34°C – 2h control group) (Figure 10D). Heat exposed specimens of the control group showed significant differences between groups, but, to the contrary, treatment groups were approaching identity.

Furthermore, temperature treated pair showed significant differences with regards to the results of CHMC (Figure 10C) ($P<0.05$). But also 28°C treatment group varied significantly from the 28°C control group ($P<0.05$) while no difference was acknowledged within 34°C groups. Nonetheless, 34°C – 2h treatment group was considered as approaching significance with a P value of 0.058.

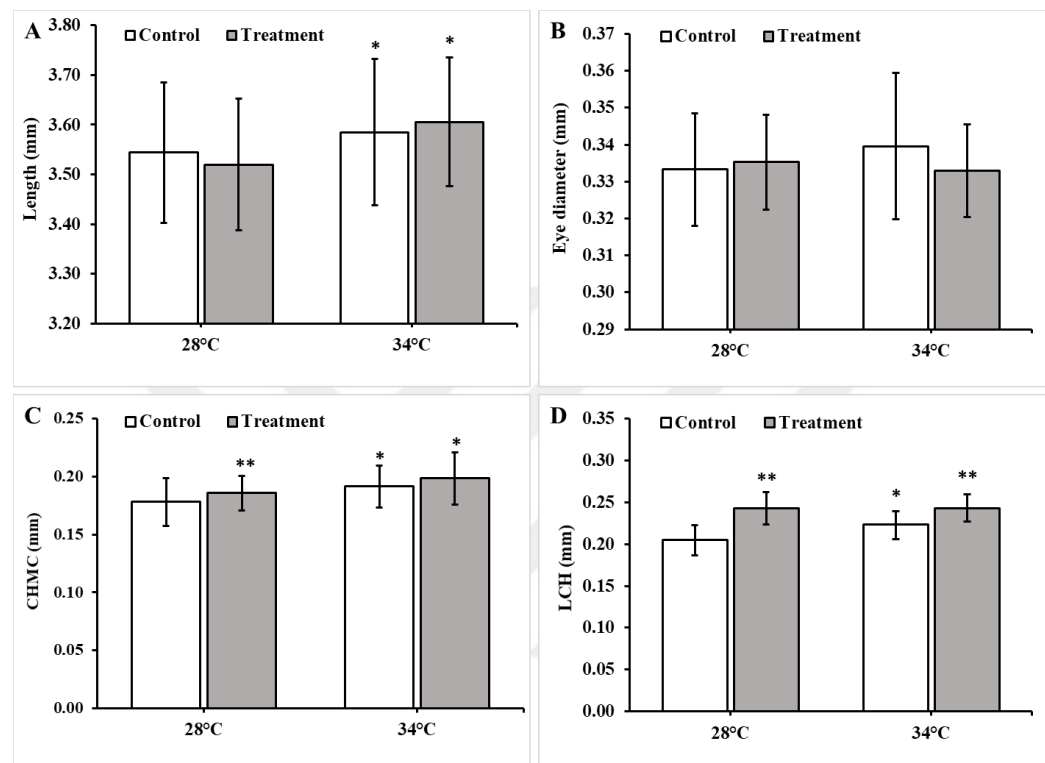


Figure 10. Bar charts (A, B, C, D) displaying morphometric analysis of the possible effects of probiotic administration to zebrafish larvae exposed to heat stress (34°C for 2h at 72hpf) at 120hpf. Measurements have taken before (A, B) and after (C, D) the alcian blue staining. CHMC: the distance between ceratohyal and meckel's cartilage. LCH: the length of ceratohyal. Asterisks indicates statistically significant differences ($P<0.05$) between-groups (* temperature) and within-groups (** treatment) determined by general linear model (ANOVA) tests followed by t-tests, kruskal-wallis is used for the not normally distributed length data. Mean A= 3.54 ± 0.14 (SE=0.013), 3.52 ± 0.13 (SE=0.012), 3.58 ± 0.15 (SE=0.016), 3.61 ± 0.13 (SE=0.012) Mean B= 0.33 ± 0.02 (SE=0.002), 0.34 ± 0.01 (SE=0.002), 0.34 ± 0.02 (SE=0.003), 0.33 ± 0.01 (SE=0.002) Mean C= 0.18 ± 0.02 (SE=0.002), 0.19 ± 0.02 (SE=0.002), 0.19 ± 0.02 (SE=0.002), 0.20 ± 0.02 (SE=0.003) Mean D= 0.20 ± 0.02 (SE=0.002), 0.24 ± 0.02 (SE=0.003), 0.22 ± 0.02 (SE=0.002), 0.24 ± 0.02 (SE=0.003) from left to right, respectively.

3.5 Gene expression

In this study, it was surfaced that the probiotic administration to the zebrafish resulted in an increase in fecundity (Figure 5). Since oocyte maturation plays a vital role in the number of eggs spawned its evident to characterize this increase by RT-PCR analysis carried out on known oocyte maturation inducer genes (*Paqr8*, *cbr1l*, *lhcgr*) and preventers (*gdf9* and *bmp15*). Expression levels of such genes normalized against *gapdh* and β -actin were given in Figure 11. *Paqr8*, *cbr1l*, *lhcgr* genes were significantly upregulated in probiotic fed treatment group compared to the control group ($P<0.05$). Not merely upregulated the inducer genes but also the preventer genes in treatment group. Nevertheless, this increase was not statistically significant ($P>0.05$). This insignificant increase in preventers had no effect on the hatching rate (section 3.3; $59.5\pm5.7\%$, $73\pm8.3\%$ control and treatment groups, respectively), the egg size (Figure 7) and fertilization rate (Figure 6). Besides the significant differences between groups, oocyte maturation inducer genes showed significant differences with-in treatment group ($P<0.05$) where the difference with-in control group was not significant ($P>0.05$) corresponding with the probiotic supplemented group's high reproductive fitness.

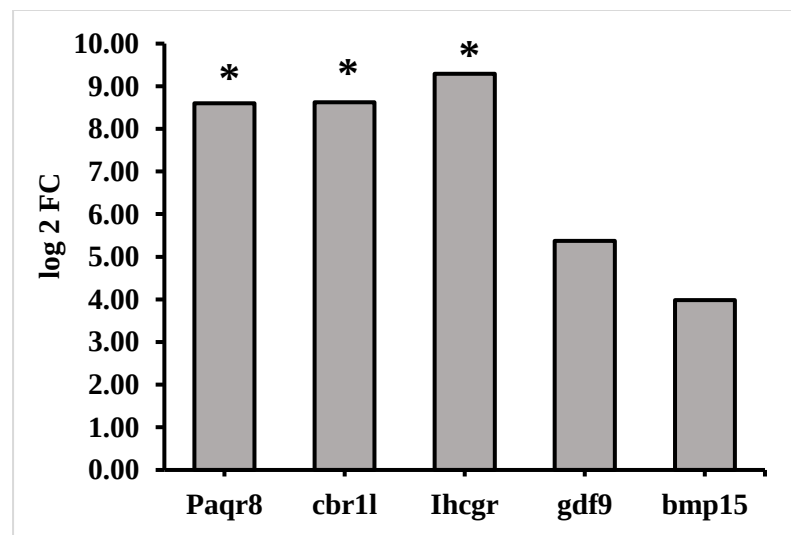


Figure 11. Bar chart illustrates fold change (FC) of oocyte maturation inducer genes (*Paqr8*, *cbr1l*, *lhcgr*) and preventer genes (*gdf9*, *bmp15*) normalized against *gapdh* and β -actin genes on a log scale with base 2. Asterisks above bars indicate the statistically significant differences between inducer and preventer genes ($P<0.05$). (8.60, 8.62, 9.30, 5.37, 3.98 left to right, respectively)

4. Discussion

The academic community have extensively explored the probiotics and their extended possible benefits to a wide range of host organisms. Such benefits are well explored and documented in the area of aquaculture, including, but not limited to; promoting growth and health (Hoseinifar et al., 2016; Ramos et al., 2015; Lin et al., 2012; Dimitroglou et al., 2011; Dharmaraj & Dhevendaran, 2010; Merrifield et al., 2010), modulating immune responses (Ramos et al., 2015; Van Hai, 2015; De et al., 2014; Lazado & Caipang, 2014b; Nayak, 2010), pathogen inhibition and disease control (Newaj-Fyzul et al., 2014; Sun et al., 2012; Gram et al., 1999), nutrient digestibility (Tapia-Paniagua et al., 2012), stress tolerance (Varela et al., 2010), water quality (Zhou, Wang & Li, 2009) and in general sense of use in aquaculture (Lazado & Caipang, 2014a; Pérez-Sánchez et al., 2014; Martínez Cruz et al., 2012).

Discussions regarding the beneficial effects of probiotic use as an additive to the reproductive performance of the host (in this case aquatic animals) have propelled to the forefront in recent years. Ghosh et al., 2007 have proffered that the administration of the probiotic (*Bacillus subtilis* in four different concentrations varied from 5×10^5 to 5×10^8 cells g^{-1}) have increased not only reproductive performance (significant increase in number of fries per female -17.81-20.23 compared to control 15.23- and an increase in fry quality – significantly low number of dead -1.04-1.36 compared to control 2.32- and/or deformed specimens -0.1-0.21 compared to control 0.32-) of live bearing ornamental fishes (*Poecilia reticulata*, *Poecilia sphenops*, *Xiphophorus helleri* and *Xiphophorus maculatus* separately stocked in 20 tanks with 60 specimens of each) but also a significant increase in weight (0.0022-0.0024g compared to control 0.0019g) and in length (6.08-6.36mm compared to control 5.82mm) of the fry. Abasali & Mohamad, 2010 have documented the effects of a commercial probiotic, Primalac (containing *L. acidophilus*, *L. casei*, *E. faecium* and *B. thermophilum* in equal proportions – 3 concentrations of diets varied from 0.04% to 0.14% is fed), on female live bearing ornamental fish sword-tail (*X. helleri*) and outlined the significantly high relative fecundity (31-42 compared to the control 28) and survival (48-65% compared to control 44%) with low rate of deformities (2.5%-6.5% compared to control 8.8%) in fry. A few more studies followed the trend and provided more information supporting the theory probiotics can improve reproductive performance of the host. Gioacchini et al., 2010

purported to show a significant increase in the number of eggs ovulated (40-250 compared to control 25-100 per female per day) and an increase in hatching rate (approx. 60% and 94% for control and probiotic groups, respectively) after the administration of a human probiotic strain *L. rhamnosus* to the zebrafish. Qin et al., 2014 demonstrated the effects of the probiotic strains of *L. rhamnosus* and *L. caseia* as a feed additive to zebrafish in reproduction by a significant increase in number of eggs laid per female (about 25% higher than control in *L. caseia* fed group) and hatching rate (about 70% and 90% for control and treatment groups, respectively). It's interesting that as a part of the experiment Qin et al., 2014 have continued feeding all experimental groups with a basal diet and observed after a week benefits of the probiotics were in a declining trend regarding number of ovulated eggs, fertilization rate and hatching rate suggesting a continuous feeding of probiotics is required to keep the reproductive fitness of a broodstock at higher levels. Mehdinejad et al., 2018 evaluated the effects of probiotic *-P. acidilactici-* supplementation with 8 experimental diet variations of probiotic (0.1%, 0.2% and 0.3%) and nucleotide (0.5%) combined or individual in goldfish *-Carassius auratus-*. Results varied for fertilization rate; $75.33 \pm 0.5\%$ to $94.33 \pm 0.5\%$, hatching rate; $73.33 \pm 0.5\%$ to $81 \pm 1\%$, relative fecundity; 176.1 ± 1.35 to 187 ± 5.32 between treatment groups, but unfortunately no data is shared regarding control group due to no spawning. This study corroborates with others findings regarding number of ovulated eggs (Figure 5.), hatching rate (Section 3.3) and growth (Figure 9.) but fails to meet fertilization rate (Figure 6.) expectations.

Topics such as temperature stress have shaped queries in the last decade (Long et al., 2015; Mininni et al., 2014; McBryan et al., 2013) and zebrafish cartilage development is well documented in prior studies (Piotrowski et al., 1996; Schilling et al., 1996). Long et al., 2012 have assessed a combined study of exposure to cold (16°C – 48h at 96hpf) and heat (34°C – 48h at 96hpf) with a control group (28°C) and assessed cartilage development of larval zebrafish. Long and his co-workers have outlined that 34°C and 28°C groups were significantly longer than 16°C groups but no significant differences are documented between heat treated and control groups. Similarly, there was no significant difference in terms of CHMC between the control and heat-treated group. And control group showed significantly higher values regarding LCH and eye diameter where cold treated group had significantly lower values in all cases. As shown in Figure 10, specimens exposed to

heat were significantly better in all measurements except eye diameter where no significance is found in this study. The difference can be explained by the exposure time. Fish exposed to heat or cold stress can develop tissue hypoxia due to a reduced cardiovascular activity (Lannig et al., 2004). The longer the exposure time the greater the damage. LCH was significantly longer in the probiotic group in both temperatures and relatively had higher means in length and CHMC (but not significant) suggesting probiotic treatment maybe can reduce the impacts of temperature stress. On the other side, both 34°C and 28°C probiotic groups had larger LCH and CHMC. This strengthens the idea that the probiotics can promote the cartilage development.

Much attention has been drawn to underlying mechanisms of reproduction in recent years especially in genomics and their relationship with probiotic administration became imminent. Gioacchini et al., 2010 have assessed the effects of the probiotic (*L. rhamnosus*) to zebrafish fecundity and documented for the first time the role of probiotic (*L. rhamnosus*) in *leptin* (a key component in modulation of *gnrh* mRNA) activation. Gioacchini and his co-workers have confirmed their findings with molecular findings and documented significantly high levels of oocyte maturation inducer genes *lhcr*, *cbr11* and *paqr8* and significantly low levels of oocyte maturation preventer genes *tgfb1*, *bmp15*, *gdf9* in probiotic fed group compared to the control group. In addition to their molecular finding, it's also reported that the probiotic fed group was significantly ovulated a greater number of eggs per day. As shown in Figure 5 and Figure 11, this study collaborates with Gioacchini and his co-workers results with the exception of oocyte maturation preventer gene (*bmp15* and *gdf9*) expression ratios which in our case was not expressed significantly different than the control group. Liu & Ge, 2007 have documented the expression levels of *gdf9* in zebrafish. Their findings showed the expression of *gdf9* is oocyte maturation stage dependent and at its highest level on earlier stages while Clelland et al., 2006 have assessed the expression levels of *bmp15* in zebrafish and found no significant differences between the oocyte stages and expression levels. Thus, not significantly lower expression levels of oocyte maturation preventer genes in our experiment can be caused by the proportion of early stage oocytes. Moreover, this difference may suggest mechanisms which probiotic supplementation indirectly effects the expression levels of genes considered as governors of reproduction can be altered by introducing single or a combined type of probiotics.

773 However, probiotic supplementation has resulted in significantly high oocyte mat-
774 uration inducer gene expression ratios suggesting a positive impact on oocyte mat-
775 uration and reproduction of zebrafish.

776 5. Conclusion

777 It's evident that findings of this study have documented the beneficial ef-
778 fects of *P. acidilactici* on growth and reproduction. Probiotic supplementation has
779 improved reproductive fitness of zebrafish with a significant increase in daily num-
780 ber of ovulated eggs per female. This was confirmed with assessing the expression
781 levels of *lhcr*, *cbr1l*, *paqr8*, *bmp15* and *gdf9* genes. A significant upregulation of
782 crucial genes such as *lhcr*, *cbr1l* and *paqr8* for reproductive performance is evi-
783 denced. In addition, bactocell administration has significantly improved growth
784 performance, cartilage development and heat tolerance of zebrafish at early and
785 post-embryonic stages.

786 There is still not much is known about the underlying mechanisms of re-
787 production and their relationship with probiotics. Further research is required to
788 identify different mechanisms such as heat and cold stress responses in probiotic
789 fed specimens in molecular levels. Changes in expression levels of stress marker
790 genes, egg and sperm quality genes in relationship with probiotics can provide es-
791 sential data in terms of reproduction to the aquaculture industry.

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