

# Comparison of fecundity and offspring immunity in zebrafish fed *Lactobacillus rhamnosus* CICC 6141 and *Lactobacillus casei* BL23

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## Abstract

To increase the knowledge of probiotic effects on zebrafish (*Danio rerio*), we compare the effects of two probiotic strains, *Lactobacillus rhamnosus* CICC 6141 (a highly adhesive strain) and *Lactobacillus casei* BL23 (a weakly adhesive strain), on zebrafish reproduction and their offsprings' innate level of immunity to water-borne pathogens. During probiotics treatments from 7 to 28 days, both the *Lactobacillus* strains, and especially *L. casei* BL23, significantly increased fecundity in zebrafish: higher rates of egg ovulation, fertilization, and hatching were observed. Increased densities of both small and large vitellogenic follicles, seen in specimens fed either *Lactobacillus* strain, demonstrated accelerated oocyte maturation. Feeding either strain of *Lactobacillus* upregulated gene expression of *leptin*, *kiss2*, *gnrh3*, *fish*, *lh*, *lhcg*, and *paqr8*, which were regarded to enhance fecundity and encourage oocyte maturation. Concomitantly, the gene expression of *bmp15* and *tgfb1* was inhibited, which code for local factors that prevent oocyte maturation. The beneficial effects of the *Lactobacillus* strains on fecundity diminished after feeding of the probiotics was discontinued, even for the highly adhesive gut *Lactobacillus* strain. Administering *L. rhamnosus* CICC 6141 for 28 days was found to affect the innate immunity of offspring derived from their parents, as evinced by a lower level of alkaline phosphatase activity in early larval stages. This study highlights the effects of probiotics both upon the reproductive process and upon the offsprings' immunity during early developmental stages.

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## Introduction

Different evolution levels and aqueous environments present the aquatic animals with specific biology, especially in immunity development (Sunyer *et al.* 1998). The teleost fish models have greatly contributed to innate immune system study (Rauta *et al.* 2012, Sunyer 2013). The fish embryonic development usually occurs outside of the maternal body in an aqueous environment, in which the embryos are potentially susceptible to microbial pathogens. Pathogen attacks during early stages in the fish life cycle naturally have a decisive impact on the survival and future reproductive performance of cultured stocks (Wang *et al.* 2008). High mortality has been recorded during larval and post-larval stages in diverse fish stocks throughout the world (Swain & Nayak 2009). In fish, the maturation of lymphoid organs and immuno-competence is delayed (Zapata

*et al.* 2006), with very limited synthesis of specific antibodies during embryonic development and early larval stages (Swain *et al.* 2002, Magnadottir *et al.* 2004). What is more, insufficient availability of high-quality animal stocks and unacceptable susceptibility to disease outbreaks limit production and may result in serious economic losses in aquaculture. Therefore, maternally derived immunity is essential in these early phases of the life cycle. However, reliance on maternally derived immunity to provide defense for embryos and larvae in turn depends on the health and immune status of the parental brood fish. Therefore, development of novel methods to improve the health of the brood fish is important and can improve their breeding performance, the quality and quantity of their offspring, and, in those species with parental care, their ability to protect their offspring (Swain & Nayak 2009).

With the development of gut commensal microbiota, their functional interactions and the corresponding

molecular mechanisms on host health are under investigation (Clemente *et al.* 2012), especially in host immunity (Cahenzli *et al.* 2013) and metabolisms (Tremaroli & Backhed 2012). As one of the main components of gut microbiota, probiotics have been described as live microorganisms that improve disease resistance (Zhou *et al.* 2012) and the microbial balance of the host's intestinal fauna (Vine *et al.* 2006, Ziaei-Nejad *et al.* 2006, Twetman & Steckslen-Blicks 2008), and also as biologically active components, or single or mixed cultures of microorganisms, capable of improving the health of the host (Ochoa-Solano & Olmos-Soto 2006, Saha *et al.* 2012). These descriptions imply the use of microorganisms or their by-products as biological controls of or modifiers to the bacterial composition of host intestines, including the aquatic animals. More recently, the effects of probiotics on fecundity have attracted attention and some beneficial influences have been observed (Gioacchini *et al.* 2010, 2012). These previous studies indicate that research into probiotic additives as dietary supplements, including the discovery of more useful probiotic strains and exploration into the molecular mechanisms underlying probiotics' beneficial effects, is essential for improving the health and fecundity of brood fish.

Over the last three decades, the zebrafish (*Danio rerio*) has emerged as an important animal model for basic physiological and biomedical research (Grunwald & Eisen 2002). As is typical in vertebrates, zebrafish reproduction is regulated by the hypothalamus–pituitary–gonadal axis. Internal and external environmental factors coordinate to stimulate the hypothalamus to release gonadotropin-releasing hormone (GNRH; Zohar *et al.* 2010). Energy balance critically influences reproductive function (Gamba & Pralong 2006, Barb *et al.* 2008). Leptin is one of the predominant cytokines signaling to the hypothalamus, the nutritional status of the body. Also, neuropeptide hormones called kisspeptins (kiss1 and kiss2) regulate the activity of GNRH-releasing neurons (Barb *et al.* 2005, 2008, Gamba & Pralong 2006, Castellano *et al.* 2009, Hausman *et al.* 2012), consequently promoting the secretion of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). Fsh stimulates estradiol production, which in turn leads to a surge in Lh hormone levels, which, through its receptor (Lhcgr; Patino *et al.* 2001, Clelland & Peng 2009), stimulates the production of maturation-inducing hormone and through its receptors (progesterin and adipoQ receptor, namely Paqr7b and Paqr8) activate the oocyte maturation processes (Hanna & Zhu 2009). In addition, the transforming growth factor  $\beta$  (Tgf $\beta$ ) superfamily also plays a role in regulating oocyte maturation (Clelland *et al.* 2006, 2007, Halm *et al.* 2008, Clelland & Peng 2009, Lessman 2009, Tan *et al.* 2009), which includes activin-A, bone morphogenetic protein-15 (Bmp15), Tgf $\beta$ 1, and growth and differentiation factor 9 (Gdf9).

In this study, we investigate the effects of administering to mature zebrafish either of two probiotic strains – the strain *Lactobacillus rhamnosus* CICC 6141, which is highly adhesive to the gut walls (Zhou *et al.* 2012), and the strain *Lactobacillus casei* BL23, which is non-adhesive or at least less-adhesive – on reproductive qualities (such as fecundity) and offspring hardiness.

## Materials and methods

### Bacteria and culture conditions

The probiotic strains of *L. rhamnosus* CICC 6141 and *L. casei* BL23 were obtained from laboratory stocks and stationarily cultivated in an MRS medium at 37 °C for 48 h. The medium (pH 6.8) contained per liter 10 g tryptone, 10 g meat extract, 5 g yeast extract, 10 g glucose, 5 g sodium acetate, 2 g diammonium citrate, 1 g Tween 80, 2 g K<sub>2</sub>HPO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.05 g MnSO<sub>4</sub>·H<sub>2</sub>O. After growing in the MRS medium for 48 h, Lactobacilli cells were collected into pellets by centrifugation (10 min, 2500 g, 4 °C). A pellet was washed in PBS buffer (pH 7.4) three times and re-suspended in 5 ml at a final concentration of 1.0×10<sup>9</sup> CFU/ml.

### Feed preparation

The basal diet consisted of 47.0% fishmeal, 24.0% soybean meal, 24.0% wheat flour, 2% soybean oil, 2.2% Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>, 0.1% vitamin C phosphate ester, 0.3% silicon-based choline chloride, 0.2% minerals, and 0.2% vitamins. The main components were analyzed to be 42.0% crude protein and 7.3% crude lipid according to Xie *et al.* (1997). The probiotic diet was prepared by supplementing the above basal diet with one of the two *Lactobacillus* strains at a final density of 1.0×10<sup>8</sup> cells/g of basal diet together with 1% skimmed milk (as a protective agent). In the control group's diet, equivalent volumes of sterile PBS and skimmed milk were added to the basal diet. The feed was air-dried at 20 °C for 15 h and kept at 4 °C. The quality of the probiotic feed was monitored by MRS plate culture, and the survival rate of the *Lactobacilli* in the experimental diet could be maintained at ~95% for at least 2 weeks. Therefore, to ensure high quality, fresh feed with probiotic additives was made every week.

### Animal subjects

Seven-month-old zebrafish (*D. rerio*) were provided by the Center of Developmental Biology and Genetics, College of Life Sciences, Peking University, China. The fish were kept in laboratory conditions for 2 weeks followed by the different experimental treatments. The animals were raised in 25.5×18.5×18.0 cm (length×width×height) tanks in a quiet water system under laboratory conditions (28±0.5 °C with a 14 h light:10 h darkness photoperiod) with the density of ten fish per tank. The water was changed completely every day. The fish (of similar size and body weight) were divided into three groups and fed either just the basal diet (the control group) or the basal diet plus the strain *L. rhamnosus* CICC 6141, or basal diet plus the strain *L. casei* BL23 (as described earlier). The fish were fed

twice per day with the given amount of diets according to the standard of 3% total body weight of fish in each tank (0830 and 1730 h). Matings were made between one female to one male per 2 l tank (21 × 10 × 10 cm, length × width × height) per pair of mating. The mating brood fish were set up weekly – at 1900 h on days 7, 14, 21, and 28 after initiating feed treatments. Apart from the mating, the male fish and female fish were divided into different tanks with ten fish per tank. At the end of 4 weeks with the indicated treatments, the fish were killed for tissue collection or switched to the basal diet. Experimental protocols with the fish were in accordance with the guidelines approved by the animal ethics committee of Feed Research Institute, Chinese Academy of Agricultural Sciences (2012-ZZG-ZF-001).

### Counts of ovulated eggs, the fertilization, and hatching rates

Counts of spawned eggs (including both fertilized and unfertilized eggs) were performed the morning after mating at 0900 h (within 1 h after the lights were turned on). The fertilization rate was taken as the ratio of fertilized eggs to total spawned eggs. The data were from ten pairs of matings for each treatment. One thousand fertilized eggs were randomly chosen to calculate the hatching rate. The eggs were kept in nursery tanks under a 14 h light:10 h darkness photoperiod in a quiet water system. One half of the water was replaced each day. After hatching, the offspring from each maternal fish were divided into four tanks of 25.5 × 18.5 × 18.0 cm (length × width × height) in a circulating water system with u.v. radiation and adequate filtration. The inlet water flow was ~0.1 l/min during larval stages and 1 l/min during juvenile stages. Feeding of all the offspring fish was initiated at 5 days post-fertilization (dpf), and fish were fed three times daily with fried paramecium during the early stages (5–20 dpf) and then brine shrimp nauplii (21–56 dpf). Offspring were killed at the indicated times to obtain whole body or intestinal tissue samples. These samples were immediately frozen in liquid nitrogen and stored at –70 °C. Later, samples from each of the groups were homogenized on ice and then centrifuged at 10 000 g at 4 °C for 15 min. The supernatant was pooled, aliquoted, and stored at –70 °C.

### Tissue collection and sample preparation

After the 28-day feeding treatment, mated females were anesthetized (500 mg/l MS-222; Sigma) and then dissected. The guts were rinsed three times by PBS to remove the intestinal contents. Guts were fixed in 2.5% glutaraldehyde for scanning electron micrograph (SEM) analysis and ovaries were fixed in 4% paraformaldehyde solution for hematoxylin/eosin (H&E) staining. To make probiotic counts in the gut, the whole guts were collected and rinsed three times by PBS (contained per liter 8 g NaCl, 0.2 g KCl, 1.42 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.27 g KH<sub>2</sub>PO<sub>4</sub>, pH at 7.4) to remove the intestinal contents and then stored in a 40% glycerol solution for probiotic MRS culture. For RNA extraction, the brains, ovaries, and guts were collected and frozen in liquid nitrogen and stored at –70 °C.

### Total RNA extract and real-time PCR

The equal amounts (by weight) of indicated tissues of two fish from the same tank were pooled to extract total RNA as one RNA replicate. Independent replicate fish from three tanks were collected to extract total RNA for the corresponding cDNA synthesis as biological triplicates ( $n=3$ ). Total RNA was isolated using TRIzol (Tian Gen, Beijing, China) following the manufacturer's specifications and treated with amplification-grade DNase I (1 U/μg RNA; Invitrogen). A total amount of 1 μg of total RNA was used for cDNA synthesis, performed with a cDNA Synthesis kit (Toyobo, Osaka, Japan) in a 20 μl volume. Two microliter-diluted (1/10) cDNA was used to perform qPCR by SYBR Green Supermix (Takara, Kyoto, Japan) according to the manufacturer description in an CFX iCycler thermal cycler (Bio-Rad) in a 20 μl volume, with final concentration of 0.5 μM forward and reverse primers. The reaction mixtures were incubated for 5 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C and finally the melt curve was performed from 65 to 95 °C with a 0.5 °C increment for 10 s. No amplification product was observed in negative control, and no primer-dimer formation was observed in the control templates. Three genes, including *rpl13* (Poulain & Ober 2011), *rps11* (Galindo-Villegas *et al.* 2012), and *ef1a* (Villamizar *et al.* 2012), were used as references. For each cDNA sample, three wells were used for qPCR amplification as the technical replicates. The sequences of specific primers that were used are presented in Table 1.

### Histological studies

Ovaries were isolated individually from three female fish as independent replicates and fixed by 4% paraformaldehyde solution for over 24 h, dehydrated in increasing concentrations of ethanol, and embedded in paraffin wax. For light microscopy, the sections of 7 μm thickness were sliced and

**Table 1** List of primers used for real-time PCR analyses.

Genes	Sequence (5'–3')	Accession no.
<i>rpl13</i>	F: TCTGGAGGACTGTAAGAGGTATGC R: TCAGACGCACAATCTTGAGAGCAG	NM_198143
<i>lep</i>	F: AGCTCTCCGCTCAACCTGTA R: CAGCGGAATCTCTGGATAA	NM_001128576.1
<i>kiss2</i>	F: ATTCTTTCATGTCTGCAATGGTCA R: TGCTTTCAGGTAAGCATCATG	NM_001142585.1
<i>gnrh3</i>	F: TTAGCATGGAGTGGAAAGGAAGGTTG R: CTTTCAGAGGCAACCTTCAGCAT	NM_182887.2
<i>tgfb1</i>	F: TCGCTTTGTCTCCAAGGACT R: TGCAAGAGAGTTGCCATTTG	NM_182873.1
<i>bmp15</i>	F: AGGGTGACCGGATCACTATG R: TGCTGCCAGACTTTTGTAGACC	NM_001020484.1
<i>lhgr</i>	F: TCGCCATCTGGTTCATCAATA R: GGCGAAGGCTAGATGGCACAT	NM_205625.1
<i>fishβ</i>	F: CAGATGAGGATGCGTGTGC R: ACCCTGCAGGACAGCC	NM_205624
<i>lhβ</i>	F: GCAGAGACACTTACAACAGCC R: AAAACCAAGCTCTGAGCAGCC	NM_205623
<i>paqr8</i>	F: CAACGAGCTGCTGAATGTGT R: GGGCCAGTTGAGAGTGAGAC	NM_183344
<i>rps11</i>	F: ACAGAAATGCCCTTCACTG R: GCCTTCTCAAACGGTTG	NM_213377
<i>ef1a</i>	F: GCTGGAGGCCAGCTCAAACATG R: ATCAAGAAGAGTAGTACCCTAGCATTAC	NM_131263

stained with H&E staining. Ten sections from each ovary paraffin sample were examined under a light microscope (Motic China Group Co., Ltd) to detect follicle maturation for Table 2. For SEM analysis, the middle and posterior intestines were isolated individually from three female fish as independent triplicates and fixed with 2.5% glutaraldehyde. Fixed intestines were cut along the horizontal axis to allow for observation of the internal surface. High-magnification SEM micrographs were analyzed with Image J 1.36 Software (National Institutes of Health, Bethesda, MD, USA).

### Alkaline phosphatase activity

To get enough tissue or whole-body sample for determining alkaline phosphatase (AP) activity, proper numbers (30–40 larvae or five to ten juveniles) of the fish from the same tank were used as one biological sample and the fish were collected from three different tanks for independent replicates ( $n=3$ ). AP activity was determined according to the report (Bates *et al.* 2006). In brief, the whole bodies of larvae, juveniles, or guts were homogenized by PBS to get supernatants by centrifugation at 4 °C, which was incubated in a *p*-nitrophenylphosphate liquid substrate system (Sigma) for 30 min following the manufacturer's specifications and then absorbance was measured at 405 nm. The concentration of total protein was measured using a BCA Protein Assay Kit (Novagen, Darmstadt, Germany) following the manufacturer's specifications.

### Assay of IgM levels in body tissue and in the intestine

The whole bodies of larvae, juveniles, or guts were homogenized by PBS to get supernatants by centrifugation at 4 °C for IgM level detection using a Fish IgM ELISA Kit (Qi Song, Beijing, China) following the manufacturer's specifications. For each treatment, fish replicate samples were made from three different tanks ( $n=3$ ). In brief, the supernatant samples were properly diluted in provided dilution buffer. Fifty microliters of all the series standards or the same volume diluted samples were added in triplicate to the Microelisa Stripplate; 100 µl HRP conjugate reagents were added in each well, followed by incubating for 60 min at 37 °C with an adhesive strip cover. After repeated washing five times, the same volumes of 50 µl chromogen solution A and chromogen solution B were added to each well with gently mixing and incubating at 37 °C at dark for 15 min. The reaction was stopped by adding 50 µl stop solution to each well and the optical density (OD) was read at 450 nm using a microtiter plate reader (Multiskan MK3, Thermo, Marietta, USA) at room temperature within 15 min.

**Table 2** The proportion of each stage follicles (%).

Treatment	Stage I–stage II follicles	Small vitellogenic follicles	Large vitellogenic follicles
Control	81.68 ± 1.40	10.80 ± 1.00	7.52 ± 0.79
6141	72.17 ± 1.56*	14.37 ± 1.16*	13.46 ± 0.66**
BL23	65.77 ± 0.74**	16.42 ± 0.76**	17.81 ± 0.25**

\* $P<0.05$  and \*\* $P<0.01$  between the experimental and control groups.

### Statistical analysis

Statistical analyses were performed using SPSS Software. Comparisons across multiple experimental groups were conducted using a one-way ANOVA followed by Bonferroni analysis. The asterisks denote significant differences existed between the compared groups, and one asterisk represents  $P<0.05$ , two represent  $P<0.01$ , and three represent  $P<0.001$ .

## Results

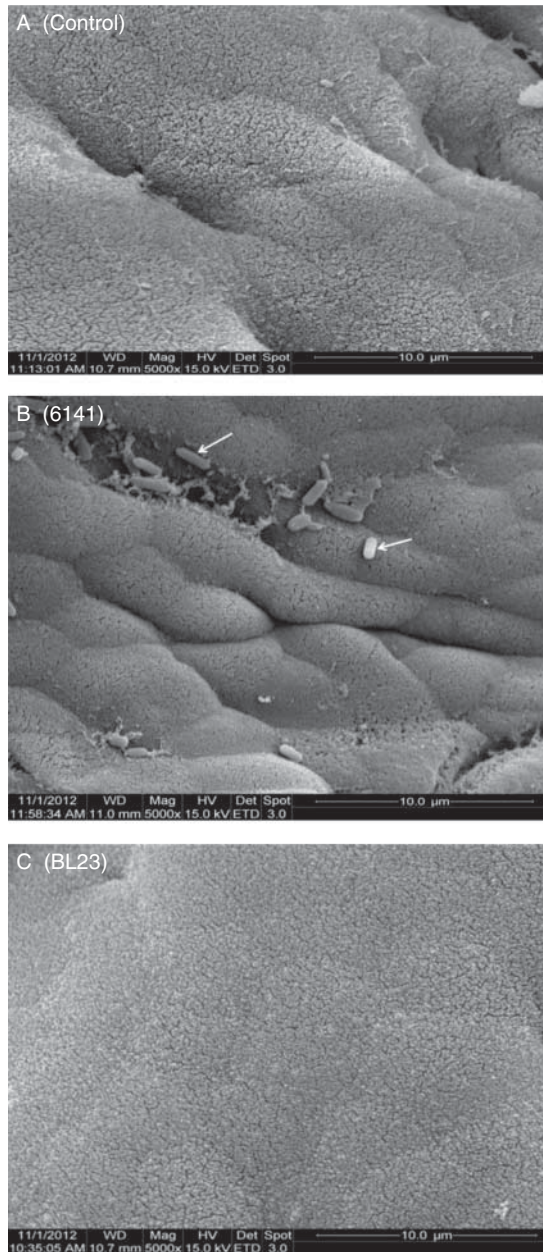
### Colonization of the intestine by the probiotics

SEM was used to estimate the number of probiotic cells adhering to the intestinal walls of maternal fish. After 28 days of the feed treatment, no clear indication of bacterial cells adhering to the intestinal walls was observed either in the control group (fed solely the basal diet) or in the group in which the basal diet was augmented with *L. casei* BL23 (Fig. 1A and C). However, in the *L. rhamnosus* CICC 6141 treatment group, obvious *Lactobacillus* colonized the inner surface of the intestine (Fig. 1B). MRS plate cultures also demonstrated the much higher adhesion rate of *L. rhamnosus* CICC 6141 when compared with *L. casei* BL23 ( $P<0.01$ , Fig. 1D), consistent with the results reported in Zhou *et al.* (2012).

### Effects of probiotics on fecundity

To evaluate the effects of probiotics on fecundity, the number of ovulated eggs, the fertilization rate, and the hatching rate were assayed (Fig. 2). At 7 days after commencement of the treatment regime, the number of ovulated eggs from females of the *L. casei* BL23 treatment group was significantly higher than that from the control group (by about 25%;  $P<0.05$ ; Fig. 2A), and this beneficial effect was maintained during the remainder of the 28-day treatment regime. There was no significant difference in this measure between the *L. rhamnosus* CICC 6141 treatment group and the control group (Fig. 2A). On the other hand, the fertilization rate (Fig. 2B) increased significantly ( $P<0.05$ ) in the *L. rhamnosus* CICC 6141 treatment group relative to the control group while the rate for the *L. casei* BL23 treatment group was unaltered. Hatching rates in both the *L. casei* BL23 and *L. rhamnosus* CICC 6141 treatment groups were significantly elevated ( $P<0.05$ , Fig. 2C) compared with the control group's rate. After 28 days of probiotics treatment, all the groups were transferred to the basal diets without the probiotics. Sustained effects from both probiotic strains on fish fecundity were observed for ~1 week after probiotic additive feeding ceased; these effects included higher counts for ovulated eggs ( $P<0.05$ , Fig. 2D), higher hatching rates ( $P<0.05$ , Fig. 2F), and slightly higher fertilization rates (Fig. 2E). These effects diminished from the second week post-additive treatment onward, with significant differences among the three test groups in the number of the ovulated

eggs, the fertilization rates, and hatching rates disappearing (Fig. 2D, E, and F). These data suggested that the continuous administration of probiotics is required to maintain the enhanced fecundity of the brood fish.



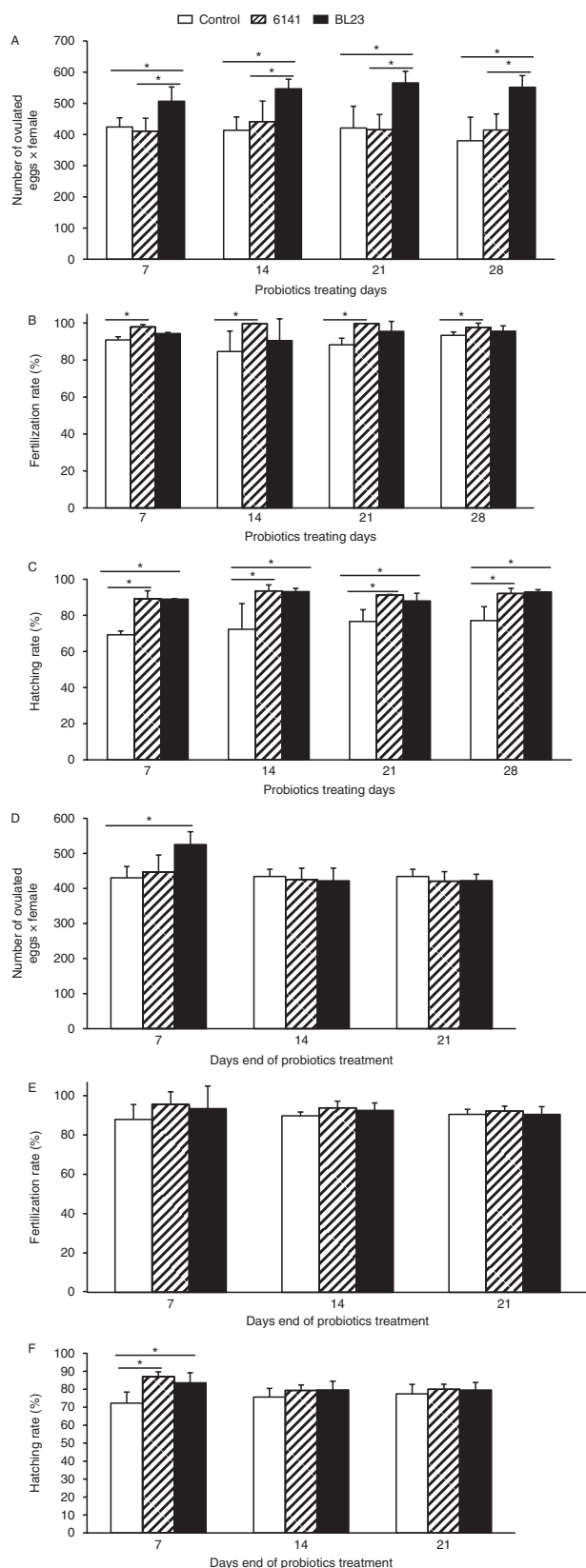
### Effects of probiotics on oocyte maturation

The ovary of an adult zebrafish may be broadly divided into zones where cells are in growth stages and zones where cells have matured. After 28 days of feed treatment, the ovarian sections in fish treated with probiotics evinced an increase in vitellogenic follicles. The percentage of the ovary occupied by both small vitellogenic follicles and large vitellogenic follicles were significantly increased in probiotic treatment groups compared with the control group ( $P < 0.05$  and  $P < 0.01$  respectively, Fig. 3A, B, and C and Table 2), with increases especially marked in the *L. casei* BL23 treatment group ( $P < 0.01$ , Table 2). These data demonstrate that the probiotics *L. rhamnosus* CICC 6141 and *L. casei* BL23 stimulate oocyte maturation.

### Effects of probiotics on the signaling pathways involved in oocyte maturation

To study the potential mechanisms of the probiotic effects on oocyte maturation, the involved molecules and pathways were analyzed (Figs 4 and 5). GNRH plays a predominant role in oocyte maturation. GNRH is itself activated by leptin and kisspeptin, and in turn increased GNRH activity leads to increased concentrations of hormones such as Lh and Fsh and subsequently regulated the expression of its downstream receptors, such as *Lhcgr* and *Paqr8*. After 28 days of probiotics administration, significant increases in the expression of the gene *leptin* were observed in specimen brains from *L. casei* BL23 treatment group ( $P < 0.05$  and  $P < 0.01$  respectively; Fig. 4A). A significant rise in the expression of the gene *kiss2* was seen in the *L. casei* BL23 group ( $P < 0.05$ , Fig. 4B) but not in the *L. rhamnosus* CICC 6141 group. The gene expression of *gnrh3* rose significantly in both *L. rhamnosus* CICC 6141 and *L. casei* BL23 treatment groups relative to the control group ( $P < 0.05$  and  $P < 0.001$  respectively, Fig. 4C). The gene expression of *fsh* ( $P < 0.01$ , Fig. 5A), *lh* ( $P < 0.05$ , Fig. 5B), *lhcgr* ( $P < 0.01$ , Fig. 5C), and *paqr8* ( $P < 0.01$ , Fig. 5D) were also significantly increased in the *L. casei* BL23 group but not in the *L. rhamnosus* CICC 6141 treatment group (Fig. 5A, B, C, and D). These data suggest that GNRH

**Figure 1** Colonization analysis of the probiotics by scanning electron microscopy (SEM) and cell counting per colony. (A, B, and C) Representative micrographs of the inner surface of the intestine from (A) control females, (B) females fed on *L. rhamnosus* CICC 6141, and (C) females fed on *L. casei* BL23. Scale bar = 10  $\mu$ m. (D) Intestinal homogenate MRS plate culture results showing the number of *Lactobacillus* cells contained in 1 g intestinal tissue (wet weight). Results are given as mean  $\pm$  S.E.M. from three fish intestines for each treatment ( $n = 3$ ). Double asterisks indicate extremely significant differences ( $P < 0.01$ ) as analyzed using ANOVA test followed by Bonferroni analysis. The arrow in panel B indicates *Lactobacillus*. Labels: 6141 = *L. rhamnosus* CICC 6141 treatment group; BL23 = *L. casei* BL23 treatment group.



signaling pathways are involved in the stimulation via probiotics of oocyte maturation. The data also point to differences in this regard between the two strains: *L. casei* BL23 affected the expression rates of *leptin*, *fish*, *lh*, *lhcg*, and *paqr8* more strongly than did *L. rhamnosus* CICC 6141.

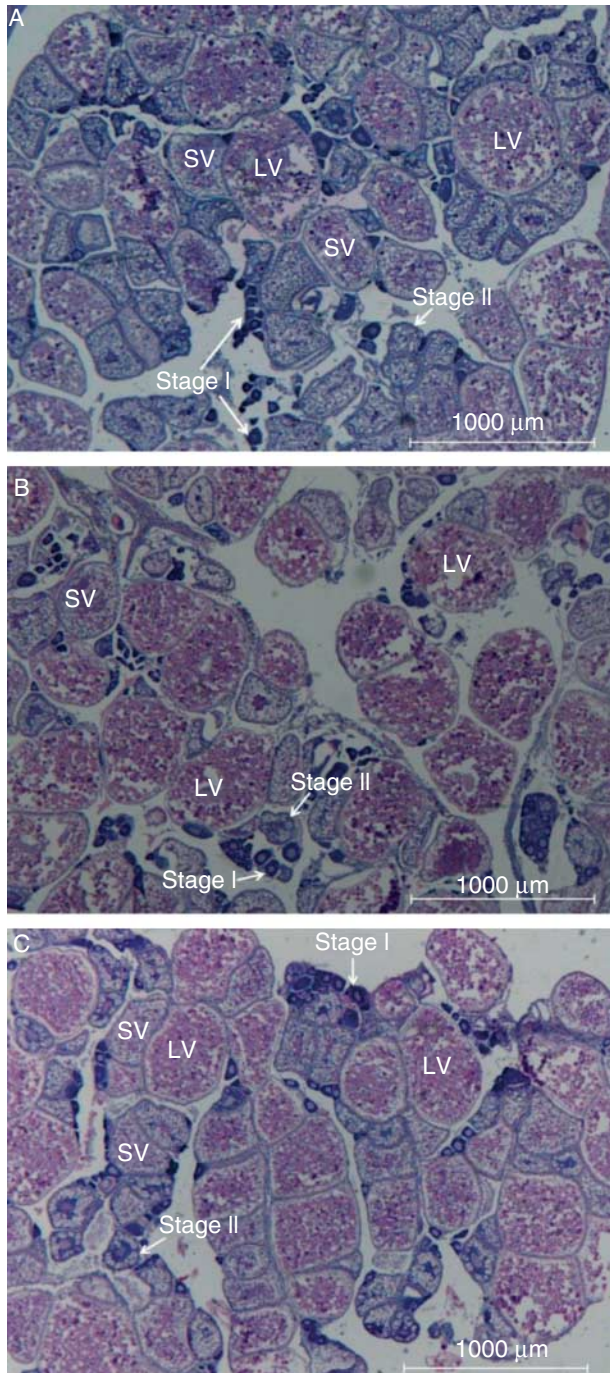
In addition, the Tgf $\beta$  superfamily has been reported to regulate oocyte maturation (Peng 2003, Knight & Glister 2006). This is consistent with our findings that the gene expression for *bmp15* and *tgfb1*, both of which are involved in the prevention of premature oocyte maturation, decreased significantly in both the *L. rhamnosus* CICC 6141 and the *L. casei* BL23 treatment groups relative to the control group ( $P < 0.05$  and  $P < 0.01$  respectively, Fig. 5E;  $P < 0.01$  and  $P < 0.001$  respectively, Fig. 5F). The administration of either *L. casei* BL23 or *L. rhamnosus* CICC 6141 clearly causes changes in the signaling pathways involved in oocyte maturation, acting to promote oocyte maturation.

### Inherited immunity of offspring

The levels of IgM and AP activity in the offspring from parents of the three different dietary groups were quantitatively compared. The IgM levels – determined either using the whole body (Fig. 6A) or just using the intestine of the offspring (Fig. 6B) – did not vary significantly among the three groups' differences. Whether the offspring were larvae or juveniles also did not matter (Fig. 6A and B). The AP activity level as ascertained using the whole body of the offspring was significantly lower ( $P < 0.05$ , Fig. 7A) during earlier stages in larvae of fish from the *L. rhamnosus* CICC 6141 treatment group (relative to the control group); however, by 28 days post-hatching (dph), the AP level in the offspring of the *L. rhamnosus* CICC 6141 treatment group became statistically indistinguishable from that of offspring from the control group. For the offspring of the *L. casei* BL23 treatment group, a significant difference in AP activity level occurred in the 0–14 dph sample, for which the AP activity level was significantly lower than that of the corresponding control group ( $P < 0.05$ , Fig. 7A). AP level comparisons resulting from sampling only intestinal tissues from the offspring generally mirrored those from the whole-body samples, although

**Figure 2** The effects of the probiotics on fecundity of brood fish. The number of ovulated eggs per week by females (A), the fertilization rate of eggs (B), and the hatching rate of fertilized eggs (C) in all treatment groups during 4-week treatment periods. The number of ovulated eggs by females per week (D), the fertilization rate of eggs (E), and the hatching rate of fertilized eggs (F) in all treatment groups when transferred to the basal diet (without probiotics). The results are obtained from ten pairs of matings and are shown as mean  $\pm$  s.e.m. for each treatment ( $n = 10$ ). A significant difference is denoted by a single asterisk ( $P < 0.05$ ). Labels: 6141 = *L. rhamnosus* CICC 6141 treatment group; BL23 = *L. casei* BL23 treatment group.

the basal levels of AP activity in intestines were much higher than those assayed from utilizing the entire body (Fig. 7). AP basal activity of the whole body generally increased as the larval zebrafish developed during 0–28 dph (Fig. 7A).

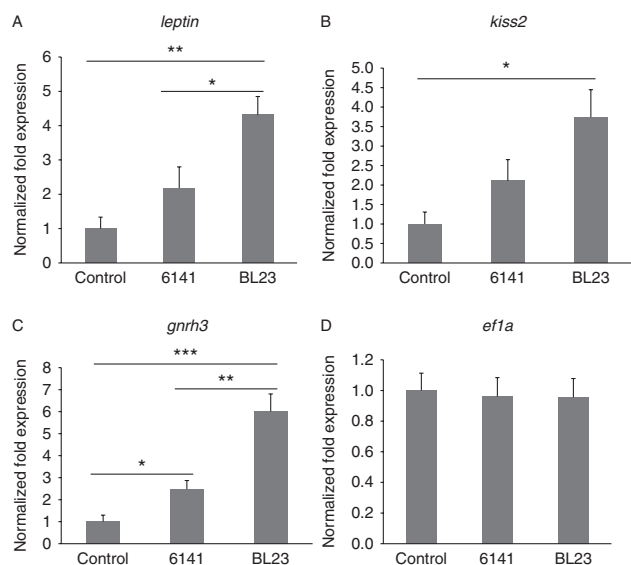


**Figure 3** Representative micrographs of ovaries from (A) control females, (B) females fed on *L. rhamnosus* CICC 6141, and (C) females fed on *L. casei* BL23. One typical micrograph is presented from triplicate brood females for each treatment by H&E staining ( $n=3$ ). SV, small vitellogenic follicles; LV, large vitellogenic follicles.

## Discussion

This study broadens research into improving the fecundity of brood fish via probiotic strains, analyzing effects from two additional strains: *L. rhamnosus* CICC 6141 and *L. casei* BL23. Very recently, Gioacchini and his group documented positive effects on fecundity from administering *L. rhamnosus* IMC 501 to zebrafish (Gioacchini *et al.* 2010, 2012, Giorgini *et al.* 2010). Administering *L. rhamnosus* IMC 501 for 10 days induced oocyte maturation, increased the number of ovulated eggs *in vivo*, and enhanced the germinal vesicle breakdown rate *in vitro* (Gioacchini *et al.* 2010). Consistent with these earlier investigations, the current study has demonstrated that the probiotic strains analyzed herein also promote increased fecundity and oocyte maturation. Interestingly, it was found that continued feeding with the probiotics was required to maintain the higher fecundity of the brood fish, which provided an important tip in the application. If the probiotic supplements were withdrawn, the beneficial effects quickly disappeared, regardless of whether the probiotic strain involved was of the adhesive or the non-adhesive type. Comparison of the effects seen in the *L. casei* BL23 and *L. rhamnosus* CICC 6141 test groups indicates that adhesion is not an indispensable characteristic when it comes to improving fecundity.

Changes in the signaling pathways involved in oocyte maturation caused by the probiotic additives could explain the observed increases in fecundity. Thus, the important molecules in these pathways could be used as biomarkers for judging the effects of environmental factors on fecundity. Reproductive capabilities are limited by the amount of usable energy stored up and/or available to the brood fish (Barb *et al.* 2008). Leptin has been demonstrated to be a pivotal regulator of the integrated energy allotments for body maintenance and for reproduction. Leptin is involved in controlling appetite, energy homeostasis, and Lh secretion (Fernandez-Fernandez *et al.* 2006, Barb *et al.* 2008). Its effects on GNRH and Lh secretion are mediated by kisspeptin (Fernandez-Fernandez *et al.* 2006, Hausman *et al.* 2012). This study has demonstrated that administering *L. casei* BL23 to zebrafish increases their fecundity. *L. casei* BL23 significantly increased *leptin* and *kiss2* expression rates, and, under the influence of leptin and *kiss2*, GNRH/Lh expression rates increased, concomitant with decreased *bmp15* and *tgfb1* expression rates (Figs 4 and 5), and these factors in turn induced oocyte maturation (Fig. 3 and Table 2). The results obtained herein are consistent with those reported by Gioacchini *et al.* (2010), wherein treatment with *L. rhamnosus* IMC 501 was also reported to modulate expression rates for genes coding for neuropeptide hormones and other metabolic signals (such as *kiss1*, *kiss2*, and leptin), increase the transcription rates of genes coding for signals that induce oocyte maturation (such as *lhcg*,



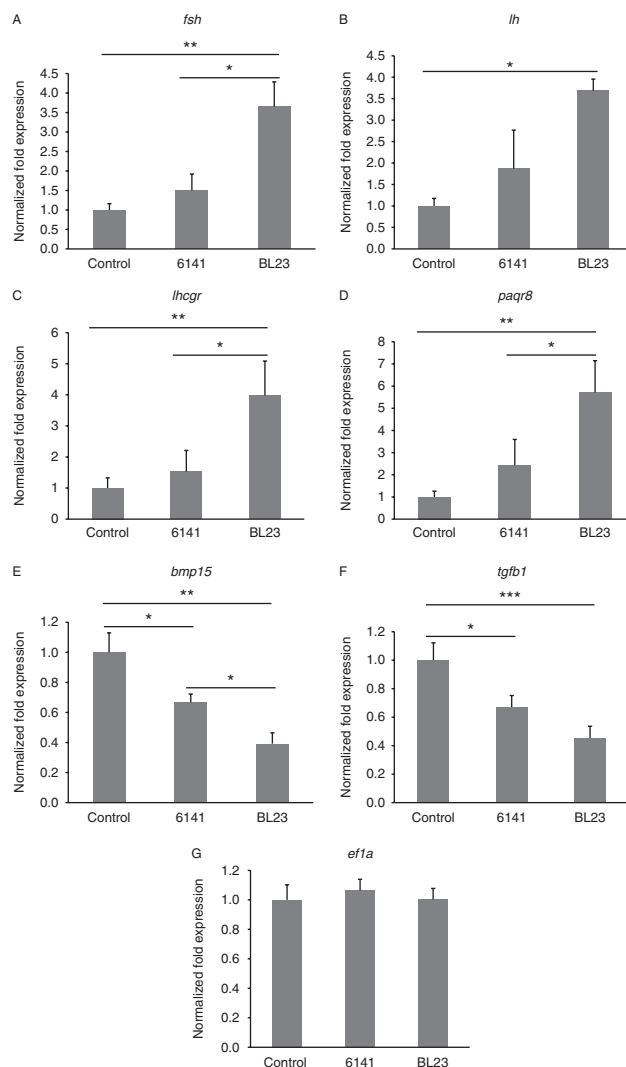
**Figure 4** The mRNA levels of (A) *leptin*, (B) *kiss2*, (C) *gnrh3*, and (D) *ef1a* in brains from treated females, normalized to the *rps11* gene. The independent triplicates of total RNA extraction and corresponding cDNA synthesis were carried out with the treated fish from different tanks ( $n=3$ ) and the results are given as mean  $\pm$  S.E.M. Single asterisk denotes a significant difference ( $P<0.05$ ), while double asterisks and triple asterisks indicate extremely significant differences ( $P<0.01$  and  $P<0.001$  respectively) as analyzed using ANOVA test followed by Bonferroni analysis. Labels: 6141 = *L. rhamnosus* CICC 6141 treatment group; BL23 = *L. casei* BL23 treatment group.

*cbr11*, and *paqr8*) and decrease expression rates of genes coding for factors preventing oocyte maturation (such as *tgfb1*, *gdf9*, and *bmp15*). Aside from this study on strain IMC 501, there appears to have been no other studies published that document how administering probiotics affects reproductive processes in the host. The current study is thus the first to compare the effects on reproduction and inherited offspring immunity for zebrafish fed different *Lactobacillus* strains (specifically, a highly adhesive *Lactobacillus* strain, *L. rhamnosus* CICC 6141, vs a less-adhesive strain, *L. casei* BL23). It is not yet known how the probiotics stimulate the secretion of leptin and kisspeptin, which in turn subsequently enhance gene expression rates downstream along the GNRH pathways promoting oocyte maturation. Quite recently, *L. rhamnosus* was also reported to accelerate backbone calcification and gonadal differentiation in zebrafish through effects on the GNRH and IGF systems (Avella *et al.* 2012).

It has been clearly demonstrated that probiotic dietary supplements can lead to improvements in feeding efficiency and growth rates in aquatic animals (Aly *et al.* 2008, Abelli *et al.* 2009, Campa-Cordova *et al.* 2009). Probiotics can generate enhanced levels of relevant digestive enzymes (like amylase, protease, and lipase) in animal intestines, which in turn would increase the digestibility of organic matter and proteins.

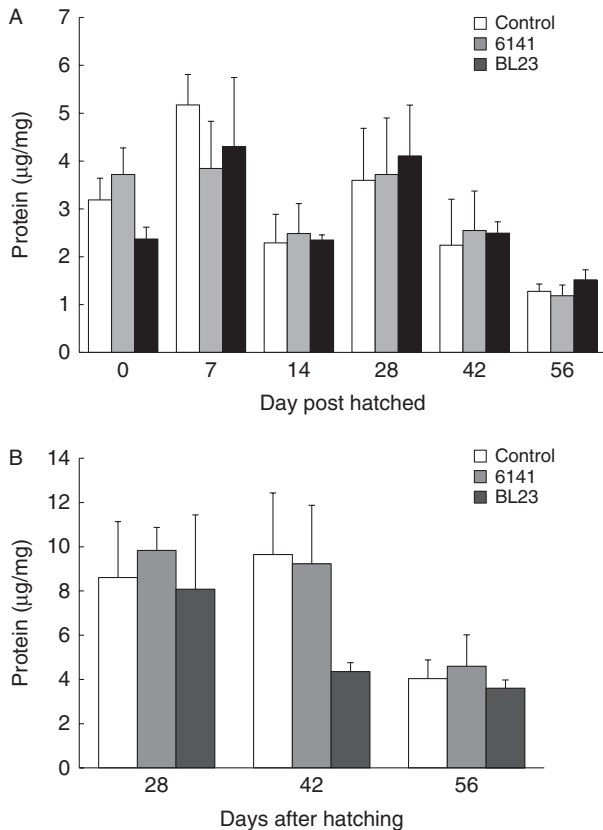
Probiotics can also provide necessary growth factors, vitamins, or amino acids (Lara-Flores *et al.* 2003, Lara-Flores 2011); affect the organism's energy metabolism and energy balance and lead to a higher growth rate (El-Haroun *et al.* 2006, Al-Dohail *et al.* 2009); and improve fecundity (Gioacchini *et al.* 2010). However, the exact molecular mechanisms by which probiotics regulate the gene expression of *leptin* and *gnrh* have yet to be uncovered.

The overall state of health of the brood fish can affect the quality of the resulting embryos and, in those species



**Figure 5** The mRNA levels of (A) *fsh*, (B) *lh*, (C) *lhcr*, (D) *paqr8*, (E) *bmp15*, (F) *tgfb1*, and (G) *ef1a* in ovaries from treated females, normalized to the *rps11* gene. The independent triplicates of total RNA samples and the corresponding cDNA synthesis were carried out with the treated fish from three different tanks ( $n=3$ ) and the results are shown as mean  $\pm$  S.E.M. Single asterisk denotes a significant difference ( $P<0.05$ ), while double and triple asterisks indicate extremely significant differences ( $P<0.01$  and  $P<0.001$  respectively), as analyzed using ANOVA test followed by Bonferroni analysis. Labels: 6141 = *L. rhamnosus* CICC 6141 treatment group; BL23 = *L. casei* BL23 treatment group.



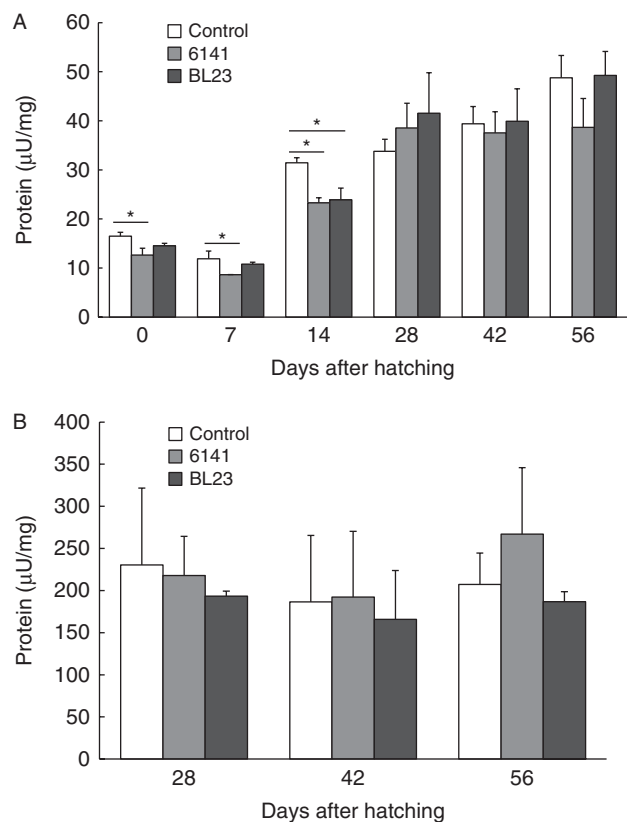


**Figure 6** IgM levels (A) in the whole-body samples of the larvae and juveniles and (B) in the gut samples of the juveniles. For each treatment, fish replicate samples were made from three different tanks ( $n=3$ ) and the results are given as mean  $\pm$  S.E.M., analyzed using ANOVA test followed by Bonferroni analysis. Labels: 6141 = *L. rhamnosus* CICC 6141 treatment group; BL23 = *L. casei* BL23 treatment group.

with parental care, their ability to protect their offspring (Swain & Nayak 2009). In most fish species, eggs are released and/or fertilized externally, exposing the resulting embryos and larvae to an aquatic environment full of potentially harmful pathogens capable of causing various diseases. Prior to complete maturation of their own immune systems, developing embryos and larvae rely on maternally inherited immune-relevant molecules for protection against invading pathogens. Previous studies on several fish species have investigated transmission to offspring of maternal immunization against pathogens (such as with antigens). In sea bream, *Sparus aurata* Linnaeus, embryos, and larvae from mothers immunized with *Photobacterium damselae* subsp. *piscicida* SK7 show higher levels of IgM activity, lysozyme activity, anti-protease activity, and total immunoglobulin (Hanif *et al.* 2004, 2005). In the Indian river carp (also known as the rohu or rohu laabeo), *Labeo rohita* (Hamilton), lower offspring mortality, and higher agglutinating activity in eggs, larvae, and fry were recorded when parental brood fish were immunized with *Aeromonas hydrophila* (Swain *et al.* 2006).

In zebrafish, higher levels of C3, Bf, and hemolytic activity along with lower mortality rates were documented in the offspring of parental brood fish immunized with *A. hydrophila* or corresponding antigens (Wang *et al.* 2009, 2012). All these studies on the transmission of maternal immunity focused on microbial pathogens, largely ignoring other possible effects on the offspring from the administration of probiotics to the mother fish. This study appears to be the first to investigate such other effects on offspring from mothers fed probiotics, as well as the first to compare the effects on offspring from administering different types of probiotics (an adhesive vs a less-adhesive strain) to the mothers. After probiotics treatment, the quality of the embryos improved, as evinced by higher fertilization and hatching rates (Fig. 2) and by lower AP activity in the offspring's early stages of development (Fig. 7).

AP can be sub-divided into body AP (BAP) and intestinal AP (IAP), and IAP activity (but not BAP activity) can be induced by LPS, the major constituent of the outer



**Figure 7** The AP activity (A) in the whole-body samples of offspring and (B) in the intestinal tissue samples of offspring in all groups in a temporal series. To get enough tissue or whole-body sample, proper numbers (30–40 larvae or 5–10 juveniles) of the fish from the same tank were used as one biological replicate and the fish were collected from three different tanks for independent triplicates ( $n=3$ ). The results are given as mean  $\pm$  S.E.M. An asterisk denotes a significant difference ( $P<0.05$ ) analyzed using ANOVA test followed by Bonferroni analysis. Labels: 6141 = *L. rhamnosus* CICC 6141 treatment group; BL23 = *L. casei* BL23 treatment group.

membrane of all Gram-negative bacteria (both pathogens and mutualists) (Beutler & Rietschel 2003, Bates *et al.* 2007). Using a germ free (GF) zebrafish model shows that during the bacterial colonization of the gut, IPA activity increases, while in GF zebrafish, IAP activity is lower than in conventionally reared fish (Bates *et al.* 2006, 2007). In this study, interestingly, a lower AP activity level (as ascertained using the whole body of the larva) was found for early-stage (1–14 dph) offspring of the CICC 6141 treatment group compared with that of offspring from the control group. Yet, at 28 dph, the AP activity level in *L. rhamnosus* CICC 6141 treatment group offspring became indistinguishable from that for the control group offspring. The mechanism causing this is unknown.

Unlike AP activity levels, IgM levels (again as ascertained with whole body samples) in the intestines of larvae and juveniles that were the offspring of mothers fed either of the two probiotic strains showed no significant differences compared with levels for offspring from the control group (Fig. 6). In early developmental stages, IgM is derived from the mother (Zhang *et al.* 2013) and thus the IgM level in larvae and early stage juveniles is determined by that of the mother fish. As probiotics, neither *Lactobacillus* strain CICC 6141 nor *L. casei* BL23, when given access into the zebrafish, can stimulate B cell synthesis of IgM. Therefore, the IgM levels in the mother fish of the probiotics treatment groups were equal to those from the control group. Consequently, the IgM levels in the offspring of mother fish of the probiotics treatment groups are also equal to those found in offspring of mother fish of the control group.

In summary, using either *L. rhamnosus* CICC 6141 or (especially) *L. casei* BL23 as a feed additive is an important avenue by which to stimulate follicle maturation, enhance fecundity, and improve egg quality in zebrafish. Furthermore, administering *L. rhamnosus* CICC 6141 may also affect the fitness level of offspring during early stages of larval development. The zebrafish has long been seen as a model subject in biological research, and so the results obtained in this study indicate the potential for using these two probiotics strains (either separately or combined) for the beneficial manipulation of stock reproductive capabilities in aquaculture and perhaps even with teleost fish.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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