Increase of fecundity by probiotic administration in zebrafish (Danio rerio)

Giorgia Gioacchini¹, Francesca Maradonna¹, Francesco Lombardo¹, Davide Bizzaro², Ike Olivotto¹ and Oliana Carnevali¹

Departments of ¹Marine Sciences and ²Biochemistry, Biology and Genetics, Polytechnic University of Marche, via Brecce Bianche, 60131 Ancona, Italy

Correspondence should be addressed to O Carnevali; Email: o.carnevali@univpm.it

Abstract

It is well known established that reproduction is sensitive to the state of energy reserves, and that there is a balance between energy homeostasis and fertility. In this view, this study examined the effects of the probiotic Lactobacillus rhamnosus, as a feed additive, on zebrafish (Danio rerio) fecundity. Ten days of probiotic treatment modulate the gene expression of neuropeptide hormones and metabolic signals, such as kiss1, kiss2 and leptin both at the CNS level and at the peripheral level. The increase in fecundity brought about by the probiotic was demonstrated by the higher number of ovulated eggs in vivo and by the higher germinal vesicle breakdown rate obtained with the in vitro maturation assay. The increase in oocyte maturation was associated with increased transcription of genes coding for signals which induce the maturation phase, such as lhcgr, cbr1l, and paqr8 genes, concomitant with a decreased transcription of genes coding for local factors which prevent oocyte maturation, such as tgifbl, gdf9, and bmp15. In conclusion, all these findings highlight the action of L. rhamnosus both on the endocrine system and at the local level by inducing oocyte maturation. The significance of the results herein obtained underlined the importance of diet in the reproductive process, supporting the hypothesis that feed additives can improve fecundity. Considering that the zebrafish has been clearly established as a vertebrate model for biomedical research, these results support the potentiality of feed additives such as probiotics, frequently used in the human diet, as a new technology to improve reproduction in all vertebrates, including humans.


Introduction

Over the past 20 years, the zebrafish (Danio rerio) has attracted considerable attention as an excellent vertebrate model system for studying genetics and development (Fishman 2001), and more recently, for understanding human disease and for the screening of therapeutic drugs (Penberthy et al. 2002, Sumanas & Lin 2004, Keller et al. 2006, Shiels et al. 2009).

Zebrafish have asynchronous ovaries, containing follicles at all stages of development (Selman et al. 1993) as well as mature eggs. The growth and maturation of the oocyte occur over a period of about 10 days (Wang & Ge 2003a, 2003b), and in laboratory conditions, eggs are spawned throughout the year.

In zebrafish, as in all vertebrates, reproduction is regulated by the hypothalamus–pituitary–gonadal axis. The hypothalamus, integrating internal and external stimuli, releases GnRH (Zohar et al. 2010). In recent years, it has been established that GnRH transcription and secretion are gated by the state of energy reserves in the organism (Hill et al. 2008). The impact of energy status on the reproductive axis is conveyed through a number of neuropeptide hormones and metabolic signals, such as kiss1, kiss2, and leptin, whose nature and mechanisms of action have begun to be deciphered only in recent years in mammals and, to a lesser extent, in fish (Fernandez-Fernandez et al. 2006, Castellano et al. 2009, Kitahashi et al. 2009). Under the influence of GnRH, the pituitary secretes FSH and LH, which act upon the gonads controlling follicle growth and maturation (Nagahama et al. 1995, Patino et al. 2001). In particular at ovarian level, LH, through its receptor (LHCGR), stimulates the production of 17α-hydroxyprogesterone that is converted (by the action of cbr1l) into 17α,20β-dihydroxy-4-pregnen-3-one, the maturation-inducing hormone (MIH) in zebrafish (Patino et al. 2001). The binding of MIH to its receptors (paqr7b and paqr8) activates the maturation processes (Hanna & Zhu 2009).

Oocyte maturation is regulated not only by the endocrine system but also by the auto/paracrine factors such as the transforming growth factor β (Tgfb) superfamily. Tgfb is a large group of oocyte maturation regulatory molecules that include positive factors such as inhibins/activins and negative factors such as tgifb1, bone morphogenetic protein 15 (bmp15), and growth

This study investigated for the first time the effects of probiotic Lactobacillus rhamnosus administration on fecundity and on the endocrine and paracrine control of follicle development in female zebrafish.

According to the WHO definition, probiotic bacteria are ‘live microorganisms which when administered in adequate amounts confer a health benefit on the hosts’. L. rhamnosus was selected in this study since it is a common constituent of the indigenous microbiota in the intestinal and urogenital tracts of mammals and other vertebrates and is able to balance the intestinal microbiota, inducing host immunomodulation and decreasing the symptoms of a wide range of gastrointestinal disorders (Manley et al. 2007, Pant et al. 2007, Szajewska et al. 2007, Verdenelli et al. 2009).

At a physiological level, a 10-day administration of L. rhamnosus had an effect on the number of ovulated eggs and on the hatching rate. At a molecular level, this administration affected the expression of molecules that control the balance between energy homeostasis and fecundity as well as the expression of molecules that control follicle maturation at brain, gut, and ovarian levels. Concomitantly with these molecular changes, the in vitro maturation assay revealed a higher rate of germinal vesicle breakdown (GVBD) in oocytes from females administered with this probiotic.

Results

Effects of L. rhamnosus on metabolic control of reproduction

Figs 1 and 2 show the endocrine control of reproduction as a result of L. rhamnosus administration. In zebrafish, both at the gut and at the brain levels, probiotic administration induced a significant increase in the gene expression of leptin, a key hormone in energy homeostasis and neuroendocrine functions (Fig. 1A and B). This increase was correlated with a significant rise in the brain gene expression of kiss1 and kiss2: these newly discovered neuropeptides can control Gnrh expression, representing the link between metabolic and reproductive systems (Fig. 2A and B). The increase in neuropeptides mRNA was concomitant with an increase in gnrh3 gene expression, as reported in Fig. 2C.

Effects of L. rhamnosus on oocyte maturation and fecundity

In zebrafish adults, L. rhamnosus administration significantly increased fecundity (Fig. 3). The number of ovulated eggs per day rose significantly as a result of the probiotic treatment, starting from the second day of administration. In particular, the number of ovulated eggs by the treated females reached the maximum on day 2, with eggs production remaining high during the following days (Fig. 3). The induction of follicle maturation was concomitant with a significant decrease in the expression of genes coding for signals involved in the prevention of premature oocyte maturation such as tgb1, bmp15, and gdf9 (Fig. 4A–C). The decrease in tgb1 and bmp15 found in the ovaries of females fed on the probiotic was concomitant with the positive control on lhcgr, cbr1l, and paqr8 gene transcription (Fig. 5A–C), all of which are important signals involved in the induction of oocyte maturation. The involvement of probiotic on follicle development was also supported by histological studies. The ovarian sections evidenced an increase of vitellogenic follicles in fish treated with probiotic (Fig. 6). The improvement in follicle maturation

![Image](reproduction-online.org)
was also confirmed by the in vitro GVBD. Stage IIIb follicles isolated from the ovaries of treated females incubated with MIH showed the greatest rate of GVBD, while probiotic administration alone had no stimulatory effect (Fig. 7).

The molecular data (Figs 4 and 5) and the significant increase in the number of ovulated eggs per day (Fig. 3) observed suggest that *L. rhamnosus* administration as a feed additive promotes oocyte maturation. Moreover, this increase in follicle maturation due to probiotic administration did not negatively affect the egg quality since the embryos produced by females fed with the probiotic showed the highest hatching rate (Fig. 8) and a faster embryo development, characterized by the fact that embryo hatching was brought forward by 4 h (Table 1).

**Discussion**

Reproduction is gated by the state of body energy reserves and is sensitive to different metabolic cues; the neuroendocrine mechanisms responsible for the tight coupling between energy homeostasis and fertility are represented by metabolic hormones and neuropeptides that integrate the hypothalamic center governing reproduction, controlling the expression and release of Gnrh (Fernandez-Fernandez et al. 2006, Castellano et al. 2009, Kitahashi et al. 2009, Zohar et al. 2010). Thus, full activation of the hypothalamic–pituitary–gonadal axis at puberty and its proper functioning in adulthood critically depend on adequate body energy stores (Hill et al. 2008). The identification of the adipose hormone leptin, which signals the magnitude of energy stores to the hypothalamic centers governing reproduction (Casanueva & Dieguez 1999, Goumenou et al. 2003), represented an important step toward understanding the mechanisms controlling this interplay.

In this study, for the first time, a possible stimulating role of *L. rhamnosus* on female zebrafish fecundity was investigated. The results suggest that the probiotic may act indirectly by activating a potent metabolic hormone such as leptin. In mammals, it was well established that leptin can regulate reproduction by acting on the hypothalamus (modulating gnrh mRNA transcription and release) and on the pituitary (modulating lh gene expression and release) as evidenced by several authors (Smith et al. 2002, Barb et al. 2005).

In this study, the expression of the *lep* gene was significantly induced after probiotic administration in the gut. Concomitantly, we also observed the induction of

---

*Figure 3* The count of number (10 days of treatment) of eggs spawned per day by females fed on *L. rhamnosus* (PROBIO) and by control females (CTRL) was performed every day at 0900 h within 1 h after lights. Data are given as mean ± s.d. (*n* = 5). Asterisks denote significant differences from the control group (*P* < 0.05) analyzed using Student’s *t*-test.

*Figure 4* (A) *tgfb1*, (B) *bmp15*, and (C) *gdf9* mRNA levels in the ovary, normalized against β-actin and *gapdh* genes in females fed on *L. rhamnosus* (PROBIO) and in control females (CTRL). Data are given as mean ± s.d. (*n* = 3). Asterisks denote significant differences from the control group (*P* < 0.05) analyzed using Student’s *t*-test.

*Figure 5* (A) *lhcgr*, (B) *cbr1l*, (C) *paqr8* mRNA levels in the ovary, normalized against β-actin and *gapdh* genes in females fed on *L. rhamnosus* (PROBIO) and in control females (CTRL). Data are given as mean ± s.d. (*n* = 3). Asterisks denote significant differences from the control group (*P* < 0.05) analyzed using Student’s *t*-test.
lepr gene transcription in the brain. These increases were correlated with a higher expression of kiss1, kiss2, and, in turn, gnrh3 genes found in the brain of treated females.

The stimulatory role of L. rhamnosus on female zebrafish fecundity may be due both to the activation of the endocrine control described above and to the direct action of leptin on the ovary. In mammals, binding of the endocrine control described above and to the peripheral levels are still unknown, and further studies are needed in order to clarify this aspect.

In conclusion, the stimulatory role of L. rhamnosus administration as a feed additive in zebrafish (D. rerio) follicle maturation. In particular, these findings suggested L. rhamnosus administration can control the expression of tgfβ1 and bmp15 mRNA levels which, in turn, can modulate cbr1l, lhcgr, and paqr8 gene expression. In fact, previous studies on zebrafish have described how treatment with recombinant human TGFβ1 inhibits oocyte maturation reducing Lh activity by decreasing lhcgr and cbr1l mRNA levels, and have shown decreased basal and hCG-induced paqr8 mRNA levels in zebrafish follicles (Kohli et al. 2005). Similar results have also been obtained with recombinant human BMP15, which also reduced hCG-induced oocyte maturation (Clelland et al. 2006). Moreover, the knockdown of bmp15 in oocytes, by antisense oligonucleotides, increased paqr8 expression (Tan et al. 2009), and the inhibition of bmp15 expression or function in class IIa follicles has been found to induce their sensitivity to MIH (Clelland et al. 2006, 2007).

The mechanisms through which L. rhamnosus stimulates lep gene expression both at central and at peripheral levels are still unknown, and further studies are needed in order to clarify this aspect.

In conclusion, the stimulatory role of L. rhamnosus administration as a feed additive in zebrafish follicle maturation, fecundity, and egg quality illustrated in this study indicates the great potential of this feed additive for ovarian physiology and presumably for embryo development.

Considering that the zebrafish has been established as a vertebrate model system that is useful for biomedical research, the results obtained here may also have a potential application in reproduction technology for all vertebrates, including mammals.
Materials and Methods

Animals and probiotic administration

Adult female and male zebrafish (D. rerio) were purchased from a local supplier, Acquario di Bologna, Bologna, Italy. The fish were acclimatized to the laboratory conditions and observed for clinical health for 4 weeks prior to start experiments. The animals were kept in 50 l glass tanks under controlled conditions (28 ± 0.5 °C and 14 h light:10 h darkness). The fish were fed on 1.5 g Tetramin (Tetra, Melle, Germany) granules twice a day.

Experiments were performed on two groups: control groups (CTRL) that were fed only on commercial diet and treated groups (PROBIO) that were fed on commercial diet mixed with lyophilized probiotic. The experiment was repeated three times.

The probiotic strain used was L. rhamnosus IMC 501, provided by Synbiotec s.r.l. (Camerino, MC, Italy) and supplied in a final concentration in the tank water of 10^6 CFU/ml as suggested by the producer.

The count of egg spawned output was performed every day at 09:00 h within 1 h after lights. The embryos were then transferred to a nursery tank and monitored till the hatching.

Histological study

Fixed gonads were prepared for histological examination using standard biological procedures. Gonads were embedded in paraffin wax and sectioned (7 μm) with a microtome (HM 355 Microm). The sections were stained using picroindigocarmine (Fluka no. 57 000) and mounted in Acolene, and then examined under a light microscope (Nikon 90i) with the software NIS element AR.

RNA extraction and cDNA synthesis

Total RNA was extracted from tissues using Minikit RNeasy (Qiagen) extraction kit following the manufacturer’s protocol. Total RNA extracted was eluted in 25 μl RNase-free water. Final RNA concentrations were determined by spectrophotometer, and the RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at −80 °C until use.

Total RNA was treated with DNase (10 U/l at 37 °C for 10 min; MBI Fermentas, Glen Burnie, MD, USA), and a total amount of 1 μg RNA was used for cDNA synthesis, employing iScript cDNA Synthesis kit (Bio-Rad).

Real-time PCR

PCR were performed with SYBR Green method in an iQ5 iCycler thermal cycler (Bio-Rad). Triplicate PCR were carried out for each sample analyzed. The reactions were set on a 96-well plate by mixing, for each sample, 1 μl diluted (1/20) cDNA, 5 μl 2 × concentrated iQ SYBR Green Supermix (Bio-Rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 μM forward primer and 0.3 μM reverse primer. The thermal profile for all the reactions was 3 min at 95 °C and then 45 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed, and it showed one single peak in all cases.

β-actin and gapdh were used as housekeeping genes in each sample to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification product was observed in negative control, and no primer-dimer formation was observed in the control templates. The data obtained were analyzed using the iQ5 optical system.

Table 1 Hatching time of embryos deriving from control or treated females.

<table>
<thead>
<tr>
<th></th>
<th>Hatching time (hpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48 ± 1</td>
</tr>
<tr>
<td>Treated</td>
<td>44 ± 1</td>
</tr>
</tbody>
</table>

Table 2 List of primers used for real-time PCR analyses.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>lep</td>
<td>AGCTTCCGGCGTCAACCTGTA</td>
<td>CAGCCGGGATACCTCTGGAATA</td>
</tr>
<tr>
<td>kiss1</td>
<td>ACAGACACTCGTGCCCACAGATG</td>
<td>CAATCGTGGACGATCTGCTGCTG</td>
</tr>
<tr>
<td>kiss2</td>
<td>ATTCCTCTATCGTCAATGACCTGA</td>
<td>TTGTTTTCAGGTTAAAGCATCATG</td>
</tr>
<tr>
<td>gnrh3</td>
<td>TTACATGAGATCAGAAGAAGGATG</td>
<td>CCTCGAGAGGCAACCTCTAGCAT</td>
</tr>
<tr>
<td>tgb1</td>
<td>TTCGTTGTTGCTCCAAGGACT</td>
<td>TGCAAGAGAGTTGCCATTTG</td>
</tr>
<tr>
<td>bmp15</td>
<td>AGGGTGACCCGGATCGACTAGT</td>
<td>TCGCTTTCTCAGGTAAAGCATCATTG</td>
</tr>
<tr>
<td>gdh9</td>
<td>CGGACCACACACCTCTTCCTC</td>
<td>TGGTCCCAGCTTCTTGACC</td>
</tr>
<tr>
<td>lhcr</td>
<td>GCGGAAAGGCTGATGCGGACAT</td>
<td>GGGCTCAGTGCTCGATGCC</td>
</tr>
<tr>
<td>cbr11</td>
<td>TCTATGCTTCTCTGCGGACAT</td>
<td>TCCCGATCTGTCATGATTA</td>
</tr>
<tr>
<td>pax6a1</td>
<td>CAAAGGACCTCTCTCTCC</td>
<td>GAGGGGTGGCTCTCC</td>
</tr>
<tr>
<td>β-actin</td>
<td>GTGACCCGGCCTGGGACTTGTG</td>
<td>GTGACCCGGCCTGGGACTTGTG</td>
</tr>
<tr>
<td>gapdh</td>
<td>GTGACCCGGCCTGGGACTTGTG</td>
<td>GTGACCCGGCCTGGGACTTGTG</td>
</tr>
</tbody>
</table>

www.reproduction-online.org
software version 2.0 (Bio-Rad). Modification of the gene expression is represented with respect to the control sampled at the same time of the treatment. In Table 2, the sequences of specific primers used were reported.

**Follicles in vitro maturation assay**

*In vitro* maturation assays were conducted as described previously (Clelland *et al.* 2006). The ovaries were teased into separate follicles using transfer pipettes (Samco Scientific Corp., San Fernando, CA, USA) without trypsinization. Therefore, stage IIIb follicles were separated according to diameters measuring (Selman *et al.* 1993, Wu *et al.* 2000) with an ocular micrometer under a dissecting microscope and sampled. Stage IIIb follicles derived from both the control and treated groups were incubated in 1 ml L15 medium at 25 °C in 24-well culture plates. For experimental purpose, incubations were carried out separately: stage IIIb follicles isolated from control groups ovary were incubated in L15 (CTRL) and L15+M1H (1 μg/ml) (MIH); stage IIIb follicles isolated from treated groups ovary were incubated in L15 (PROBIO) and L15+M1H (1 μg/ml) (PROBIO+MIH). Maturation was scored after 18 h of incubation. Follicles that underwent GVBD could be identified by their acquired translucency. Each experiment was conducted in four wells with ~20 follicles per well, and all the experiments were repeated three times.

**Statistical analysis**

Data presented are mean±s.d. for the number of experiments. Student’s *t*-test was used for comparison between the two experimental groups. *P*<0.05 was considered significant.

In *vitro* maturation results were examined by one-way ANOVA followed by the Bonferroni’s multiple comparison test, using a statistical software package Prism5 (GraphPad Software Inc., San Diego, CA, USA) with significance set at *P*<0.05.

**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.

**Funding**

This work was supported by ‘Fondi d’Ateneo 2009’ grant to O Carnevali.

**Acknowledgements**

The authors wish to thank Dr Cecilia Totti and Dr Chiara Pennesi for their technical assistance and support with microscopy; Madame Chantal Cuty, director of the plateau technique d’histologie of INRA-SCRIBE Rennes-francia for the support for the histological study; Synbiotech for providing probiotic; and OceAN soc coop for the technical support at the facilities.

**References**


Received 18 March 2010
First decision 3 June 2010
Revised manuscript received 28 July 2010
Accepted 8 September 2010