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Original Paper

Beneficial Bacteria Affect *Danio rerio* Development by the Modulation of Maternal Factors Involved in Autophagic, Apoptotic and Dorsalizing Processes

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Key Words
Probiotics • *Ambra1* • *Beclin1* • *Caspase3* • *Lc3* • *Bcl2* • *Bax* • Dorsalization • *Goosecoid* • *Chordin*

Abstract
Background/Aims: Probiotic strains have been recognized to exert important roles in many biological systems, including immune response, growth, development and reproduction. However, to date, no studies have focused either on the relation among probiotics and maternal factors or on probiotics' ability to qualitatively and/or quantitatively modulate maternal transcripts. ***Methods:*** In this study, the effects of *Lactobacillus rhamnosus* administered to parental fish on the control of maternal factors involved in autophagic, apoptotic and dorsalizing processes during zebrafish embryo development were assessed through q-PCRs, WMISH and TUNEL assay. ***Results:*** The results we obtained show that probiotic induced significant changes in both maternal and zygotic mRNA levels involved in embryo development. The maternal autophagy-regulating genes herein investigated -*ambra1a*, *ambra1b*, *beclin, lc3-*, as well as those involved in the apoptotic process –*caspase3, bcl2, bax*- were modulated in disfavor and favor of the treated group, respectively. Also, the key transcripts ruling the dorsalizing process –*goosecoid* and *chordin*- were subject to a significant regulation of their gene expression. ***Conclusion:*** The results we acquired demonstrated that parentally administered *Lactobacillus rhamnosus* is able to modulate important physiological processes involved in zebrafish embryo development.

Introduction

In zebrafish, as well as in every living organism, the very first processes occurring in the embryo are directed by maternal factors including mRNAs produced during oogenesis and stored into the mature oocyte. During this period, which is characterized by a species-specific

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length, mRNAs and proteins provided by the mother drive the development. Their presence, abundance and localization can control cell fate and patterning [1], regulate nuclear and cellular divisions and carry out processes related to cellular metabolism [2].

Many authors have described the role of maternal factors as being fundamental. They supervise the first life stages of the zygote and lay the foundation for a proper embryo development. Indeed, maternal factors are crucial for the early stages of animal development, as demonstrated either in invertebrate models such as *Drosophila* [3] and *Caenorhabditis elegans* [4] or lower vertebrates like ascidians [5], Xenopus [6] and zebrafish [2].

Maternal transcripts continue exerting their influences until the zygotic genome is activated and the newly formed individuals are then able to progressively control their own gene expression in an autonomous way. This is the so-called maternal-to-zygotic transition (MZT), also referred to as the mid-blastula transition (MBT). Throughout the period ranging from egg activation (in zebrafish, the egg is activated after fertilization) to MZT, the contribution of maternal factors gradually decrease, and they are destabilized by means of 3'-UTR deadenylation as the ultimate step of a cascade triggered either by egg activation [7] or embryonic miRNA [8]. Kane and Kimmel [9] reported that in zebrafish the mid-blastula transition begins at cell cycle 10, which roughly corresponds to 3 hours post-fertilization. Considering the biological importance and functions of the maternal control, it is clear that this process must be very finely regulated in order not to induce severe damages. To our knowledge, though, very little research has investigated the possible roles of either exogenous or environmental factors in its modulation. Specifically, probiotics were never tested in relation to maternal control, even though many studies have already demonstrated their supplementation to positively interfere with normal biological processes, in fact affecting the transcription of hundreds of genes ascribable to different molecular pathways.

The so-called beneficial bacteria have been studied extensively in terms of biological properties [10], genres composition and habitat preferences [11-13], and in some cases were found to be naturally present in the fish gastrointestinal microbiota [12]. Importantly, the ecological relations among gastrointestinal bacteria are responsible for benefits to the immune system [13-20], nutrient metabolism [21], growth [22], stress tolerance [23, 24], vertebral column deformities, bone calcification and density [25-28], development [29, 30] and reproduction [31-34], all deriving from their administration.

Taking into account the widely known potentialities of probiotics, we wondered whether the maternal control mechanism in *Danio rerio* could be affected too. In the present study, we evaluated the possible effects of the probiotic *Lactobacillus rhamnosus* on zebrafish maternal control with the aim of investigating whether its administration, as food supplement, provided to parental fish could eventually result in a difference of either the quality or quantity of transcripts loaded by the mother into the oocytes and therefore inherited by F1 fish.

Herein, we report our findings concerning the changes of the expression and localization patterns of some genes that are commonly recognized as biomarkers of the autophagic, apoptotic and dorsalizing processes in zebrafish embryos ranging from the 1-cell (0.2 hpf) to the long pec stages (48 hpf) -stages nomenclature as to [35]- when probiotic was provided to the parents.

Materials and Methods

Fish maintenance and embryo collection

Adult male and female zebrafish specimens purchased from a local supplier (Acquario di Bologna, Bologna, Italy) were acclimated to laboratory conditions and their health state was monitored for 4 weeks prior to the beginning of the experiments.

Parental fish were divided into a control group (CTRL), which was fed with commercial food, and a probiotic-treated group (PROBIO), that received a commercial diet containing the lyophilized probiotic at a final concentration of 10^6CFU/g. The probiotic strain, *Lactobacillus rhamnosus* IMC 501® (Synbiotec S.r.l., Camerino, MC, Italy) was mixed into the diet prior to providing fish with the food. All fish were served with

a quantity of food ranging from 1.5 to 2% of their bodyweight per day for at least ten days. The experiment was conducted in triplicate.

Procedures were performed in accordance with the Guidelines on the Handling and Training of Laboratory Animals by the Universities Federation for Animal Welfare (UFAW) and with the Italian animal welfare legislation (D.L. 116/92).

Fish tank parameters were kept constant in terms of water temperature (28 °C) and photoperiod (14/10 hours of light and dark, respectively).

Embryos obtained by natural spawning from each of the two groups were maintained in the same environmental conditions as adults and collected at 0, 2, 4, 8, 12, 24 and 48 hours post fertilization (hpf). Samples were taken for q-PCR, whole mount *in situ* hybridization analyses and TUNEL assay in triplicates consisting of 20, 10 and 10 individuals, respectively.

RNA extraction and cDNA synthesis

Total RNA was extracted from 20 embryos at the desired stage of development using RNAzol® RT reagent (SIGMA-ALDRICH®, R4533) according to the manufacturer's instructions. RNA was then eluted in 15 µl of RNAse-free water. Final RNA concentrations was determined using the Nanophotometer TM P-Class (Implem GmbH, Munich, Germany) while its integrity was verified by GelRed staining of 28S and 18S ribosomal RNA bands on 1% agarose gel.

RNA was stored at -80 °C until use. A total amount of 1 µg of RNA was used for cDNA synthesis employing Tetro Reverse Transcriptase cDNA synthesis kit (Bioline, BIO-65050) and the nucleic acids were then kept at -20 °C until use.

Quantitative Polymerase Chain Reaction (q-PCR)

q-PCRs were performed in an iQ5 iCycler thermal cycler (Bio-Rad, 179-8891) with SYBR green method. All samples were analyzed in triplicates and the final volume of each reaction was 20 µl. The single reaction mixture consisted of 2 µl of diluted cDNA (1/10), 10 µl of 2x concentrated iQ TM SYBR Green Supermix (Bio-Rad,170-8882) containing SYBR Green as a fluorescent intercalating agent, 0.3 µM of forward primer and 0.3 µM of reverse primer.

Real-time PCR conditions were optimized after various trials at different times and temperatures. Eventually, the reaction conditions were as follow: i) enzyme activation at 95°C for 3 min, ii) 45 cycles of denaturation (30 sec at 95°C) followed by the annealing stage (30 sec at 55°C for*18S*, 30 sec at 54°C for *ambra1a* and *ambra1b*, 30 sec at 59°C for *goosecoid,* 30 sec at 60°C for *beclin1, lc3, caspase3, bcl2, bax and chordin* and extension step for 20 sec at 72°C), iii) final hold at 4°C. Monitoring of fluorescence occurred at the end of each cycle. The extension phase of the last cycle was prolonged by 10 min. Primer specificity and the absence of primer-dimer formation during real-time PCR analysis was indicated in each data file by the presence of a single peak in the dissociation (melt) curve at the end of the amplification program. Primer sequences are reported in Table 1. The internal reference, chosen with the aim of standardizing the results by eliminating variations in mRNA and cDNA quantity and quality, was the *18S* rRNA. The obtained data were processed by the iQ5 optical system software version 2.0 (Bio-Rad) including GeneEx Macro iQ5 Conversion and Genex Macro iQ5 files.

Whole Mount in situ Hybridization (WMISH) and microscopy analyses

Zebrafish embryos were collected and fixed overnight in 4% paraformaldehyde (PFA, P6148, Sigma) in phosphate-buffered saline (PBS) at the desired developmental stages. When necessary, pigmentation was

Table 1. List of primers used to amplify selected genes through q-PCRs

	Sequence (5' - 3')		
	Forward	Reverse	Acc. number
18S	TCGAATGTCTGCCCTATCAACT	AGACTTGCCCTCCAATGGATC	AF308735
ambra1a	CTGCTGCTCATTGCCACC	CGCATCTCCACACTGTCC	HE602022
ambra1b	GCATACCACGTCAGACTCG	CCTACCATCACATAGCAGC	FR846230
beclin1	GGACCACTTGGAACAACT	CCGAAGTTCTTCAGTGTCCATC	AB266448
lc3	GAGAAGTTTTTGCCGCCTCT	ACCTGTGTCCGAACATCTCC	NM_199604.1
caspase3	GTGCCAGTCAACAAACAAAG	CATCTCCAACCGCTTAACG	NM_131877
bcl2	CCTTCAATAAAGCAGTGGAGGAA	CGGGCTATCAGGCATTCAGA	AY695820.1
bax	GGCTATTTCAACCAGGGTTCC	TGCGAATCACCAATGCTGT	AF231015
chordin	CTGTGGATTCTGCTGTCCGT	CCGAAGGAGCAACCGGATAA	NM_130973
goosecoid	TCCAGCGCCGAACTTACAAT	GCGCTGTCATAACCTGTAGGAA	NM_131017

Table 2. List of markers used in the Whole-Mount *in situ* Hybridization analyses

Gene	Reference	GenBank cDNA reference	Vector	Endonuclease and RNA polymerase
chd	Miller-Bertoglio *et al*., 1997.	AF034606	pBluescriptKS(+)	*Spe*I, T7
gsc	Schulte-Merker *et al*., 1994.	NM_131017	pBS SK	*Bam*HI, T7
becn1	-	NM_200872	pGEM	*Apa*I, Sp6
z-am1a1-3'-UTR	Benato et al., 2013	HE602022	pGEM	*Apa*I, Sp6
z-am1b-3'-UTR	Benato et al., 2013	FR846230	pGEM	*Sal*I, T7

removed by hydrogen peroxide treatment according to [36]. In all cases, embryos were then washed in PBT (PBS plus 0.1% Tween 20 – Sigma, P1379), dechorionated with forceps and stored in methanol at -20°C until use.

WMISH was performed on embryos deriving from both experimental groups according to the guidelines reported by [36]. All riboprobes for WMISHs are listed in Table 2.

TUNEL

Embryonic apoptotic cells were detected by the TdT-mediated fluorescein-dUTP nick-end labeling (TUNEL) assay. The TUNEL assay was performed using alkali stable digoxigenin-dUTP and TdT (terminal Deoxynucleotidyl transferase) (Roche, 03 333 574 001). Embryos were fixed in 4% PFA (overnight, 4°C), treated with methanol and stored at -20°C.

Methanol-stored embryos were rehydrated in methanol/PBS series and permeabilized by proteinase K (10 µg/ml). Then, embryos were washed in PBT (5 × 5 min, RT) and in ethanol/acetic acid (2:1) (20 min RT). After incubation with TUNEL buffer (30 min, RT), they were incubated in 100 µl TUNEL reaction mixture (overnight, RT), which was then washed, and therefore stopped, with PBT/EDTA 1 mM (2 × 1 h, RT). After that, samples were processed as for the whole-mount *in situ* hybridization and stained with the AP substrate Fast Blue [Fast Blue BB 4-benzoylamino-2,5-diethoxybenzenediazonium chloride hemi (zinc chloride) salt, Sigma, F3378] plus NAMP (3-hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate, Sigma, N5000). Stained embryos were mounted in 80% glycerol in PBT and examined with Leica SP5 confocal microscope.

Apoptotic nuclei were estimated within the same area in six embryos of each experimental condition. Quantification of numbers of apoptotic nuclei has been performed with ImageJ software by integrated density calculations on normalized and thresholded images. All data have been represented as the mean ± SD.

Statistical analysis

As far as results obtained with q-PCRs are concerned, the two experimental groups have been processed with a Two-Way ANOVA. Values were compared regardless of experimental group and developmental stages. Multiple comparisons were corrected with the Tukey test and the confidence interval was set at 95% ($p < 0.05$). Asterisks in the graphs represent the statistical difference between the two experimental groups at a given developmental stage, while letters above histograms symbolize the statistical difference within the same experimental group at consecutive developmental stages.

With regards to data deriving from the TUNEL analyses, the statistical difference was tested by the Student's t-test. Groups were considered significantly different if $p < 0.05$.

Concerning the outputs of the survival rates analyses, data were plotted as percentages but values were transformed with the arcsen function and tested for statistical significance by Student's t-test. Groups were considered significantly different if $p < 0.05$.

Results

L. rhamnosus on gene expression during zebrafish development

The administration of the probiotic *Lactobacillus rhamnosus* on zebrafish adult fish produced evident effects on both maternal and zygotic levels of transcripts herein considered as biomarkers of the autophagic, apoptotic and dorsalizing processes.

As far as the autophagic process is concerned, four well-established biomarkers of the process were investigated. A specific set of primers was designed to amplify all the isoforms of *ambra1a* [37], *ambra1b, beclin1* and *lc3*.

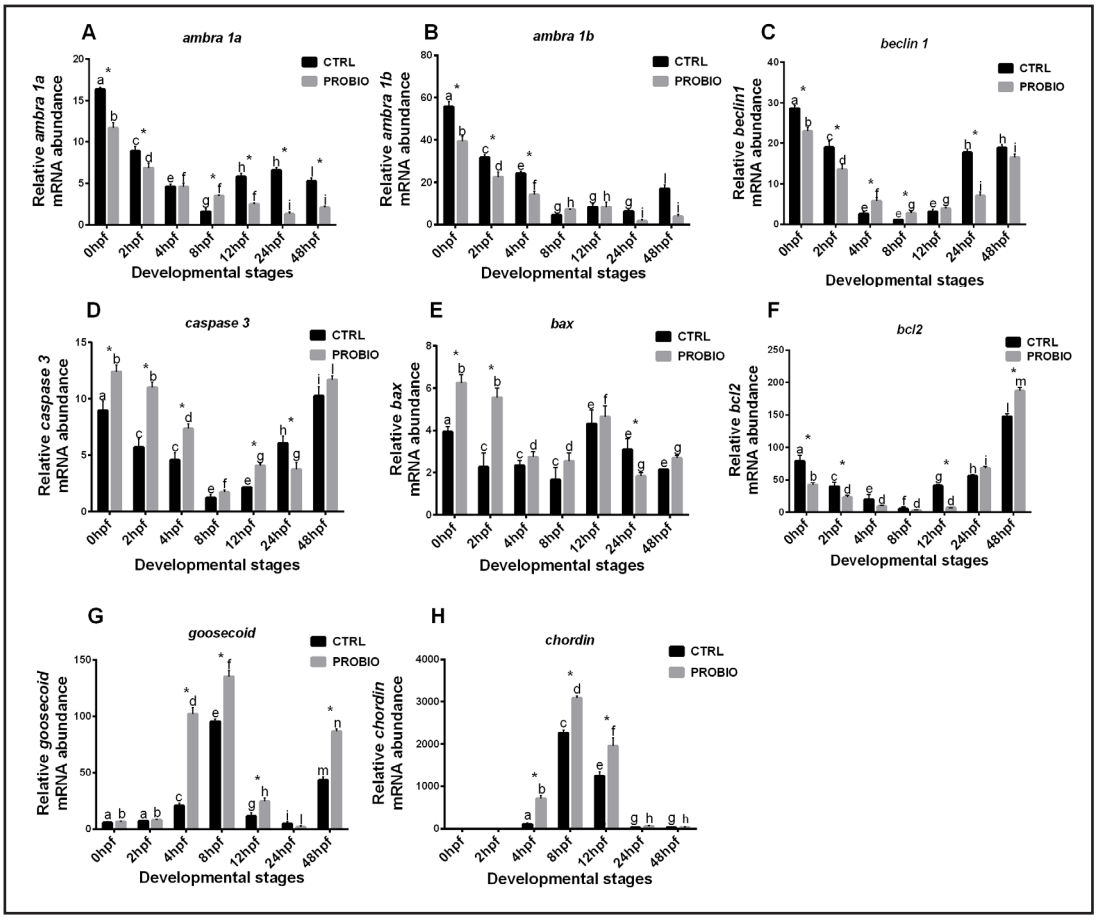

Fig. 1. q-PCRs graphs reporting the temporal gene expression of *ambra 1a1*, *ambra 1b*, *beclin 1*, *caspase 3*, *bax, bcl2*, *goosecoid* and *chordin*. mRNA levels normalized against 18S for the control (CTRL) and treated (PROBIO) groups. Error bars indicate mean ± S.D. Data were processed with a Two-way ANOVA followed by Tukey multiple comparison test. Confidence interval was set at 95% ($p < 0.05$).

Results obtained through q-PCR analyses exhibit the typical pattern of the maternally inherited transcripts throughout the first eight hours of development for both experimental groups (Fig. 1A,1B,1C,2A). The gradual exploitation of mRNAs, and therefore the constant decrease of their expression levels, is clearly deduced in all four cases (Fig. 1A,1B,1C,2A). Evidently, in the majority of the developmental stages ranging from 0 hpf to 4 hpf, except for *beclin1* and *ambra1a* levels (the latter of which do not show any statistical difference at 4 hpf), embryos descending from probiotic-treated fish have a statistically significant lower availability of autophagy-related transcripts (Fig. 1A,1B,1C,2A). Conversely, an opposed scenario was detected at 8 hpf, a stage at which treated embryos showed a higher quantity of all genes with respect to controls. It must be pointed out, though, that those differences were supported by statistical significance only in *ambra1a* and *beclin1* cases.

A situation similar to those uncovered for the first four hours of development (i.e. higher levels of autophagy-related transcripts in control embryos than in treated ones) could be identified in the remaining developmental stages (12, 24 and 48 hpf) for *ambra* and *beclin 1* genes. *Beclin1* at 12 hpf was the only exception (Fig. 1C). In particular, differences of *ambra1a* expression levels between the two experimental groups were always statistically significant for the three above-mentioned stages (Fig. 1A). *Ambra1b* expression differences among groups were never significant, even though controls had higher transcript levels (Fig. 1B), while a statistical difference in *beclin1* mRNA abundance was found only at 12 hpf (Fig. 1C).

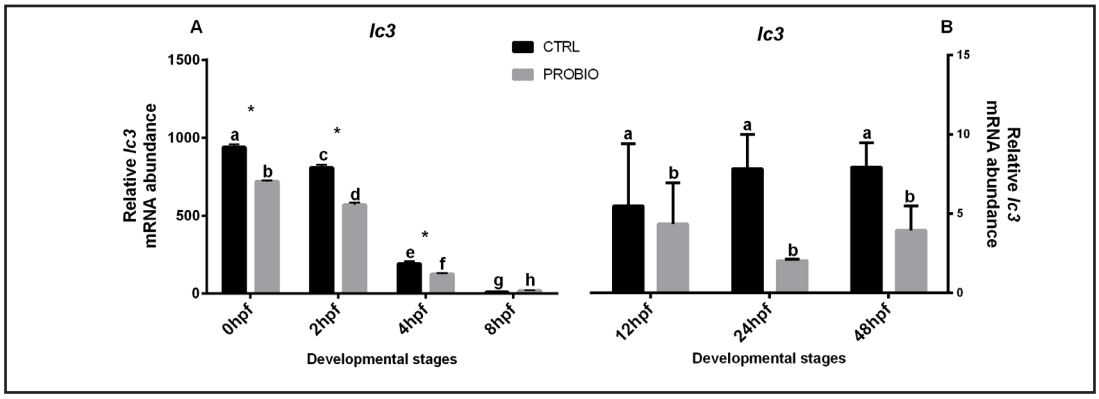

Fig. 2. q-PCRs graphs reporting the temporal gene expression of *lc3*. mRNA levels normalized against 18S for the control (CTRL) and treated (PROBIO) groups. Error bars indicate mean ± S.D. Data were processed with a Two-way ANOVA followed by Tukey multiple comparison test. Confidence interval was set at 95% ($p < 0.05$).

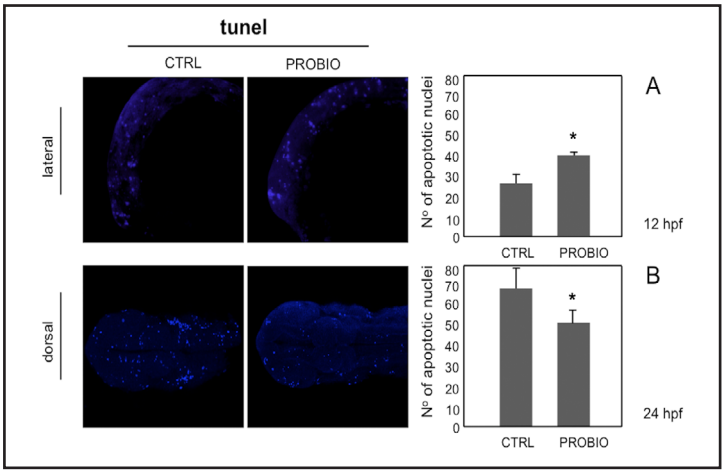

Fig. 3. TUNEL analysis to detect apoptotic nuclei in probiotic treated and control embryos at 12 (A) and 24 hpf (B). Values represent the mean ± SD (n = 6). Asterisk indicates significantly different number of apoptotic nuclei between experimental groups.

Lc3 deserves to be separately taken into account, since its transcriptional level has a very different range before and after the 75%-epiboly developmental stage (Fig. 2). In any case, however, Lc3 level is always statistically lower in the PROBIO group.

In addition, while *ambra1a* and *beclin1* maternal transcripts were evidently replaced by zygotic messages after the 8 hpf (Fig. 1A,1C), *ambra1b* and *lc3* expressions after the MZT was generally maintained to levels as high as those present at the 75%-epiboly stage (Fig. 1B,2B).

Concerning WMISH, no clear differences were detected between the two groups for each of the three autophagy-related transcripts analyzed (data not shown). Although it is important to point out that such technique is not quantitative but instead gives us qualitative evidences, the discrepancy among q-PCR results before the MZT stage could be also due to the small expression disparities at those developmental stages. After MZT, the widespread embryonic expression of these genes prevents from the possibility to observe differences clearly.

Regarding apoptotis, we chose to investigate some of the most known biomarkers of the process, the *caspase3*, *bax* and *bcl2.* Similarly to autophagic biomarkers, these apoptotic proxies were reported to follow the typical tendency of maternally controlled genes, as depicted in the gene expression graphs (Fig. 1D,1E,1F).

Despite a declining trend of transcripts belonging to the two different biological process is maintained, the administration of *L. rhamnosus* led to very different consequences in the case of apoptotic markers. Indeed, the beneficial bacterium positively interfered with the relative abundances of *caspase3* and *bax* messages since the biomarkers' relative quantities, at most stages, were higher in embryos descending from probiotic-treated fish than controls',

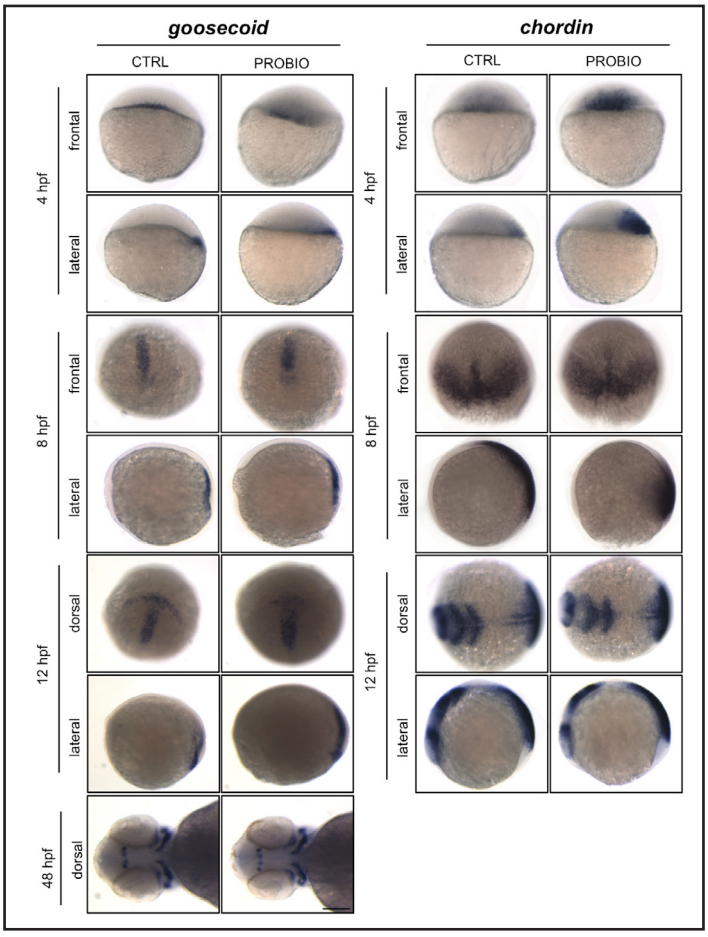

Fig. 4. Whole Mount *in situ* Hybridization analysis of *goosecoid* and *chordin* expression in probiotic treated and control embryos. Analysis of *goosecoid* expression in embryos treated with the probiotic *L. rhamnosus* compared to control embryos at 4, 8, 12 and 48 hpf and of *chordin* expression in the same groups at 4, 8 and 12hpf. According to q-PCR results both markers are up-regulated at 4 hpf in probiotic treated embryos compared to controls, although the over-expression is more evident for *chordin*. At 8 hpf *chordin* expression seems to be reduced ventrally in probiotic treated group compared with controls. No clear differences were detected at the other developmental stages (scale bar: 200 μm).

except for 24 hpf (Fig. 1D,1E). This cannot apply to *bcl2,* which is an anti-apoptotic signal, since it generally exhibited a trend opposed to that of the *caspase3* (Fig. 1F).

These results were supported by the TUNEL assay performed at two sampling times, 12 and 24 hpf (Fig. 3). The abundance of apoptotic nuclei, hence the number of cells undergoing programmed death, was highlighted in light blue at both lateral and dorsal views. At 12 hpf, PROBIO embryos were characterized by a greater number of apoptotic nuclei with respect to wild type embryos (Fig. 3A), while at 24 hpf the scenario was opposed and we could appreciate a higher amount of them in the control embryos than in the probiotic-treated ones (Fig. 3B).

In order to further investigate the consequences of *L. rhamnosus* administration on the modulation of *D. rerio* gene expression, a pivotal process such as the dorsalizing process was taken into account. The biomarkers we selected to analyze it were *goosecoid* and *chordin*.

These genes, distinctly from the ones previously described, did not exhibit any maternal trend throughout the first eight hours of development and, in fact, they are commonly referred to as early developmental genes (Fig. 1G,1H).

The probiotic, though, provoked severe changes on their expression pattern. Both were strongly up regulated throughout the embryonic development, starting from 4 hpf. At this sampling time, the fold change of *goosecoid* and *chordin* expressions was extremely up regulated in favor of the treated group, being equal to approximately 5 and 6, respectively (Fig. 1G,1H). At this developmental stage, the up-regulation of these genes could be appreciated also in the WMISH analysis (Fig. 4) whereas, later on, the differences are still present but less evident, in agreement with the lower transcriptional increase and the technique's low efficacy of quantification. Furthermore, *goosecoid* exhibited a significant increase at 48 hpf, in both experimental groups, with statistically higher rates of gene expression still belonging to the probiotic-treated one (Fig. 1G). *Chordin* did not show this peculiar expression pattern

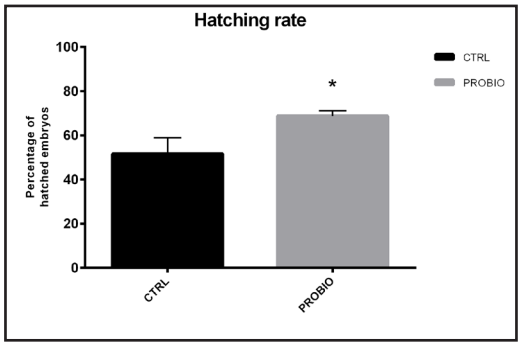

Fig. 5. Hatching rates of the two experimental groups. Percentage values were transformed with the arcsen function and submitted to Student's t-test statistical analyses ($p < 0.05$).

and its relative abundance after the 12 hpf reverted to the same levels of those present at the first two developmental stages (Fig. 1H). By a qualitative point of view, WMISH did not show evident changes in the expression domain of the two dorsalizing genes analyzed. However, at 75% of epiboly (8 hpf), *chordin* expression seems to be reduced ventrally in PROBIO group compared with WT.

Remarkably, no statistically significant evidence could be appreciated among groups at 0 and 2 hpf for any dorsalizing-related messages. Hence, in these cases, probiotic administration did not influence the maternal storage of transcripts (Fig. 1G,1H).

At last, the hatching rates of the two groups were assessed. We found that the 68.8% of PROBIO embryos, compared to the 51.7% of wild types, successfully hatched after nearly 60 hours post-fertilization (Fig. 5).

Discussion

In this study we assessed the effects of the probiotic *L. rhamnosus* on zebrafish's transcriptional maternal control. We specifically focused to identify the temporal and spatial expression patterns of some genes that have been recognized to play a key role in the autophagic (*ambra1a*, *ambra1b*, *beclin1, lc3*), apoptotic (*caspase3, bcl2, bax*) and embryonic dorsalizing processes (*goosecoid*, *chordin*) in embryos descending from fish supplemented with such beneficial bacteria.

Previous experimental findings have asserted that probiotic administration is beneficial to the i) IGF system and the gonadal development [26], ii) body growth [19-22, 38, 39], iii) immune system [18,40, 41] and iv) reproduction [31, 32, 37].

Our initial hypothesis, based on the higher levels of autophagic signals that Gioacchini and collaborators [33] found in class III and IV zebrafish oocytes sampled from *L. rhamnosus*-treated females, was to expect an increase of the autophagy-related signals in embryos belonging to the treated group. This hypothesis was not supported by the results here obtained. Even though autophagy was recognized as a process of pivotal importance [42], its regulating genes, which have been described by numerous studies [43-46], were found to be less expressed in the embryos of the PROBIO group than in controls. We could therefore speculate that some fundamental, yet unknown, process influencing the amount of both autophagic and apoptotic messages has happened during oogenesis at class V zebrafish oocytes.

The expression of *ambra1a*, *ambra1b*, *beclin1* and *lc3* was generally lower in almost every analyzed developmental stage, except for the 8 hpf, a step at which the differences among the two groups were statistically significant in favor of treated organisms, at least for the isoform *ambra1a* and for *beclin1.* These differences could be appreciated also at 4 hpf for *beclin1*.

Noteworthy, Benato et al. [37] report that in zebrafish the expression of the autophagic genes is driven by maternally controlled transcripts stored into the oocytes during the oogenesis. Such transcripts follow the typical maternal tendency, being firstly very abundant, then progressively exploited and eventually replaced by correspondent message RNA by the zygotic machinery itself. Such transcripts appear to be present in high copy numbers during

the very first hours of development while we could observe a gradual decrease of their levels until a basal level at 8 hpf is evident [37].

However, it must be kept in mind that He and collaborators [47] did not find any evidence of autophagy in zebrafish until 32-48 hpf, a developmental time at which the authors observed the conversion of Lc3-I to Lc3-II. Therefore, the transcripts in question -*ambra1*, *beclin1* and *lc3*- may have an active role in the developmental process other than the autophagic one. This is in agreement with what was proposed by Benato and collaborators [37], whom have uncovered the severe alteration of the nervous system arising in Ambra1a and b-deficient zebrafish embryos trough knockdown experiments, and Skobo and co-workers [48], that have suggested the involvement of the protein isoforms in the zebrafish skeletal muscle development.

Considering the higher hatching rates found in the present study for the embryos belonging to the treated experimental group and the lack of body plan alterations at a morphometric level, we can assume that *ambra1* relative abundances, although lower than those of controls, were still adequate to ensure a correct embryonic development. In fact, distinctly from the knockdown described by Benato and co-workers [37], in the present study, *ambra1* was far from being completely abolished and we can speculate that its lower levels were still able to carry out its functions.

Regarding *lc3*, we found a peculiar transcriptional trend. It consisted in the presence of a high copy number in the first three analyzed developmental stages, which demonstrates the maternal deposition and therefore control of the signal, and in a very low expression levels in all the remaining sampling times. Again, it is important to point out that Lc3 message is present at a stage at which the autophagic process is likely inactive, leaving the field open for speculations. Nevertheless, Lee and co-workers [49] demonstrated the presence of autophagic activity through a variety of techniques in 16 hpf-old zebrafish embryos and 6 hpf-old embryos cell cultures. The results we obtained –lower levels of *lc3* transcripts as well as other autophagic markers, in the PROBIO group- could be explained by a nutrient-richer yolk which may allow a reduced deposition of this transcript by the mother.

Very interesting is the information we acquired at 8 hpf for *ambra1a* and *beclin1* genes. The differences among embryos of the two groups are statistically significant, with the treated group showing a higher gene expression. Since in zebrafish the zygotic transcription machinery is activated after the maternal to zygotic transition which occurs in a time lapse ranging from 4 to 8 hours post fertilization, we can speculate that embryos deriving from fish that had been fed with probiotic were able to gain the full control of their own gene expression in shorter times compared to controls. At this regard, in the future, additional morphological studies could be useful to reveal possible biological differences among the two groups.

Moreover, embryos pigmentation was slightly anticipated, as eye and body pigmentations appeared first in embryos descending from probiotic-treated fish but not in wild types. To find this peculiarity solely in one of the two groups led us to hypothesize the existence of important effects deriving from probiotic administration that could also support the idea of an accelerated embryonic development [26, 27, 29].

We can then speculate that *L. rhamnosus* supplementation brought differences in maternal mRNA storage process from mothers to oocytes during the last phase of oogenesis. However, we cannot exclude a role of the probiotic on sperm chromatin structure affecting the embryo epigenome, leaving this field open for further investigations.

Calling the attention on the apoptotic process during embryo development, we chose to monitor the transcriptional temporal pattern of expressions to see how beneficial bacteria would modulate the Caspase3, Bax and Bcl2 genes. These genes are important element of the apoptotic machinery [50, 51], but just the former of the three acts as apoptotic effecter by targeting substrates for proteolytic cleavage specifically at aspartyl residues [52, 53] and carries out its functions during organogenesis. The caspase cascade requires an accurate regulation, for the proteolytic process, unlike most other post-translational modifications, is irreversible [54]. For this reason, every molecule implicated in the cascade is translated

as pro-enzyme, which is then activated either by proteolytic cleavages through the action of other caspases or through an autocatalytic process [55].

Despite a similar declining trend of messages was observed in the two groups among 0 and 8 hpf, probiotic administration substantially up-regulated the levels of both maternal and zygotic *caspase3* and *bax* expression, while *bcl2*, which is an anti-apoptotic molecule involved in the regulation of apoptosis [56], showed an opposed tendency, its transcriptional activity being generally higher in the control group than in the PROBIO.

These findings, taken together with the information resulting from the TUNEL assay, suggest us the existence of a higher rate of apoptosis in embryos of the PROBIO group with respect to the development in the control.

Apoptosis is usually linked to the notion of programmed cell death. Despite the negative acceptation of the concept, a number of papers report its essential role in processes such as cellular differentiation [57] and development [50]. In a paradoxical way if the words cell and death are kept in mind, caspases were found to be required for sperm differentiation, and therefore initiation of life, in Drosphila [58]. Caspase8, in addition, may act a function in cellular differentiation, since knockdown experiments carried out on mouse conducted the experimental model to defects insurgence of the cardiac muscles anatomy [58].

Wang and Lenardo [50] highlighted the essential role of Caspase3 and 9 in the mouse brain development. They found that the survival rates of *caspase*-deficient mouse embryos were extremely low and that they were usually led to death after three weeks from birth because of significant defects in the brain anatomical structures.

Considering these studies, we would speculate on the fact that finding higher expression of the transcripts directly related to the apoptotic process in embryos belonging to the treated group, especially in the first hours of life and therefore development, could be a positive factor. Indeed, the most critical morphological and functional modifications happen in that time lapse, and cellular degradation and proliferation support differentiation and development [59].

As last resort, we focused our attention on the dorsalizing process. We did that in order to seek for data that could confirm the hypothesis that was taking shape and, considering the higher apoptotic rates, we wished to assess whether the embryonic development of the PROBIO group could be somehow accelerated by the probiotic administered to their parent. Among the many molecular signals implicated in embryonic dorsalization and body axis formation, we chose *goosecoid* and *chordin* as its proxies.

The results collected from q-PCR confirmed our suggestion. *L. rhamnosus*, mostly between 4 and 12 hours post fertilization, was able to significantly and positively interfere with the biomarkers' expression pattern.

Goosecoid and *chordin* established themselves as key factors in the formation of the body plan in the mid-nineties: the former was described as a dorsal-specific gene [60] induced in its expression by, at the highest level of a molecular cascade, the Nieukoop Center and the Spemann organizer. The latter was localized in the dorsal region and was demonstrated to inhibit, together with *noggin* and *follistatin*, the dorsal genes repressor Bone Morphogenetic Proteins [61].

According to data emerged in literature [62, 63], these signals are referred to as immediate early genes and are stated to be necessary starting off from the first life stages of the embryos. They did not exhibit any maternal trend throughout the first hours of development; however, in agreement with Stachel's results [64], very low level of maternal *goosecoid* transcripts have been detected at 0 and 2 hpf.

Regarding *goosecoid* temporal expression, we were able to confirm all of the evidences already reported on the matter by Schulte-Merker and co-workers [62] in 1994.

In relation to *chordin*, its biological importance as inhibitor of ventralizing signals was thoroughly investigated in the past years [63, 65]. Sasai and collaborators [66] and Jones and Smith [67], independently on one another, published data revealing the relation between the homeobox gene *goosecoid* and *chordin*, whose expression was demonstrated being highly subjected to, and actually starting in Spemann's organizer after that of *goosecoid*.

Therefore, their work strongly linked the just-mentioned molecular signals. The information we obtained seem to be consistent with these findings, since, data from q-PCRs showed how *chordin*'s maximal temporal expression was slightly shifted compared to that of *goosecoid*, presenting the highest values at 8 and 12 hpf, but anyhow triggered in its start at the same developmental stage.

From a qualitative point of view, WMISH results did not show evident changes in the expression domain of these two dorsalizing genes and they basically correspond to those previously described for *goosecoid* [62, 64] and *chordin* [68].

Dorsalization-wise, the results of the analyses showed the outstanding up-regulation of the dorsalizing key factors' gene expression found in fish derived from probiotic-treated parents.

Taken together, these data suggest us that the supplementation of *Lactobacillus rhamnosus* induced remarkable changes in the maternal and zygotic control of F1 fish, enabling them to undergo a faster and more successful embryonic development. Nevertheless, considered the novelty of this research field, the transcriptional profiles and the results of TUNEL assay we herein reported could represent a starting point on which further biochemical and morphological analyses should be based to better elucidate the role of such beneficial bacteria in zebrafish maternal control.

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