Debaryomyces hansenii CBS 8339 promotes larval development in *Seriola rivoliana*

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Abstract

The present study aimed to know the effect of the administration of *Debaryomyces hansenii* yeast on growth, development and skeletal deformities, through the quantification of molecular and morphophysiological indicators in longfin yellowtail larvae. Larvae from the yeast group were fed with the rotifers and *Artemia* metanaupli half enriched with *D. hansenii* (50% of live prey) and half enriched with Origreen® (50% of live prey) and were compared to control (100% Origreen®) from 5 to 30 days post-hatching (DPH). Each treatment had two methodological replicates. Studies on bone mineralization, skeletal deformities, intestinal histological analysis, digestive enzyme activity, differential gene expression (RT-qPCR) and transcriptome analysis (RNA-Seq) were performed in whole larvae. Results showed that larvae fed *D. hansenii* encapsulated within enriched live preys had higher survival and growth, higher intestinal mucin secretion, as well as higher activity of alkaline phosphatase, pepsin and α-amylase. The degree of bone mineralization in the cranial and caudal fin complex was higher in larvae fed *D. hansenii* and a lower incidence of deformities at the vertebral column was also observed. In general, the gene expression throughout the development fluctuated between the control group and the yeast treatment; however, at 30 DPH, a higher expression of the bone morphogenetic protein type 2 (*bmp2*), collagen type 1α1 (*col1α1*) and proliferating cell nuclear antigen (*pcna*) genes was detected in larvae fed with yeast. The transcriptomic analyses using RNA-Seq revealed that the main genes related to bone mineralization degree and digestive tract maturation, were overrepresented in the *D. hansenii* treatment, 1,25-dihydroxyvitamin D (3) 24-hydroxylase (*cyp24*), cytochrome P450 family 27 subfamily A (*cyp27a*), protein 5 related to the low-density lipoprotein receptor (*lrp5*), myocyte-specific enhancing factor 2C (*mef2*), enterokinase (*entk*), *pepsin* and *alkaline*

phosphatase. Based on the results, *S. rivoliana* larvae supplemented with the yeast *D. hansenii* presented higher growth and survival, a higher degree of maturation of the digestive tract, a higher degree of bone mineralization and a reduction in skeletal deformities, for which the continuous use of yeast is recommended as a food supplement to larvae from 5 days post-hatching onwards.

Keywords: longfin yellowtail, probiotic, transcriptomic, digestive enzyme, bone.

1. Introduction

The need for sustainable aquaculture has encouraged the scientific research community to use probiotics as an eco-friendly health strategy (Carnevali et al., 2017). In the last decades, the use of probiotics for fish larvae and juveniles has gained popularity, mainly with the use of lactic acid bacteria (LAB) (Ringø and Gatesoupe 1998, Hoseinifar et al., 2014; Ringø et al., 2018, 2020). Although yeasts have been less studied as probiotics, several studies have suggested that they could be a good option to be used in aquaculture (Navarrete et al., 2014; Raggi et al., 2014; Hernández-Contreras et al., 2020; Reyes-Becerril et al., 2021).

Yeasts have been identified as part of the normal microbiota of both wild and farmed fish, and their role in fish health and nutrition has been addressed in the literature, as yeasts have been used either alive to feed live food organisms or after processing as a feed ingredient after demonstrating artificial colonization of the intestinal host (Navarrete and Tovar, 2014). In addition, yeast secretory metabolites have been proposed as major contributing factors of gut microvillus morphology, cell maturation and differentiation, gut maturation, and reduced stress status of animals, which are desirable probiotic attributes (Angulo et al., 2020).

In this context, several marine strains of the yeast *Debaryomyces hansenii* have shown probiotic and immunostimulatory effects on aquatic animals (Angulo et al., 2019). *D. hansenii* is a non-pathogenic, extremophilic yeast of the family Saccharomycetaceae that can be found in many habitats with a low water activity (a_w), such as in seawater from which it was initially isolated; although it has been also isolated in cheese, meat, wine, beer, fruits, soil, as well as in products rich in sugars (Breuer et al., 2006). Furthermore, *D. hansenii* CBS 8339 is a polyamine-producing yeast isolated from the digestive tract of rainbow trout (*Oncorhynchus mykiss*), which is also capable of adhering to the intestinal mucus of other teleost species and has proven probiotic attributes in fish (Reyes-Becerril et al., 2008; 2021). The longfin yellowtail (*Seriola rivoliana*, Carangidae) is a fish species candidate for aquaculture because of its rapid growth and high market value (Kissinger et al., 2016). Although some countries have implemented commercial production of this warm water fast-growing species, limited information on larval development is available, and the larviculture of this species is still a bottleneck in its production process (Roo et al., 2012; Mesa-Rodríguez et al., 2014; 2016; Teles et al., 2017, 2019).

At hatching, *S. rivoliana* larvae present an incipient digestive tract, which takes 3 to 4 weeks (Teles et al., 2019) until it becomes fully functional. During this period, there are high mortality rates due to the fragility and non-maturation of the digestive tract. The most common indicators of digestive maturation in fish larvae are the enzymes secreted by the exocrine pancreas, as well as those found in the brush border membrane of enterocytes (Tovar et al., 2004). Besides digestive tract maturation, skeletogenesis is a key morphogenetic event in the embryonic and post-embryonic development of vertebrates by which the skeletal structures are formed (Fernández et al., 2017). In this regard, it is well known that for both commercially produced finfish and diversification candidates for aquaculture, the normal

pattern of skeletogenesis may be altered under aquaculture operations, causing skeletal deformities. Similarly, to other *Seriola* species, skeletal anomalies constitute one of the most important bottlenecks during the hatchery phase, taking this into account, skeletal studies are important sources of basic knowledge to characterize and understand the osteological development and mineralization process, which in turn would help to understand and prevent the appearance of skeletal abnormalities (Mesa-Rodriguez, 2014; 2016).

Recently, we observed the humoral immunity of skin mucus improvement of longfin yellowtail juveniles fed *D. hansenii* CBS 8339 (Hernández-Contreras et al., 2020); however, more studies are needed during larval stages to improve quality and survival., In this sense, this study aimed to examine the effect of administration of live yeast *D. hansenii* CBS8339 through live prey enrichment on *S. rivoliana* larval development, with special emphasis on the functionality of the digestive system and skeletal quality.

2. Material and methods

2.1. Egg production and larviculture of S. rivoliana

Eggs were obtained by natural spawning from a batch of *S. rivoliana* breeders (5 males and 5 females) confined in Kampachi Farms at Centro de Investigaciones Biológicas del Noroeste (CIBNOR) in La Paz, Baja California Sur (Mexico). Fertilized eggs (89% fertilization rate) were transferred to four larviculture tanks (two tanks for the control group and two tanks for the experimental group) with 540 L of water volume at a density of 100 eggs L^{-1}. The temperature was maintained at 23.0 ± 0.3 °C (mean ± standard deviation, SD), dissolved oxygen concentration at 6.8 ± 0.8 mg L^{-1}, and salinity 37.0 ± 1.0 g L^{-1}. Photoperiod changed along larviculture; in particular, 24 Light (L) from incubation to 9 DPH (days post-hatching),

from 9 to 14 DPH 18:6 L:D, and 13:11 L:D on day 15 afterward. At hatching and during the first 5 days, water was not renewed in the rearing tanks, after that, water exchange progressively increased by 20% until 600% (per day) at 30 DPH. The experiment complied with the Guidelines of the European Union Council (2010/63/EU) and the Mexican Government (NOM-062—ZOO-1999) for the production, care, and use of experimental animals, and with the ARRIVE guidelines (Percie du Sert et al., 2020).

For evaluating the impact of the administration of yeast on *S. rivoliana's* development and performance, one group was fed with live food enriched with Origreen® (Control) and another group with live food enriched with Origreen® and yeast (YE).

The feeding protocol is shown in Table1, larvae were fed from 2 to 18 DPH with enriched rotifers (*Brachionus plicatilis*) with a density of 20 rotifers mL^{-1} in a green water system with fresh microalgae *Nannochloropsis oculata* at a density of 0.5 x 10^6 cells mL^{-1}. Rotifers and *Artemia* metanauplii were enriched with a commercial artificial diet Origreen® (Skretting; crude proteins 43 %, crude lipids 30 %, ashes 12 %), following the manufacturer's instructions. From 12 to 15 DPH nauplii of *Artemia* Biogrow® (Proveedora de Insumos Acuícolas, S.A de C.V.; protein 70.2 %, lipid 20.8 %, ash 6.1 %) were provided to larvae (0.2-2 nauplii mL^{-1}). From 15 to 30 DPH, larvae were fed with enriched *Artemia* metanauplii (2 to 5 mL^{-1}). A compound dry diet was first offered at 18 DPH with inert diet Otohime® (Reed Mariculture Inc; protein 56.3 %, lipid 15.9 %, ash 13.5 %, particle size 250-840 μm). The acceptance of live preys was daily examined under a binocular microscope using the level of gut fullness in their transparent digestive tract (*n=10*), and food ratios were adjusted according to the feeding protocol.

2.2. Live Food Enrichment

138 *D. hansenii* yeast (CBS 8339) were cultivated at 30 °C for 24 h in Yeast extract peptone
139 Dextrose (YPD) medium (Y1375; Sigma-Aldrich, Madrid, Spain) supplemented with
140 ampicillin (50 mg L^{-1}) (Sigma-Aldrich, Madrid, Spain), then, the CFU were grown in 150
141 mL YPD broth, stirred at 150 rpm at 30 °C for 24 h. For scaling up the production, incubation
142 was made in 2 L Erlenmeyer with YPD broth and stirred for 24 h. The cells were recovered
143 from YPD broth by centrifugation (5 min, 1,000 x *g*, 4 °C).
144 Rotifers were harvested from their culture tanks, washed with filtered seawater and
145 concentrated into a bucket (15 L), enriched with Origreen® (Skretting) 0.25 g x 10^6 rotifers,
146 for 2 hours, then harvested and washed. One part was provided immediately for the larvae
147 and another part was reconditioned in coolers at 4-10 ° C, until the next feeding (4 h later).
148 Exclusively for the *D. hansenii* treatment, half of the rotifers were enriched with commercial
149 enrichment as mentioned above and the other half was enriched with yeast in the following
150 way: rotifers were harvested from the culture tank, filtered and washed with filtered seawater,
151 once they were clean and concentrated in a bucket with aeration, yeast was added at the ratio
152 of 1 g x 10^6 of rotifers. They were incubated for 15-20 min (Burgoin, 2015); then, washed,
153 filtered, concentrated and distributed to the larviculture tanks. Yeast enrichment was also
154 performed with *Artemia* at the same proportion (50% commercial enrichment: 50% yeast).
155 Incubation with *Artemia* metanauplii followed the same procedure as for rotifers; however,
156 the enrichment time lasted for 45 min. This time was previously determined by fluorescence
157 microscopy in which yeast was labeled with the 5-DTAF (5- (4,6 dichlorotriazinyl)
158 aminofluorescein).
159
160 *2.3. Larval sampling and growth measurements*

Variable numbers of larvae (*ca*. 3 to 200 larvae, depending on the type of analysis) were collected with a 200-µm diameter net before early morning feeding. For growth measurements larvae were sampled at 0, 5, 7, 10, 20, 25 and 30 DPH, for gene expression at 6, 15, 25 and 30 DPH, for enzymatic activity, mucin production and transcriptome analysis were collected at 15 and 30 DPH, whereas for evaluating the ossification degree they were just collected at 30 DPH. Larvae were euthanized in an ice bath; rinsed with distilled water and stored, until analytical analysis, at -80 °C for gene expression, transcriptome analysis and enzymatic analysis. For histological analysis and double staining technique, larvae were fixed in Davidson´s solution for 24-48 h, and then preserved in EtOH 70% until analysis.

Using a binocular microscope, standard length (SL) was measured to the nearest 0.01 mm, from the tip of the maxilla to the posterior end of the notochord. Larval growth was determined as the absolute growth rate (AGR) as mm day^{-1} and specific growth rate (SGR) as % day^{-1}: $AGR = [(SL_f - SL_i) / \Delta t]$ and $SGR = [100 (\text{Ln } SL_f - \text{Ln } SL_i) / \Delta t]$ (Hopkins 1992); where SL_f is final standard length, SL_i is initial standard length, and Δt is the time interval (days) between sampling points. Larval development was scaled to age (days post-hatching, DPH) and thermal age units (cumulative degree-days after hatching, CTU) were used for comparisons between closely related species. Survival rate (%) = 100 × (final fish number/initial fish number).

2.4. Enzymatic assays

Two different groups of enzymes were assayed: extracellular enzymes and brush border enzymes. The extracellular enzymes assayed were trypsin, chymotrypsin, pepsin-like (acid proteinases like enzymes belonging to the aspartic peptidases family, such as cathepsin D

184 and E), α-amylase and lipase. The brush border (BB) enzyme assayed was the alkaline
185 phosphatase.
186 Enzymatic extracts were obtained by homogenization of three pooled samples of larvae for
187 each sampling day in Tris–HCl 50 mmol L^{-1}, $CaCl_2$ 20 mmol L^{-1}, pH 7.4 buffer solution (1:5
188 w/v) using a Teflon pestle in an ice bath until complete tissue disintegration and frozen at -
189 80 ºC until analysis. Enzymatic activity was expressed as U $larva^{-1}$ after photometric (pepsin
190 and α-amylase) and fluorometric (trypsin, chymotrypsin, lipase and alkaline phosphatase)
191 analyses. Trypsin, chymotrypsin and lipase activities were assayed by the fluorometric
192 method as described by Toledo-Cuevas et al. (2011). The pepsin-like activity was evaluated
193 according to Anson (1938) and α-amylase was assayed according to Vega-Villasante et al.
194 (1993), both enzymes determined by spectrophotometric method. The measurements were
195 carried out on a Varioskan fluorometer (Thermo Fisher Scientific, USA) in triplicate. All
196 these activities were expressed as specific activities (U mg $protein^{-1}$).
197 Protein content was measured in the homogenates as described by Bradford (1976) using
198 Bio-Rad Protein assay dye reagent (BioRad 500- 0205, Hercules, USA) and bovine serum
199 albumin (BSA, A7906; Sigma-Aldrich, Madrid, Spain) as the standard. Samples were
200 assayed in triplicates in 96-well and read at λ = 595 nm.
201
202 *2.5. Histological analysis*
203 For histological analyses, larvae were fixed for 24 h in Davidson's solution after being
204 euthanized by anesthesia in ice-cold seawater. Fixed specimens were dehydrated in ethanol,
205 cleared in xylene, and embedded in paraffin (Paraplast X-Tra, Mc Cormick Scientific, San
206 Diego, USA), then individually embedded in paraffin blocks and sectioned in serial sagittal
207 sections (5 μm), using a rotary microtome. Periodic acid- Schiff (PAS) was used to stain

neutral mucins and alcian blue (AB) at pH 2.5 was used to stain acidic mucins (carboxyl-rich glycoconjugates). Tissue sections were permanently mounted (Entellan, Merck Millipore, Darmstadt, Germany). To quantify the production of mucins in the middle intestine (MI), a total of 240 images were evaluated. Mucin coverage area in the tissue (mucin inside the cells and secreted by the cells) was calculated following the formula proposed by Rosero-García et al. (2019): MI = [mucin coverage area (μm^2)/image area (μm^2)]*100. Histological sections were randomly examined under a light microscope and analyzed with Image-Pro Premier software 9.2 (Media Cybernetics, Rockville, USA).

2.6. Incidence of skeletal deformities and level of bone mineralization

To identify and quantify the incidence of skeletal deformities, 40 larvae per treatment were fixed 24 h in Davidson´s solution and then preserved in 70% ethanol. Animals were stained with Alizarin red (AR) for bone and Alcian blue (AB) for cartilage using a modified method described by Darias et al. (2010). Briefly, preserved larvae were first stained in AB pH 1.3 (10 mg L^{-1}), then neutralized in 100% ethanol plus KOH 0.5%. Then, larvae were digested by trypsin 1%, bleached in H_2O_2 3%:KOH 0.5% solution (1:9), followed by bone staining in 1 mL AR solution (5 g L^{-1}) diluted in 49 mL KOH 0.5%. Once stained, specimens were washed (distilled water and KOH 0.5%), dehydrated by serial grade KOH 0.5%:glycerol solutions (2:1; 1:1; 1:2), then finally preserved in 100% glycerol. Skeletal structures were identified according to Mesa-Rodríguez et al. (2014; 2016). Skeletal deformities in the cranium, vertebral column were evaluated; for mineralization degree, structures of the cranium, vertebral column and caudal fin complex were evaluated (Supplementary figure). For the vertebral column evaluation, besides the 23 vertebrae, the urostyle was also considered.

232

233 *2.7. Gene Expression*

234 To understand the pattern in the expression of certain genes of interest throughout larval
235 development, gene expression on pooled larvae was determined by reverse-transcriptase
236 quantitative PCR (RT-qPCR) as follows. Total RNA of whole larvae was extracted using
237 TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.
238 Quantity and quality of isolated RNA were determined with Nanodrop 2000 (Thermo Fisher
239 Scientific, USA) and on a 1.2% agarose gel, respectively. The cDNA was made from 2.5 µg
240 of DNAase treated RNA (RQ1 RNase-Free DNase kit – M6101 Promega, Madison, USA)
241 and synthesized using ImProm-II™ Reverse Transcriptase (Promega, Madison, USA). The
242 *ef1α* and *18S* genes were used as housekeeping after checking their stability. The primers for
243 the candidates' genes bone morphogenetic protein type 2 (*bmp2)*, collagen type 1α1
244 (*Col1α1*), insulin growth factors type 1 and 2 (*igf I*, *igf II*), growth hormone (*gh)* and
245 proliferating cell nuclear antigen (*pcna*) are shown in Table 2. RT-qPCR was performed in
246 CFX 96 Touch™ Real-Time Detection System (BioRad, Hercules, USA). RT-qPCR
247 measurements were performed in a total volume of 15 µL, containing 1.0 µL of each primer,
248 10 µL of 2× concentrated SYBR Green (BioRAD, Hercules, USA), 2 µL of cDNA and 6 µL
249 DEPC-water. The thermal-cycling conditions used for qPCR were as follows: 95 °C for 2
250 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s. Standard
251 curves were generated using four different dilutions (in quadrupled) and the amplification
252 efficiency (E) was analyzed as follows: $E = 10^{(-1/\text{ slope})-1}$ (Jothikumar et al., 2006). The
253 amplification efficiencies of all genes were approximately equal and ranged from 100 to
254 105%. The expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method as

255 described by (Livak and Schmittgen, 2001), normalized with the reference genes and the
256 control group expression was set at 1.
257
258 *2.8. Transcriptomic analysis*
259 *2.8.1. RNA extraction, preparation of cDNA libraries and sequencing*
260 For RNA extraction, the Trizol method was used as previously described. A second
261 extraction was performed with phenol: chloroform: isoamyl alcohol (25: 24: 1 pH 4-5) to
262 guarantee the purity of the RNA, the sample was homogenized in a vortex and then
263 centrifuged at 12,000 x *g*. Once the RNA was extracted, 5 µg aliquots were preserved from
264 each sample in RNAstable® (Sigma-93221-001). Samples were sent to sequence at the
265 Institute of Biotechnology of UNAM (IBT, Cuernavaca, Mexico). The integrity of the RNA
266 (RIN) of the samples was determined in Bioanalyzer Agilent 2100 (Agilent Technologies).
267 To study the transcriptome of the whole *S. rivoliana* larva, a pool of larvae corresponding to
268 day 15 (n = 20) and day 30 (n = 3), a total of 8 cDNA libraries (4 per treatment) were
269 constructed using the TruSeq RNA Sample Preparation Kit (Illumina®, San Diego, USA)
270 from 5µg of the total RNA of each sample according to the manufacturer's protocol. Libraries
271 were sequenced in both directions (paired-end) on Illumina's NextSeq 500 platform,
272 employing 2x75-cycle sequencing chemistry. Fragment sizes were analyzed with
273 Bioanalyzer Agilent 1000 (Agilent, Santa Clara, USA). Once the samples were sequenced,
274 they were purified by strict quality criteria and the adapters were removed with the
275 Trimmomatic tool (Bolger et al., 2014).
276
277 *2.8.2. Quality of readings and genome de novo assembly*

278 A total of 16 files from 8 libraries were obtained (2 per library) in fastq.gz format. 94,030,579
279 pairs of 75 bp sequences were obtained, in a range of 9,511,035 to 14,113,052 pairs of
280 readings per sample. The readings of each file passed through a quality filter in which they
281 eliminated adapters, readings of less than Q20 (Phred quality score) and readings of less than
282 50 bp. In addition, the percentage of GC, the percentage of nucleotides per cycle and the
283 percentage of under-represented readings were verified. As at the moment of the present
284 study analysis, a *S. rivoliana* whole genome reference was not available, a *de novo* assembly
285 was performed. The purified sequences were assembled using the Bruijn algorithm
286 implemented in the Trinity software (Grabherr et al., 2011). Conceptual translations were
287 generated with the software Transdecoder
288 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3875132/) and the transcripts and their
289 conceptual translations were annotated by similarity to the SwissProt, Pfam and Gene
290 Ontology database.
291
292 *2.8.3. Differential gene expression and GO enrichment analysis*
293 The 8 libraries were used for the analysis of differential expression. On day 15, four libraries
294 were used (two replicates of the yeast treatment and two of the control) and on day 30, the
295 other four were used (two replicates of the yeast treatment and two of the control). The
296 analysis was performed with the Bioconductor EdgeR package (Robinson et al., 2010) that
297 uses a generalized linear model in which it is assumed that the expression of each gene
298 follows a negative binomial distribution. Differentially expressed contigs were selected with
299 a Fold change> 4 and an adjusted P-value <0.001 (multiple test correction according to the
300 False Discovery Rate (FDR) method of Benjamini and Hochberg, 1995).

301 The enrichment analysis was performed using the goseq R Bioconductor package (Young et
302 al., 2010) with the Wallenius approximation method to correct the length bias of the
303 transcripts. Only those GO terms with FDR <0.1 were considered significantly enriched.
304
305 *2.9. Statistical analysis*
306 For growth parameters, enzyme activity, percentage mucin secretion, percentage skeletal
307 deformities and mineralization degree, the results were expressed as mean ± standard
308 deviation (SD). Relative gene expression was expressed by mean ± standard error (SE). The
309 Student's t-test was applied to observe the statistical differences between the treatments ($P <$
310 0.05). For transcriptomic analysis, differentially expressed contigs were selected with a fold
311 change> 4 and a value of P <0.001 (Benjamini-Hochberg FDR correction).
312
313 **3. Results**
314 *3.1. Growth and survival*
315 Significant differences in survival and final weight were observed between the yeast and
316 control treatments ($P < 0.05$). Larvae fed the diet including *D. hansenii* were heavier (94.0 ±
317 2.0 mg) than those fed the control feeding protocol (65.0 ± 2.0 mg) ($P < 0.05$). Survival rates
318 were almost five times higher in larvae (2.6 ± 0.3%) from the YE group than the control
319 group (0.6 ± 0.2%) (Table 3; $P < 0.05$). No significant differences were observed between
320 treatments for final standard length (SL) or SGR ($P > 0.05$). The standard length throughout
321 the development of *S. rivoliana* larvae in the different treatments is available as
322 Supplementary material.
323
324 *3.2. Enzymatic activity*

The total activities of selected pancreatic, intestinal and stomach enzymes were analyzed at 15 and 30 DPH and their results are represented in Figure 1. At 15 DPH, no differences were observed between both treatments for any of the digestive enzymes considered ($P > 0.05$). However, differences between the control and yeast groups were observed at 30 DPH for α-amylase, pepsin and alkaline phosphatase. In particular, α-amylase activity was below the detection level in the control group, whereas in larvae from the yeast treatment the activity was 5.79 ± 2.8 U larva $^{-1}$ (Fig. 1a, $P < 0.05$). Pepsin activity was lower in the control group (576.3 ± 46.1 U larva $^{-1}$) when compared to the yeast treatment (1,001.48 ± 170.7 U larva $^{-1}$) (Fig. 1e, $P < 0.05$). The activity of alkaline phosphatase was 722.58 ± 44.5 U larva^{-1} and 1,340.40 ± 162.3 U larva^{-1} in the control and yeast treatments, respectively (Fig. 1f, $P < 0.05$).

3.3. Mucin production in the intestine

At 30 DPH, there was an increase in the mucin coverage area (%) in the intestine concerning day 15 (Fig. 2), mucous cells and secretion were stained in purple, which indicated the presence of both neutral and acidic mucins. At 15 DPH no significant difference between the C and YE groups was observed; however, at day 30 larvae from YE dietary treatment presented a higher percentage of mucin production (2.90± 1.1 %) than the C group (1.49 ± 0.3%) ($P < 0.05$).

3.4.Bone mineralization and skeletal deformities

At the end of the experiment, larvae from the YE group presented a higher degree of mineralization in the cranial structures (Fig. 3) and those from the caudal fin complex (Fig. 4). In particular, 44% of larvae from the YE group presented the caudal fin complex structures fully mineralized in comparison to the 5% from the C group ($P < 0.05$; n = 40).

These changes in the mineralization degree were mostly associated with the three epurals and the five hypurals of the caudal fin complex. Regarding the cranial region, the observation of the dentary, nasal, coracoid, infraorbital, premaxilla, scapula and lateral ethmoid were carried out. The YE group presented 48% well-mineralized structures in comparison to 5% from the C group. However, in the vertebral column, no significant differences were observed between treatments regarding the level of mineralization in both prehemal and hemal vertebrae along the vertebral column.

No differences in the incidence of cranial deformities (mainly retrognathism and prognathism) were found between both dietary groups ($P > 0.05$). However, the incidence of deformities affecting the vertebral column was lower in larvae from the YE group (10 ± 0.0%) in comparison to the C group (27.5 ± 2.5 %) from control ($P < 0.05$). Fusion and compression of vertebral bodies, mainly from the cephalic and prehemal regions, were the most common deformities found along the vertebral column (Fig. 5a).

3.5. Gene expression

The normalized relative expression of the selected genes involved in cell differentiation and proliferation in this study did not show significant differences between treatments at 6 and 25 DPH. At 15 DPH, up-regulation of *col1α1* (Control group) and *igf1* (YE group) were observed, however no differences between treatments were observed in the other genes at this age. At 30 DPH, *bmp2*, *col1α1* and *pcna* were up-regulated in *S. rivoliana* larvae fed the YE in compared to the C group ($P < 0.05$) (Fig. 6).

3.6. Transcriptomic analysis

From the 8 libraries assembled, a total of 125,286 transcripts were obtained, of which 84,048 genes were identified. In particular, 50% of the contigs (N50) had an average length of 2,035 bp. We identified a total of 45,690 proteins with annotation against the SwissProt database and 19,154 proteins with the assigned ontological terms (GO) (Table 4). The enrichment of the GO terms over-represented in the differentially expressed genes was evaluated to identify the main processes between the two stages of development (15 and 30 DPH) in larvae from the YE group (Tables 5 and 6).

The estimation of transcript abundance comparing both dietary treatments is depicted in Figure 7. In particular, the major difference in gene expression between the treatments was observed at 15 DPH. In the YE group, a total of 398 over-expressed genes were detected, whereas, at 30 DPH, only 283 over-expressed genes were found between both groups.

The correlation of all differentially expressed transcripts between treatments, their replicates and the different sampling points (15 and 30 DPH) were higher in larvae from the YE group at 15 DPH with an average correlation of 0.87, whereas the lowest correlation was observed in the control group at 30 DPH (Fig. 8).

4. Discussion

4.1.Growth and survival

The oral administration of the yeast *D. hansenii* within live prey (enriched rotifers and *Artemia* metanauplii) positively affected the growth and survival of *S. rivoliana* larvae reared at 22ºC under standard larviculture conditions. In the current study, SL values were similar to those reported by Roo et al. (2014) in which the authors reported total length at day 30 of 15-16 mm with an average cultivation water temperature of 24 °C. Given the above-mentioned, it can be inferred the larvae of the present study had similar growth patterns when

related to previous studies on the same species. Concerning survival, the control treatment was similar to that obtained by Roo et al. (2014), in similar density conditions (intensive culture) the authors obtained 0.5% survival. However, the yeast treatment presented a survival five-fold than control (2.6 ± 0.3%), this result was similar to that observed by Roo et al. (2014) in a semi-intensive system (3.6 larvae L^{-1}). It is worth mentioning that in the present study the larval density was 80 L^{-1} larvae, which shows the advantage of using *D. hansenii* as a probiotic to *S. rivoliana* larvae, and its benefits in intensive culture systems.

The positive effect of probiotics on growth and survival of fish larvae and juveniles has been extensively reported in different studies (Burgoin, 2015; Frouël et al., 2008; Leyton et al., 2017; López et al., 2016; Suzer et al., 2008; Tovar-Ramírez et al., 2002, 2004 among others). In this sense, Banu et al. (2020) using different doses of *Saccharomyces cerevisiae* (0, 0.5, 1.0, and 1.5 g kg $diet^{-1}$) in *Mystus cavasius* fry increased survival between the treatments and control, which the best survival was observed in the 1.0 g kg^{-1} versus the control. In terms of growth, the authors also observed that fry treated with the probiotic yeast (1.0 g kg $diet^{-1}$) had the highest final length and weight when compared to the control group. The authors attributed the positive results of the probiotics as they might have increased digestive enzyme activity, improved feed intake, digestion, and enhanced protection against harmful bacteria.

Tovar-Ramírez et al. (2002) used the yeast *D. hansenii* in microdiets for *D. labrax* larvae which improved larval survival when compared to control and *S. cerevisiae* treatment. In addition, the inclusion of *D. hansenii* in microdiets for *D. labrax* promoted larval growth (Tovar-Ramírez et al., 2004), the authors suggested that the positive effects of live yeasts may be due to yeast-produced molecules such polyamines (spermine, spermidine and putrescine) which are considered as a natural growth factor and play a role in promoting intestinal maturation and increasing the ability of enterocytes to absorb nutrients (Angulo et

al., 2020; Hernández-López et al., 2021). Polyamines are ubiquitous molecules that have been reported to have a biological role in cell metabolism and proliferation by stimulating DNA, RNA and protein synthesis and particularly spermine and spermidine are involved in the differentiation and maturation of the intestinal tract in mammals (Vohra 2016).

The Growth hormone (GH)/insulin-like growth factor (IGF) axis have a key role in the regulation of body size in growing animals (Triantaphyllopoulos et al., 2019), however, although larvae from the YE group had higher weight gain, the expression of *igf*1, *igf*2 and *gh* did not show having influence between treatments and fluctuated between the different ages. On the other hand, *pcna* (proliferating cell nuclear antigen) gene expression was up-regulated in YE group, which may explain, in part, the better whole development of the larvae such as growth, digestive tract maturation and skeletal mineralization. *Pcna* plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery (Kelman 1997) which plays a central regulatory role in cell growth and development and has been reported to be expressed exclusively in the actively proliferating cells, which it is considered as a molecular marker with its predictive capacity for cell proliferation (Yu et al., 2022).

4.2.Digestive tract maturation

Many studies have focused more specifically on the detection of pancreatic enzymes before the first feeding; enzymatic maturation of the brush border and detection of pepsin activity in fish with a stomach that indicates the transition to juvenile digestion, which is the same as that of adults of the species to be studied (Yúfera et al., 2018). Regarding the effects of the dietary administration of yeast on the digestive physiology in *S. rivoliana* larvae, at 15 DPH, there was no difference in the enzymatic activity between the YE group and that of the

445 control; however, the larvae from the YE group on day 30, besides showing higher growth,
446 also showed higher activity levels on several digestive enzymes like alkaline phosphatase
447 and α-amylase.
448 In the present study, the YE group larvae showed higher α-amylase activity at 30 DPH
449 compared to the larvae of control treatment. These results were similar to those reported by
450 Tovar-Ramírez et al. (2002), who observed an increase in α-amylase activity in *D. labrax*
451 larvae fed a microdiet supplemented with *D. hansenii* ($7x10^5$ CFU g^{-1}) at 27 DPH. In
452 carnivorous fish, the decrease in α-amylase in prejuveniles is an indicator of maturation of
453 the digestive tract; however, the detection of α-amylase in 30 DPH larvae from the YE group,
454 in this case, does not indicate that the larvae were not mature from a digestive point of view,
455 but that the larvae had better growth once α-amylase hydrolyzed both dietary starch and
456 glycogen. This allows us to suggest that the larvae mobilized their glycogen reserves to use
457 them as energy, obtaining better use of their reserves and growth improvement when fed with
458 yeast. Furthermore, *D. hansenii* yeast possess high polysaccharide content as biochemical
459 composition such as α; β-glucans and mannans (Morales-Lange et al., 2022) and the presence
460 of glycosidic chains that could stimulate the α-amylase activity, hypothesis that must be
461 proved in the future.
462 Concerning the pepsin-like activity, its presence depends on the rate of development of each
463 species, which could be influenced by larvae size, the temperature of production and its
464 development pattern itself. In this sense, it is known that species with early pepsin-like
465 presence are better prepared to develop effective protein digestion in the early stages of
466 development and this should be stimulated by a similar behavior of other enzymes also
467 involved in the digestion process (Yúfera et al., 2018). YE group presented improvement of
468 the pepsin-like activity, which is associated with a more mature digestive function and a more

developed stomach in which there is an increase in the digestive capacity of the gastrointestinal system due to the production of pepsin and secretion of hydrochloric acid by the gastric glands, which implies the establishment of acid digestion and, consequently, a more efficient extracellular protein digestion, improving intracellular function in the posterior intestine (Galaviz et al., 2012). In particular, under normal rearing conditions, the maturation of the digestive function (luminal digestion) is characterized by a decrease in the activity of alkaline proteases concomitantly to an increase in pepsin-like enzyme levels characterized by an acid environment that is generally considered as an optimal point for larval weaning onto microdiets in fish hatcheries (Nolasco-Soria et al., 2020). Additionally, through the transcriptome analysis, it was possible to verify the gene that encodes Pepsin A, showing up-regulation in larvae from the YE group, that could explain the higher activity of this enzyme in the yeast group. In addition, genes involved in digestion and maturation such as *entk* (enterokinase) and *ppb* (alkaline phosphatase) were up-regulated in the larvae from the yeast treatment.

Major intestinal maturation was observed in larvae from the YE group, where the alkaline phosphatase activity was higher than in the larvae from the control group. The same was observed by Tovar-Ramírez et al. (2004) when used *D. hansenii* was included in microdiets for *D. labrax* larvae. According to Cahu and Zambonino-Infante (1995), present results indicated that larvae from the YE group had a more developed intestine as data from alkaline phosphatase indicated since this enzyme is a marker of gut maturation.

Askarian et al. (2011) suggest that the main modes of action and the beneficial effects of probiotics are the prevention of intestinal disorders, and the pre-digestion of the anti-nutritional factors present in the food ingredients, moreover, may stimulate appetite, improve nutrition by synthesizing and providing essential nutrients and enzymes, detoxifying

493 potentially harmful compounds in the diet and by the breakdown of indigestible compounds into
494 simpler compounds (Ghosh et al., 2008; Hemaiswarya et al., 2013).
495 In addition, yeasts secrete and provide the host with molecules of physiological interest such
496 as polyamines, free amino acids, fatty acids, β-glucans, among others, that can positively
497 affect the development of the larvae, as it was already described concerning gut maturation.
498 Currently, several evidences indicate yeast can promote growth and survival due to intestinal
499 maturation, larval conformation and stimulation of the immune system by a possible
500 involvement of endoluminal polyamines secreted in the host by yeast (Navarrete and Tovar-
501 Ramírez, 2014). Likewise, Tovar-Ramírez et al. (2004) suggested that the beneficial effects
502 of live yeasts on seabass larval development could be attributed to the role of polyamines in
503 promoting intestinal maturation and increasing the ability of enterocytes to absorb nutrients.
504
505 *4.3.Skeleton deformities and bone mineralization*
506 The benefits of probiotics on ossification degree and incidence of skeletal deformities have
507 been observed in different studies with fish larvae and juveniles (Tovar-Ramírez et al., 2004;
508 Aubin et al., 2005; Avella et al., 2009; Maradonna et al., 2013; Carnevali et al., 2017;
509 Ljubobratovic et al., 2017).
510 Yeast inclusion affected the degree of ossification and incidence of deformities in *S. rivoliana*
511 observed at 30 DPH larvae, from which it was observed that most of their skeleton was
512 completely ossified. In this sense, Tovar-Ramírez et al. (2004) obtained similar results with
513 *D. labrax* larvae, in which larvae fed with a supplement of *D. hansenii* reduced skeletal
514 deformities when compared to the control. Aubin et al. (2005) when feeding juvenile rainbow
515 trout (*Oncorhynchus mykiss*) with diets supplemented with LAB or probiotic yeast observed
516 a reduction in the incidence of vertebral deformities. It is worth mentioning that, in the

present study, the larvae of the control treatment showed slower growth, which is consistent with previous studies where the same pattern was presented in the different treatments in which the animals with less growth also had less maturation of the digestive tract and a higher incidence of skeletal deformities (Boglino et al., 2012; Darías et al., 2010; Fernández et al., 2008; Kjorsvik et al., 2009).

Besides, the yeast treatment on day 30 showed higher expression of genes involved in bone and cartilage formation, as in the case of *bmp2* and *col1α1*. The *col1a1* gene is mainly expressed in connective tissues and is abundant in bone, cornea, dermis, and in two types of cells, osteoblasts and fibroblasts; *col1a* is a component that comprises 90% of the proteins of the extracellular matrix in skeletal tissues, it is expressed during osteoblast differentiation together with some non-collagenous proteins and serves as a useful marker of early mineralization (Fernández et al., 2014; Riera-Heredia et al., 2018). It is crucial for the mechanical properties of bone, however, its relationship with malformation development in the vertebral column of juvenile fish is still unclear (Boursiaki et al., 2019), notwithstanding, as *col1a1* is expressed in osteoblasts, it could be associated with delayed ossification and skeletal deformities in the spine due to its downregulation in the control group. Patel et al. (2015) found the down-regulation of *col1a1* gene in jaw deformed *S. lalandi* larvae, the authors attribute it to the downregulation of *bmp2,* since as *bmp2* is known to coordinately induce the expression of type I collagen as well as other extracellular matrix proteins in osteoblasts (Shen et al., 2007). Han et al. (2021), analyzed the expression of genes associated with the development of chondrocytes and osteoblasts in the vertebrae of juvenile *Trachinotus ovatus* and compared the transcriptional levels of related genes in the spines of juvenile fish with the normal phenotypes and with vertebral fusion. The levels of *col1a1*, *bmp2*, and other genes related to bone development were down-regulated in fish with

vertebral fusion compared to fish with normal phenotypes. A similar result was observed with *Salmo salar* parr, in which the treatment with the highest incidence of fused vertebrae had down-regulation of *bmp2*, however, there was no difference in *col1a1* expression between treatments (Vera et al., 2019).

Bone morphogenetic proteins (BMPs) are very powerful cytokines belonging to the transforming Growth Factor beta (TGF-β) superfamily that induce bone and cartilage formation, their main functions are to induce the differentiation of mesenchymal cells towards the lineage of osteoblasts and promote their maturation and function (Milat and Ng, 2009; Nishimura et al., 2012). Specifically, *bmp2* recruits mesenchymal cells surrounding the initial cartilage condensations into chondrogenic fate (Li and Cao, 2006), which could be related to poor mineralization of the skeletal structures of chondral formation in larvae from the control group, such as skull region and caudal complex.

Up-regulation of genes involved in the parathyroid hormone metabolism (important hormone for calcium and phosphate homeostasis) were observed in the YE group by transcriptome analysis, like 1,25-dihydroxyvitamin D (3) 24-hydroxylase (*cyp24a1*), Protooncogene Raf-1, serine/threonine kinase (*raf*), Myocyte-specific enhancing factor 2C (*mef2c*), Protein binding to GTP related to Rho / RhoA-C (*rhoa*) and Protein 5 related to the low-density lipoprotein receptor (*lrp5*), these directly related to bone formation and regulation of bone mass.

The *raf* gene codes for an enzyme part of the metabolic pathway Extracellularly Regulated Mitogen-Activated Protein Kinase (ERK). A study by Provot et al. (2008), demonstrated that this gene was highly expressed in mature hypertrophic chondrocytes. The authors suggest that *raf* may be responsible for ERK activation in endochondral development and its activation may play an important role in hypertrophic differentiation of chondrocytes. The

565 *rhoa* protein is a small GTPase protein in the Rho family. The gene that encodes for this
566 protein participates in different cellular processes such as transcriptional control,
567 organization of actin, cellular development, and maintenance of the cell cycle, being an
568 important component in the signaling of parathyroid hormone in osteoblastic cells (Radeff et
569 al., 2004). The *mef2c* gene is a transcription factor commonly associated with the
570 development and differentiation of the heart and skeletal muscle. However, Stephens et al.
571 (2011) demonstrate that the expression of the *mef2c* gene is dynamically regulated during
572 osteoblast differentiation and the silencing of this gene is associated with highly significant
573 decreases in alkaline phosphatase activity, osteoblastic gene expression, and extracellular
574 matrix mineralization, demonstrating the important role in the differentiation of osteoblasts.
575 *cyp24a* has an important role in the strict regulation of calcitriol and, therefore, in the control
576 of intracellular degradation of this and the maintenance of normal calcaemia and bone health
577 (Veldurthy et al., 2016). Calcitriol is the active form of vitamin D, furthermore, calcitriol
578 significantly increases the expression of *lrp5*, a gene product that stimulates osteoblast
579 proliferation through canonical Wnt signaling and is therefore anabolic to bone (Milat and
580 Ng, 2009).
581 Additionally, the molecular analysis coincided with the double staining technique in that the
582 larvae from the YE group had a lower incidence of deformities in the vertebral column and
583 a higher degree of bone mineralization in the cranial and caudal fin complex.
584
585 *4.4. Mucin production*
586 The intestinal mucous gel layer is thought to be a vital component of the gut barrier which
587 constantly selects and transport essential materials through the intestinal epithelium and is
588 composed, in part, of a family of glycoproteins known as mucins, high-molecular-weight

589 glycoproteins that forms a net-like polymer mucus gel that protects the intestinal epithelium
590 by acting as insoluble mucous barrier from chemical, enzymatic, mechanical, and microbial
591 damage (Mattar et al., 2002; Catalán et al., 2017; Midhun et al 2019). In the present study,
592 the production of mucin was stimulated by the yeast and we also found, through
593 transcriptome analysis, the up-regulation of the *muc-2* gene as well, which suggests a
594 promotion in intestinal epithelial barrier function in the fish larvae from the YE group.
595 Among the nine mucin genes expressed in the vertebrate GI tract, *muc-2* is the major gel-
596 forming one and it is the chief structural component of the mucus gel and plays a role in the
597 protection of gut barrier, the regulation of microbiome homeostasis and the prevention of
598 diseases (Midhun et al., 2019; Liu et al., 2020).
599
600 *4.5. Transcriptome analysis*
601 In this study, the *de novo* transcriptome was performed to identify processes involved in
602 digestive maturation and bone mineralization and the incidence of skeletal deformities in *S.*
603 *rivoliana* larvae from both dietary groups sampled at 15 and 30 DPH as discussed above.
604 The quality of the assembly could be observed in that 50% of the contigs (N50) presented an
605 average length of 2,035 bp, for the *S. lalandi* species this value was 2356 bp (Patel et al.,
606 2015). The number of proteins with the assigned ontological term (GO) were similar for *S.*
607 *rivoliana* and *S. quinqueradiata*, 19,154 and 18,575, respectively (Yasuike et al., 2018), and
608 in the case of *S. lalandi* 15,744 proteins with assigned ontologically (Patel et al., 2015), these
609 authors attribute the small number of GO proteins assigned to the limitation of the NCBI
610 number protein database, which does not have an extensive annotation.
611 When the data is thrown against the KEGG database, overexpressed genes in yeast treatment
612 can be observed participating in 216 metabolic pathways.

5. Conclusion

In conclusion, *D. hansenii* CBS 8339 is a viable option as a probiotic to improve the production of *S. rivoliana* larvae, since it improves the growth, survival, and digestive tract maturation, besides, promoting skeleton mineralization and reducing skeletal deformities. The health of the intestine of fish fed supplemented yeast could be demonstrated with histological analysis of mucin production and gene expression, reinforcing the probiotic effect of yeast. Besides, the transcriptomic approach showed an overview of the development of *S. rivoliana* larvae, as well as insight on genes related to skeletal deformities and digestive maturation and the relationship with the *D. hansenii* probiotic effect that will be useful as a tool for subsequent studies. Further studies should be conducted on the effect of *D. hansenii* on immune response against bacterial infection in *S. rivoliana* larvae and juveniles as well as the modulation and interaction with the host microbiota.

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Yúfera, M., Moyano, F.J., Martínez-Rodriguez, G., 2018. The Digestive Function in Developing Fish Larvae and Fry. From Molecular Gene Expression to Enzymatic Activity 51-86 pp In: Emerging Issues in Fish Larvae Research Ed. Yúfera M. Springer.

907 Table 1. Feeding schedule and food types during *Seriola rivoliana* larval rearing.

Food	**Type**	**Amount**	**DPH**
Microalgae	*Nannochloropsis oculata*	0.5-1.0x10^6 cell mL^{-1}	0-18
Enriched rotifer	*Brachionus plicatilis*	20 rotifers mL^{-1}	2-18
Yeast	*Debaryomyces hansenii*	1 g x 10^6 rotifers	4-18
		1 g x 150.000 *Artemia*	15-30
Artemia nauplii	*Artemia* sp.	0.2-2 naplii mL^{-1}	12-15
Enriched *Artemia* metanauplii	*Artemia* sp.	2-5 metanauplii mL^{-1}	15-30
Compound dry feed	Otohime	*Ad libitum*	18-30

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913 Table 2. Oligonucleotide primers used for the qPCR analysis of target genes of *Seriola rivoliana*
914 larvae.

Gene	Specific Oligonucleotide Primers		
	Forward 5' to 3'	Reverse 5' to 3'	Bp
col1α1	ACCAGGAAGACCTCTGTCTC	ATCTGCGAGGACACAACA	147
gh	CCAATCAGGACGGAGCAGAG	TTTAGCCACCGTCAGGTACG	174
pcna	ATCATCACCCTCAGAGCAGAAG	CCAGGTCCATCAGTTTCATCTC	106
bmp 2	AAGGTGCTACGGGACTTATCC	TGAGAAGCCGAAGCTCAAAC	114
igf l	GAGCTGCCTTGCTAGTCTT	TGTGGAGAGAGAGGCTTTTA	154
igf ll	TGGGACTTCCTGTTTTAGTG	AGTGTTGTTTCCGTAGCTGT	134
ef1α	TGGTGTTGGTGAGTTTGAGG	CGCTCACTTCCTTGGTGATT	173
18s	CTGAACTGGGGCCATGATTAAGAG	GGTATCTGATCGTCGTCGAACCTC	165

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Table 3. Growth performance in body weight and standard length, and final survival of *Seriola rivoliana* larvae. Data are represented by means ± SD The asterisk denotes the existence of statistical differences between both experimental groups (t-test, $P < 0.05$).

Treatment	Final SL (mm)	Final weight (mg)	SGR	Survival (%)
Control	11.35 ± 1.1	65.0 ± 2.0	4.44 ± 0.24	0.55 ± 0.2
Yeast	13.0 ± 1.6	94.0 ± 2.0*	4.89 ± 0.57	2.60 ± 0.3*

923 Table 4. Descriptive statistics obtained from the *de novo* assembly of the *Seriola rivoliana*
924 transcriptome.
925

Assembled transcripts	125,286
Identified genes	84,048
N50 transcripts	2,035 bp
Identified proteins	81,258
Complete proteins	41,998
Internal partials	11,978
Partials 5 '	21,079
Partials 3 '	6,203
Proteins with SwissProt annotation (e-value < 1e-5)	45,690
Proteins with an assigned ontological term	19,154

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929 Table 5. Main processes annotated and over-represented on day 15 in larvae treated with
930 yeast.

Process (GO)	**GO ID**	**# Genes**
Digestion	GO:0007586	3
Aspartic-type endopeptidase activity	GO:0004190	2
Aspartic-type peptidase activity	GO:0070001	2
Hydrolase activity	GO:0016787	4
Hydrogen:potassium-exchanging ATPase activity	GO:0008900	1
Extracellular region	GO:0005576	3
Cell wall chitin metabolic process	GO:0006037	1
Cell wall polysaccharide metabolic process	GO:0010383	1

Endopeptidase activity	GO:0004175	2
Lysozyme activity	GO:0003796	1
Cell wall macromolecule metabolic process	GO:0044036	1
Production of molecular mediator involved in inflammatory response	GO:0002532	1
Positive regulation of chemokine secretion	GO:0090197	1
Catalytic activity	GO:0003824	4
Sodium:potassium-exchanging ATPase activity	GO:0005391	1

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936 Table 6. Main processes annotated and over-represented on day 30 in larvae from the yeast
937 group.

Proceso (GO)	**GO ID**	**# Genes**
Response to lipids	GO:0033993	5
Response to ketones	GO:1901654	3
Response to organic substances	GO:0010033	6
Response to oxygen-containing compounds	GO:1901700	5
Response to hormones	GO:0009725	4
Response to chemicals	GO:0042221	6
Cellular response to hormone stimuli	GO:0032870	3

Cellular response to ketones	GO:1901655	2
Response to alcohols	GO:0097305	3
Response to fatty acids	GO:0070542	2
25-hydroxycholecalciferol-24-hydroxylase activity	GO:0008403	1
1-alpha,25-dihydroxyvitamin D3 24-hydroxylase activity	GO:0030342	1
Vitamin D 24-hydroxylase activity	GO:0070576	1
Mitochondrial intermembrane space	GO:0005758	2
Organelle envelope lumen	GO:0031970	2
Vitamin D catabolic process	GO:0042369	1
Negative regulation of integrin activation	GO:0033624	1
Negative regulation of eosinophil migration	GO:2000417	1
Regulation of eosinophil extravasation	GO:2000419	1
Negative regulation of eosinophil extravasation	GO:2000420	1
Cellular response to tumor cell	GO:0071228	1
Vitamin D receptor signaling pathway	GO:0070561	1
Response to endogenous stimulus	GO:0009719	4
Mitochondrial part	GO:0044429	4
Response to glucose	GO:0009749	2
Response to hexoses	GO:0009746	2

Response to monosaccharides	GO:0034284	2
Oxidative phosphorylation uncoupler activity	GO:0017077	1

Figure legends

Figure 1. Yeast effect on a) α-amylase, b) lipase, c) trypsin, d) chymotrypsin, e) pepsin and f) alkaline phosphatase activities in *Seriola rivoliana* larvae fed a diet supplemented with the yeast *D. hansenii* CBS 8339. Control diet (not supplemented with the yeast): black bars; Yeast-supplemented diet: gray bars. Data are represented in means ± SD. Asterisks indicate significant differences between treatments (t-test, $P < 0.05$; n = 4).

Figure 2. Effect of yeast on mucin production in the intestine of *Seriola rivoliana* fed a diet supplemented with the yeast *D. hansenii* CBS 8339 at 15 and 30 days after hatching. Control diet (not supplemented with the yeast): black bars; Yeast-supplemented diet: gray bars. Data are shown as mean ± SD. Asterisks indicate significant differences between treatments (t-test, $P < 0.05$; n = 20).

Figure 3. Bone mineralization level in the craniofacial region in *Seriola rivoliana* larvae fed a diet supplemented with the yeast *D. hansenii* CBS 8339. Examples of mineralization categories used: a) larva with mild mineralization; b) larva with partial mineralization; c) larva with a mineralized skull. Asterisk indicates significant differences between treatments according to the degree of mineralization (t-test, $P < 0.05$).

Figure 4. Bone mineralization level in the caudal complex and vertebral column region in *Seriola rivoliana* larvae fed a diet supplemented with the yeast *D. hansenii* CBS 8339. Examples of mineralization categories used: a) larva with mild mineralization; b) larva with partial mineralization; c) larva with mineralized caudal complex. Asterisk indicates significant differences between treatments according to the degree of mineralization (t-test, $P < 0.05$).

Figure 5. Incidence of skeletal deformities in *Seriola rivoliana* larvae. Some examples of most common skeletal deformities found: a) larva presenting compression and fusion of prehemal vertebrae; b) larva presenting retrognathism and deformation of the dentary bone;

973 larva presenting mild prognathism with a shortening of the premaxillary and maxillary bones.
974 The asterisk indicates significant differences between treatments (t-test, $P < 0.05$).
975
976 Figure 6. Effect of yeast on the expression of genes involved in cell differentiation and
977 proliferation in *Seriola rivoliana* larvae fed a diet supplemented with the yeast *D. hansenii*
978 CBS 8339. Control diet (not supplemented with the yeast): black bars; Yeast-supplemented
979 diet: gray bars. Data are represented as means ± SE. Asterisks indicate significant differences
980 between treatments (t-test, $P < 0.05$; n = 4).
981
982 Fig 7. Multidimensional analysis for each comparison of the differential expression *of Seriola*
983 *rivoliana* larvae. Differentially expressed genes ($P < 0.001$) are represented by red dots; the
984 other genes are represented by black dots. **a** represents yeast treatment vs control at day 15.
985 **b** represents yeast treatment vs control at day 30. Data were graphed by fold-change (Y-axis;
986 logarithmic scale) and by expression levels (count of readings; X-axis, scale logarithmic).
987
988 Figure 8. Correlation matrix of *Seriola rivoliana* larvae fed a diet supplemented with the
989 yeast *D. hansenii* CBS 8339 at 15 and 30 days after hatching.
990
991 Supplementary figure. Cranial and axial regions and skeletal structures to evaluate
992 mineralization degree and deformities in *Seriola rivoliana* larvae. Ce, cephalic vertebrae; Co,
993 coracoid; D, dentary; Ep, epural; H, hemal vertebrae; Hy 1+2, hypurals 1+2; Hy 3+4,
994 hypurals 3+4; Hy 5, hypural 5; Inf, infraorbitals; N, nasal; Ph, prehemal vertebrae; Pm,
995 premaxilla; Sc, Scapula; Ur, urostyle.
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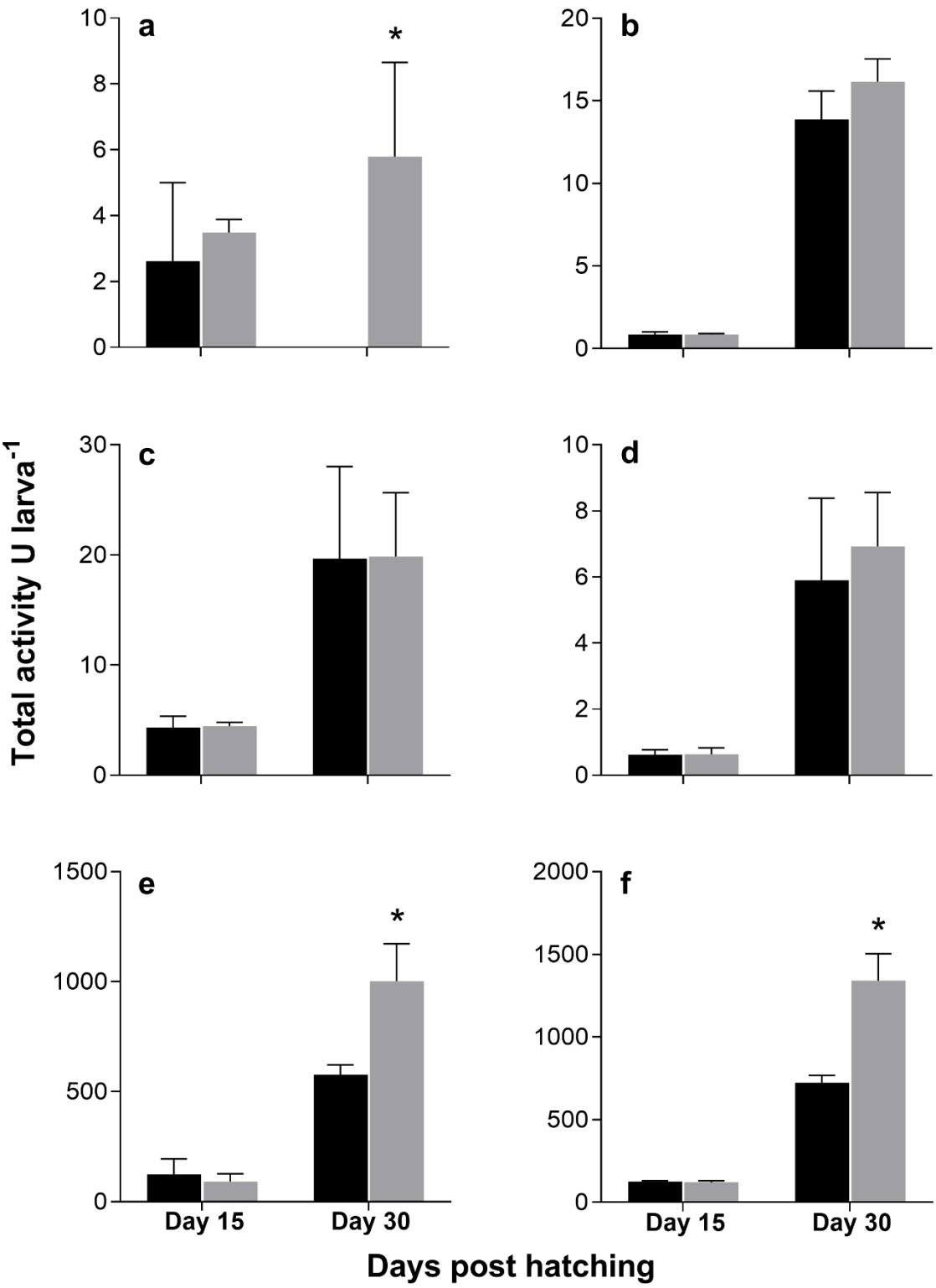

1005

1006 **Figure 1.**

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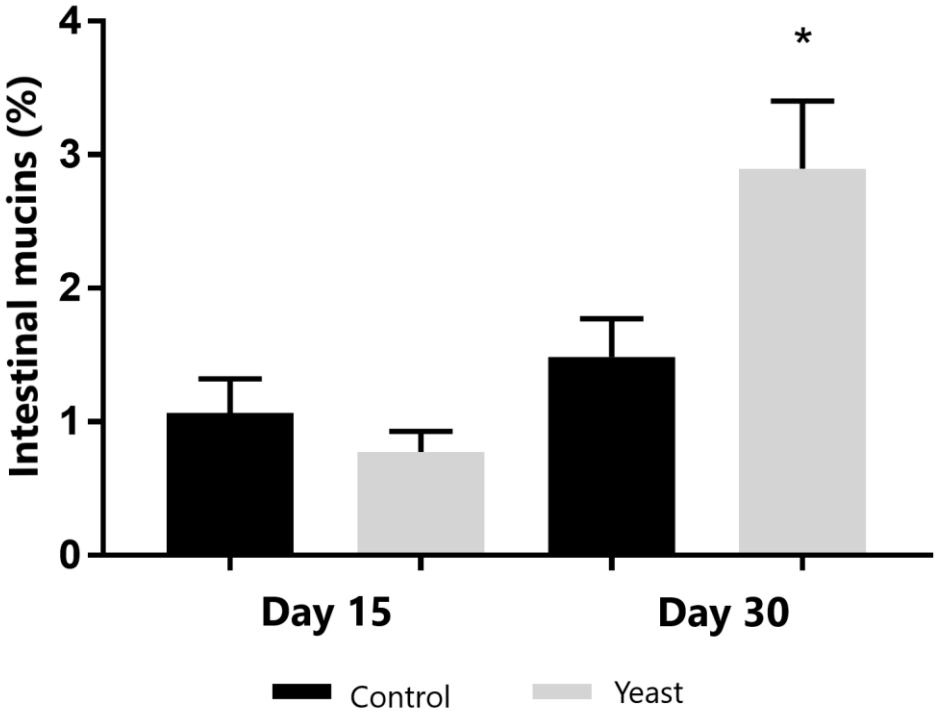

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1009 **Figure 2.**

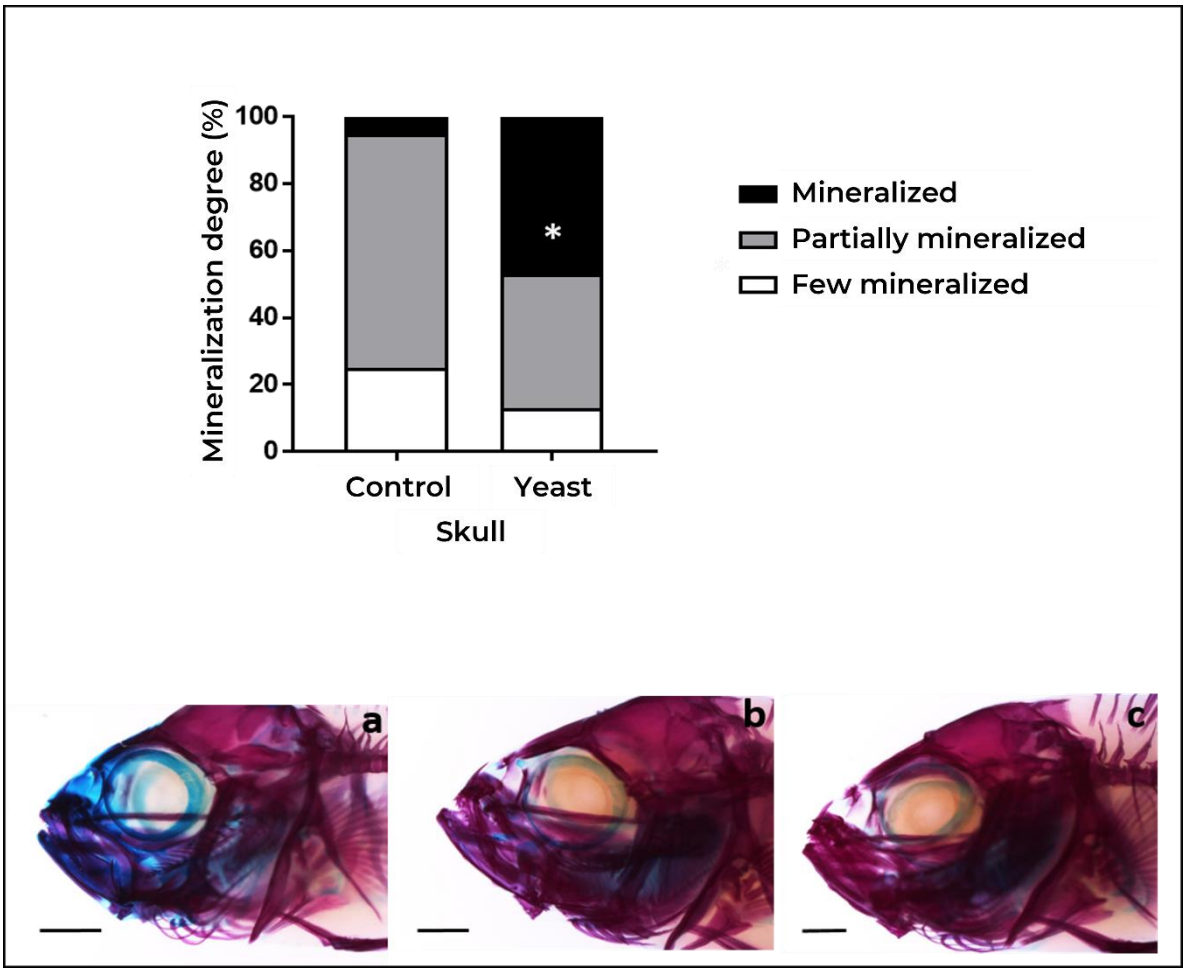
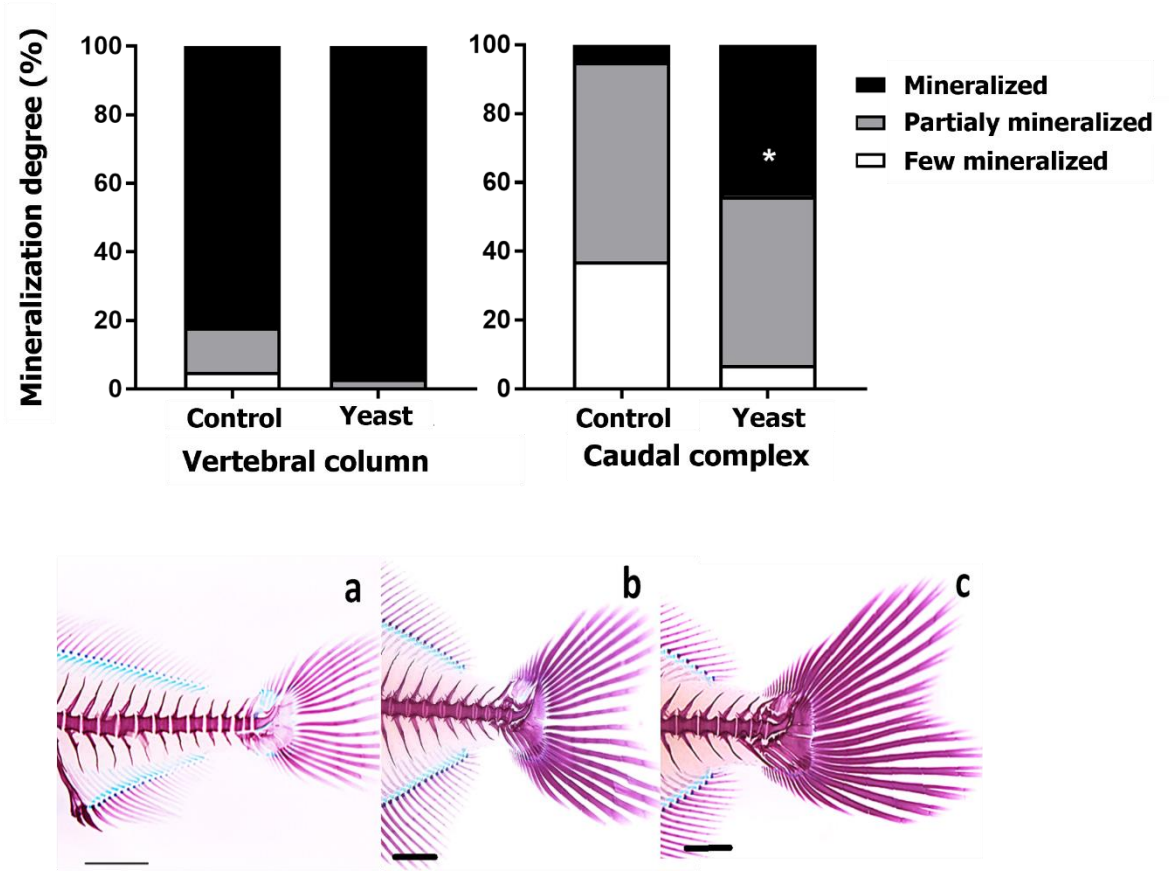

Mineralization degree (%)
100
80
60
40
20
0
*
Control
Yeast
Skull
Mineralized
Partially mineralized
Few mineralized
a
b
c

Figure 3.



1013

1014 **Figure 4.**

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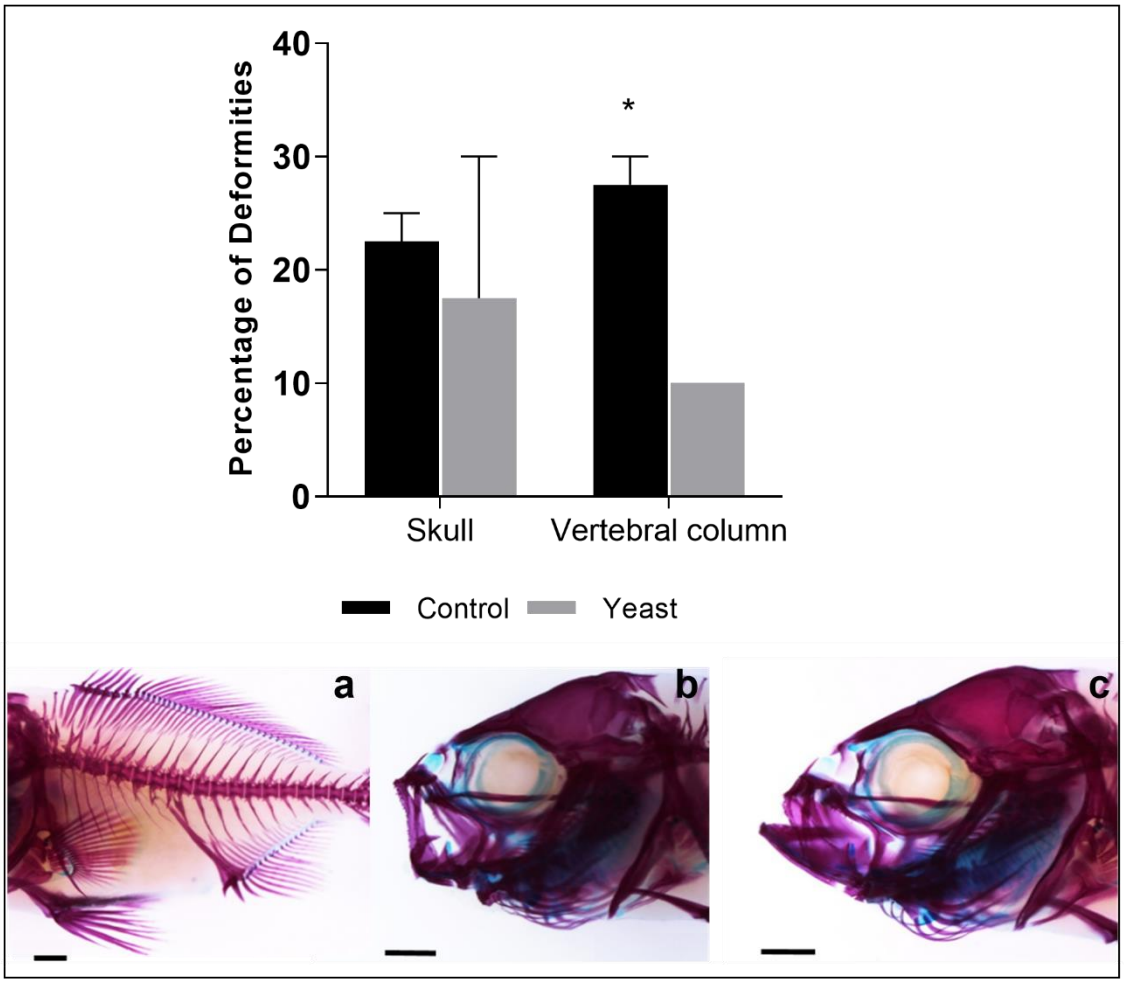

Percentage of Deformities
40
30
20
10
0
*
Skull
Vertebral column
Control
Yeast
a
b
c

Figure 5.

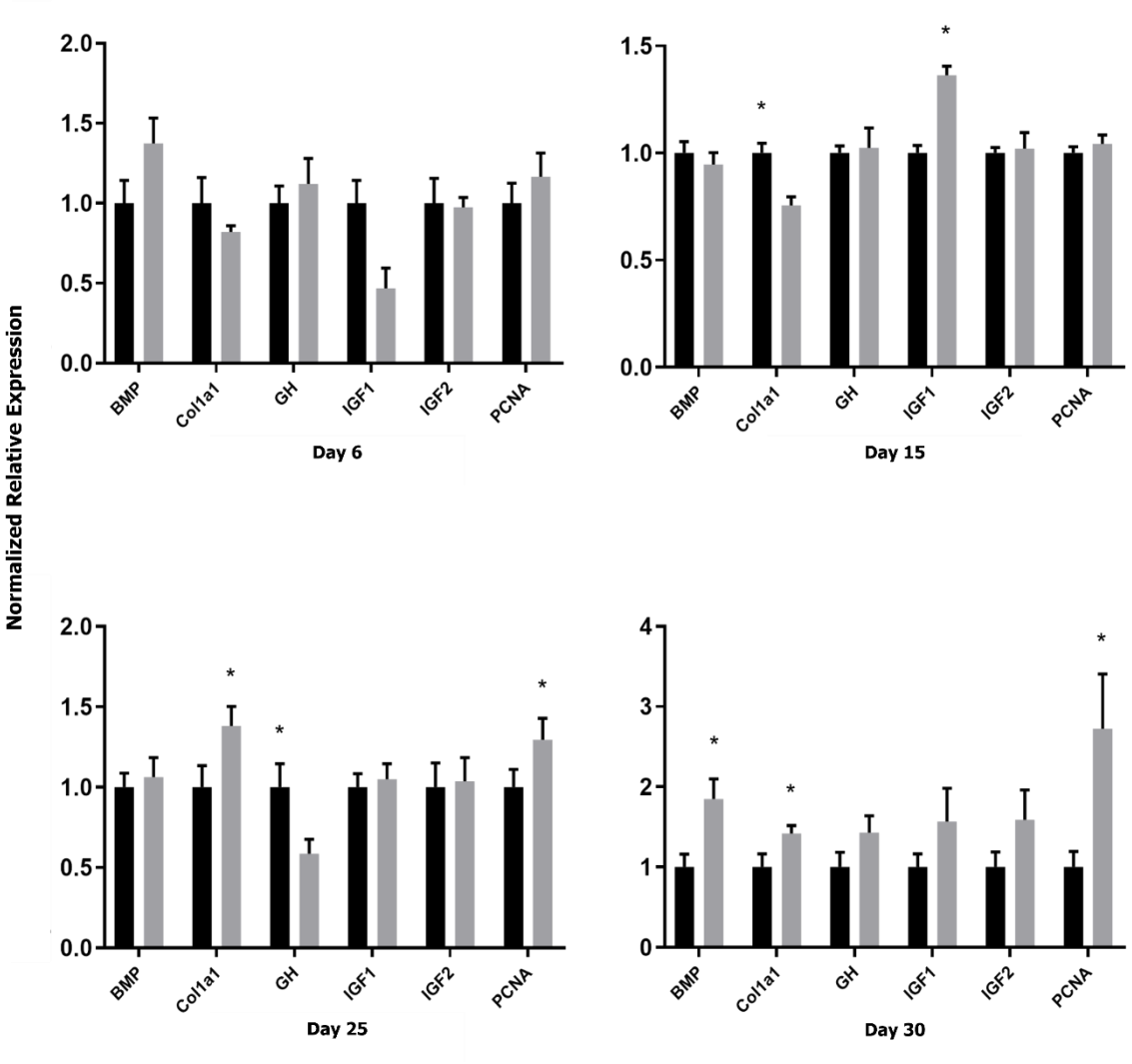

Normalized Relative Expression
Day 6
Day 15
Day 25
Day 30
BMP
Col1a1
GH
IGF1
IGF2
PCNA

Figure 6.

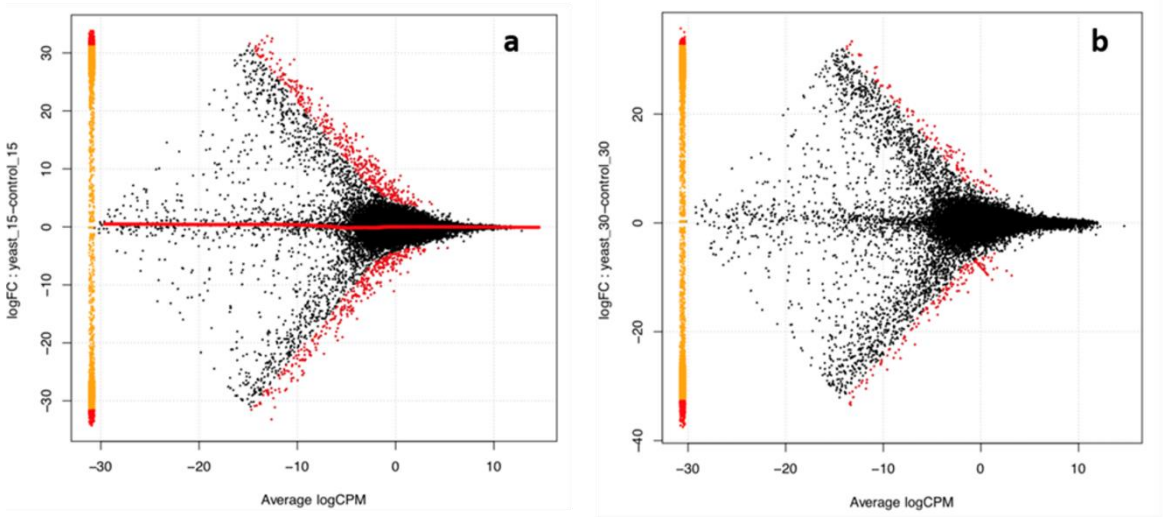
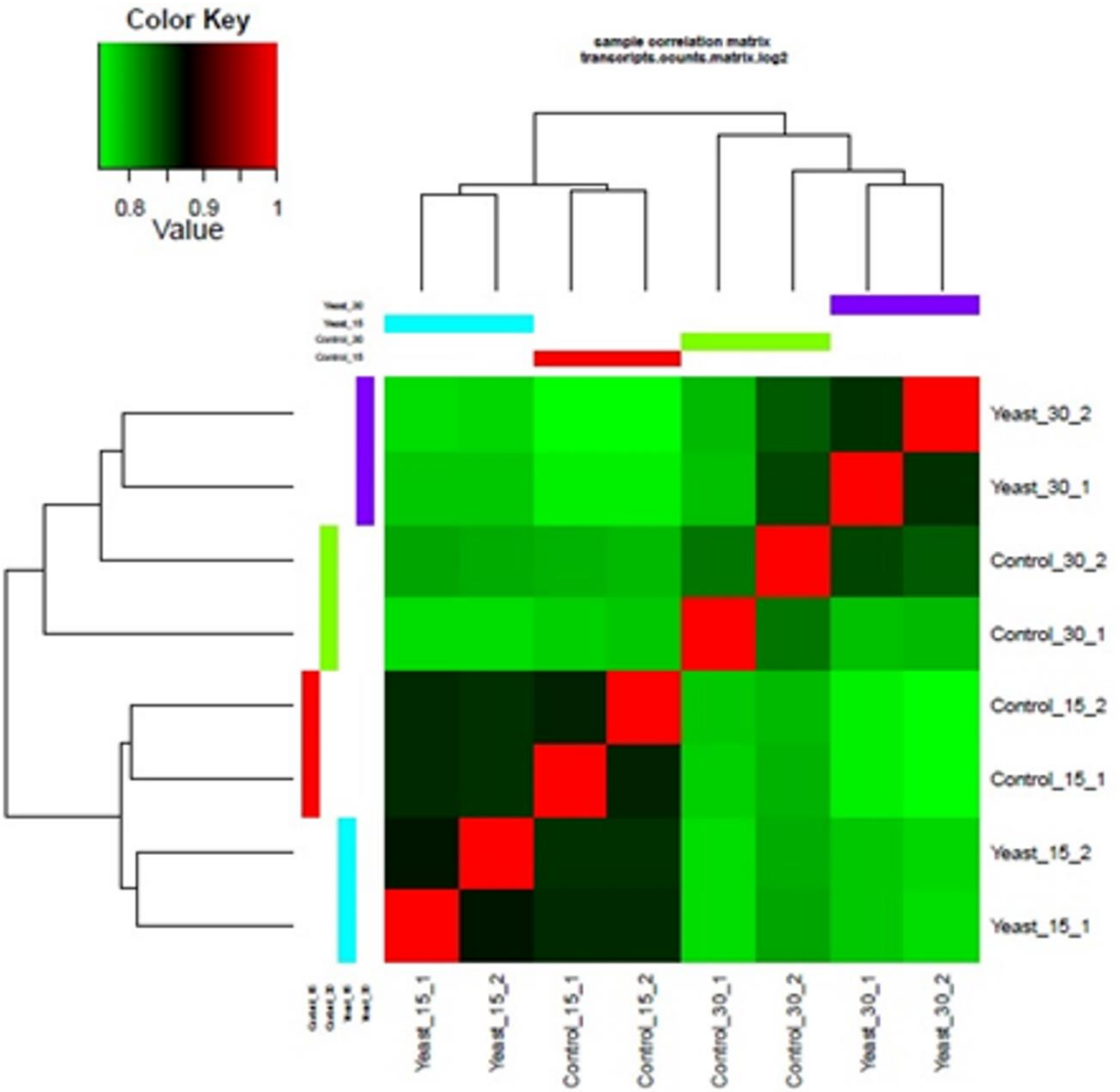

a
b
logFC : yeast_15−control_15
logFC : yeast_30−control_30
Average logCPM
Average logCPM

Figure 7.



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1026 **Figure 8.**

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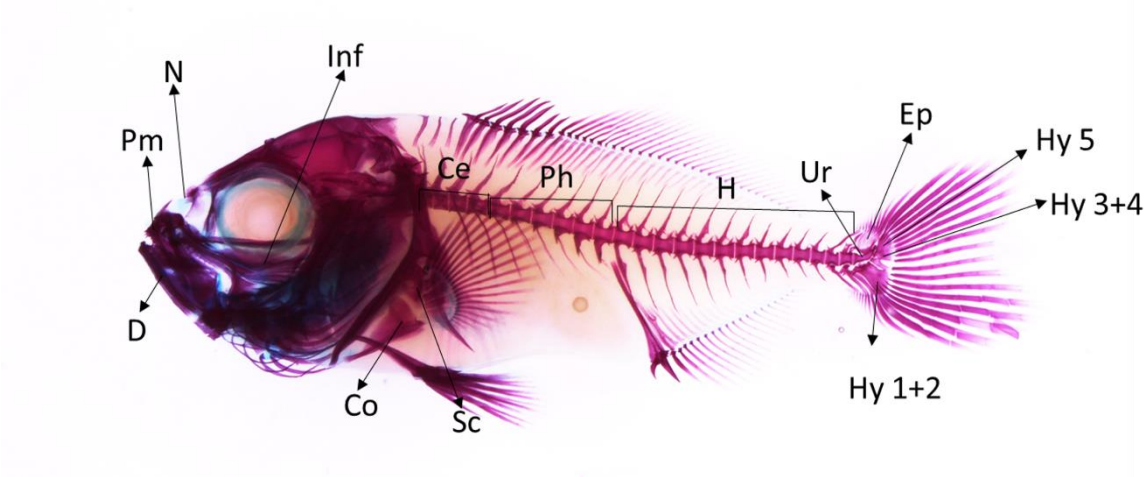

Supplementary figure.

Supplementary Table

Standard length of *Seriola rivoliana* larvae throughout development.

	Days post-hatching						
Treatment	0	5	7	10	20	25	30
Control	2.9±0.14	3.51±0.19	4.03±0.12	4.12±0.24	6.44±0.74	7.14±0.61	11.35±1.1
Yeast	2.9±0.14	3.8±0.17	4.20±0.23	4.50±0.33	6.61±0.85	9.94±1.37	13.0±1.6

Data are represented by means ± SD.