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Research Article

First Assessment of Prebiotics, Probiotics, and Synbiotics Affecting Survival, Growth, and Gene Expression of European Eel (*Anguilla anguilla*) Larvae

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European eel, *Anguilla anguilla*, larval culture faces a bottleneck during the transition to exogenous feeding. To stimulate gut-priming, in the present study, prebiotics (AgriMOS, mannan-oligosaccharides, and β-(1,3 and 1,6)-poly-D-glucose), probiotics (Bactocell, *Pediococcus acidilactici*), and synbiotics (AgriMOS + Bactocell) were administered to European eel larvae during the endogenous prefeeding stage. Eel larvae were reared in 2 L incubators with an initial stocking density of ~200 larvae/L. Each treatment (pre-, pro-, synbiotics, and control), represented by 3 replicated incubators, was connected to a separate recirculating aquaculture system. The gut-priming agents were introduced directly into the rearing water. Results revealed increased mortality when larvae were introduced to synbiotics and impaired growth in connection to probiotics and synbiotics. Larvae receiving prebiotics showed similar survival and growth to larvae reared without gut-priming agents. The immune gene expression revealed a lag phase between maternally inherited protection (*c3, igm, and il10*) and the gradual buildup of the larvae's own immune system (*il1β, irf7)*. The lack of treatment-related immune (*c3, igm, il10, il1β*, and *irf7*) and stress/repair (*hsp70, hsp90*) responses revealed an immature immuno-readiness. Digestion (*try, ctra, ctrb, tgl,* and *amyl*), food intake (*cck*), and appetite (*ghrl*)-related genes were expressed at basal levels already on 4 days post-hatch, which combined with phenotypic plasticity of the appetite-regulating ghrelin (*ghrl*), indicated a prospective adaptive capability towards earlier maturation of the larval digestive capacity. Overall, we contemplate that the application of gut-priming agents in water has merit; however, as no beneficial effect was observed, we conclude that the regimen applied is not recommendable in the present form and needs to be customized for future eel larval culture. As such, water management strategies and rearing options need to be further explored to establish prefeeding and feeding regimens, targeting optimized culture conditions, and the production of healthy eel offspring.

1. Introduction

Catadromous anguillid eels have continental juvenile stages followed by oceanic reproductive and larval stages, where so-called silver eels and larvae (leptocephali) travel thousands of kilometers to complete their life cycle [1]. For the European eel (*Anguilla anguilla*), knowledge about natural reproduction and spawning habitats is limited, while insights regarding ecophysiology and nutrition of their offspring are negligible. However, significant progress has recently been made towards closing the life cycle of European eel in culture, enabling a steady production of high-quality gametes, embryos, and yolk-sac larvae [2]. In particular, early life challenges and preferences in relation to light, salinity, and temperature have been addressed [3–5], but metamorphosis to the leptocephalus stage remains

a challenge mainly due to bottlenecks throughout the first-feeding period [6, 7].

Hatchery reared European eel larvae reach the first-feeding stage at 10–12 dph [4]. During this period, encompassing the yolk-sac stage, eel larvae rely solely on endogenous utilization of the yolk sac and cannot eat exogenous feeds as the feeding apparatus and the gastrointestinal tract are still developing and not fully functional [6–8]. In nature, the diet of the earliest stages is not known; while in culture, once larvae reach the feeding stage, they tend not to prey on live organisms but feed on paste-like liquid feeds provided to the bottom of each rearing tank [9, 10]. Currently, research efforts focus on exploring pre-feeding options, where especially considering the long yolk-sac period, eel larvae might potentially benefit from dietary nutrients and gut-priming agents through "drinking."

Fish nutrition does not only depend on the feed source and availability but also on the digestive potential, which is linked to underlying genetic mechanisms [11–13]. Feeding and digestion functionality also relies on the microbial communities inhabiting the gastrointestinal tract [14], where gut microbiota support several functions within the host concerning the regulation of ingestion and metabolism [15, 16], as well as immune defense [17]. However, the early life microbiome is rather limited, as the larval gastrointestinal tract will progressively be colonized by microbes originating from the egg epibiota, the rearing water, and the first feed [15], which highlights the importance of which type of microbes fish larvae get to interact with during early life [18, 19]. As such, gut microbial steering, to prime and assist or support digestion and health, has become of great interest.

In this regard, microbial steering in fish can be performed through controlled maturation of bacterial communities in recirculating aquaculture systems [19] but also by the use of specific products in the diet [20] or even direct applications into the rearing water [21]. For instance, a β-glucan (prebiotic) bath during the embryonic development increased the larval size of Nile tilapia, *Oreochromis niloticus* [22], while treatments with probiotics as water additives, such as *Bacillus* coagulans B16 and *Rhodopseudomonas palustris* G06 [23] or AquaStar® and EM® [24], have previously shown to enhance health status, improve water quality parameters, and increase growth performance of the same species. However, within the European Union, the only probiotic authorized for use in aquaculture is "Bactocell," with the active ingredient *Pediococcus acidilactici* CNCM I-4622 (MA 18/5M) [25]. On the other hand, several mannan-oligosaccharides (MOSs) have been applied as prebiotics to stimulate growth performance and nutrient digestibility as well as immuno-readiness [26–28]. When applied individually, both probiotics and prebiotics have shown encouraging results in improving the growth performance and health status of several aquaculture species [29–31], but the effects depend on the product, dose, time of supplementation, and way of administration [27, 32]. In addition, a plethora of combinations of different prebiotics and probiotics, defined as synbiotics, have been reviewed in Huynh et al., [33]; where it is described that they can act synergistically or complementarily. Here, synbiotics based on combinations of the aforementioned *Pediococcus acidilactici* with different types of prebiotics (β-glucan among others) have been suggested to influence growth, survival, and gene expression in aquaculture species [33].

In the case of eel, the expression profiles of genes linked to larval food intake and digestion-related processes indicated the potential adaptive capacity towards an earlier maturation of the ingestion and digestion functionality [7]. In combination with the early drinking capability [34], this may facilitate the "gut-priming" principal even before the onset of exogenous feeding. As such, in the present study, prebiotics that include b-glucan (AgriMOS: Mannan-oligosaccharides (MOS) and β-i(1,3 and 1,6)-poly-D-glucose), probiotics (Bactocell: *Pediococcus acidilactici*), and synbiotics (AgriMOS + Bactocell) were administered to European eel larvae during the endogenous (pre)feeding stage. The aim was to explore the applicability of introducing those gut-priming agents directly into the rearing water and evaluate the ability of those products in promoting gut-priming and supporting digestive potential in European eel larvae. For this, larval survival and biometrics were measured, and the expression patterns of genes related to appetite, food intake, and digestion as well as stress/repair and immune-related processes were investigated.

2. Materials and Methods

2.1. Ethical Statement. All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, and Agriculture and Fisheries (permit number: 2020-15-0201–00768). In brief, adult eels were anesthetized using ethyl p-aminobenzoate (benzocaine) before tagging and handling. European eel larvae were anesthetized prior to handling and euthanized prior to sampling by using tricaine methanesulfonate (MS-222).

2.2. Broodstock Management. Female broodstock were wild-caught from Saltbaekvig, Denmark (55°44′51.1″N 11°08′28.3″E), while all males were raised from the glass eel stage at a commercial eel farm (Royal Danish Fish, Hanstholm, Denmark). After collection, broodstock were transferred to the EEL-HATCH facility (DTU Aqua, Hirtshals, Denmark), where they were maintained in ~1250 L polyethylene tanks integrated into a recirculating aquaculture system (RAS) under a continuous flow rate per tank of ~10–15 L/min, low intensity light (~20 lux), and 12 h light/12 h dark photoperiod. Acclimatization took place over three weeks to reach a salinity of 36 ppt and a temperature of 20°C. As eels naturally undergo a fasting period from the onset of the prepubertal silvering stage, they were not fed during this period. Prior to experimentation, eels were anesthetized (ethyl p-aminobenzoate, 20 mg/L; Sigma-Aldrich Chemie, Steinheim, Germany), tagged with a passive integrated transponder, and length and weight were recorded.

2.3. Gamete Production and Embryonic Incubation. To induce vitellogenesis, female eels received weekly injections of salmon pituitary extract (Argent Chemical Laboratories, USA) at 18.75 mg/kg body weight [35]. To stimulate follicular maturation and induce ovulation, female eels received an additional injection of 17α,20β-dihydroxy-4-pregnen-3-one (Sigma-Aldrich, St. Louis, USA) at 2.0 mg/kg body weight [36, 37]. Then, within 12–14 h, eggs were strip-spawned. Males received weekly injections of human chorionic gonadotropin (hCG, Sigma Aldrich Chemie, Germany) at 1.5 IU/g initial body weight. Prior to fertilization, they were given an additional injection and milt was collected ~12 h thereafter [38]. Milt was pipetted into an immobilizing medium at a concentration of 1 : 99 [8] and used for fertilization within 4 h of collection [39].

Eggs from each female were "crossed" with a pool of milt from 3–5 males to create different ($n = 3$) family crosses [40]. Eggs from each female were stripped into dry plastic containers and gametes were swirled together. Artificial seawater (~20°C), prepared by using reverse osmosis filtration (Vertex Puratek 100 gpd RO/DI, Vertex Technologies Inc., USA) and salted to 36 ppt (Aquaforest Reef Salt, Poland), was added for a gamete contact time of 5 min [8, 39]. The fertilized eggs were then incubated for 2 h in 20 L containers filled with 15 L of the abovementioned artificial seawater before the buoyant eggs were transferred to 60 L black conical incubators, supplied with conditioned filtered seawater [7] at a flow through rate of ~350 mL/min. Gentle aeration was added after ~4 h post fertilization (hpf), while temperature was lowered to ~18°C for better embryonic development [4]. Light was kept at a low intensity below ~10 lux [3] and sinking dead eggs were purged from the bottom valve of each incubator in regular intervals. At ~48 hpf, aeration was stopped, and larvae hatched at ~56 hpf.

2.4. Experimental Design and Conditions. Especially considering the potential variability among batches, the experiment was repeated 3 times, each time using a different family cross ($n = 3$), to reduce effects of errors and increase reliability of results. For each family cross ($n = 3$), different rearing treatments ($n = 4$) of probiotics, prebiotics, synbiotics, and control were targeted, while each treatment was represented in replicated ($n = 3$) 2 L incubation jars (2 L brine shrimp jars, Ø 13 cm, height: 30 cm, jug desk-type, Taipei, Taiwan). At two days post-hatch (dph), ~200 larvae/L were gently transferred into each acrylic incubator, featuring bottom inlets with flow rates of ~150 mL/min and a 250 μm mesh subsurface outlet. The rearing jars were randomly divided into four groups (4 treatments × 3 replicates) and connected to four RAS units, each representing the 4 treatments (pre-, pro-, synbiotics, and control). In total, 3 family crosses × 4 treatments × 3 replicates = 36 experimental units × ~400 larvae = ~14.400 larvae.

Each RAS unit consisted of a 50 L biofilter filled with RK bioelements, a protein skimmer (Wavereef, China), a 100 L reservoir hosting the main pump, and a 180 L header tank. Three jars were connected to each system, while within each jar, a steady upwelling flow created enough turbulence to keep the larvae in suspension and maintain optimal oxygen levels for rearing. To keep the bacteria level under control, each system was connected to a UV-C lamp (11W JBL ProCristal UV-C, Compact, Germany) turned on from 9 pm to 9 am. Water temperature was kept at 19 ± 1°C and salinity was progressively reduced from 36 to 18 ppt over a period of 4 days to improve larval survival [5, 41, 42]. Prebiotics (AgriMOS: mannan-oligosaccharides (MOSs), and β-(1,3 and 1,6)-poly-D-glucose), probiotics (Bactocell: *Pediococcus acidilactici*), and synbiotics (Bactocell + AgriMOS) were added daily according to the suppliers recommendations (Table 1) to the reservoir tank of the corresponding RAS (Figure 1). One RAS received no additives (control). Approximately, 10% of water was exchanged daily.

3. Data Collection

3.1. Survival. Considering each family cross ($n = 3$), treatment ($n = 4$), and replicate ($n = 3$), dead larvae were counted daily and removed from all experimental units. Additionally, all larvae at the end of the experiment as well as all sampled larvae from each experimental unit were enumerated and recorded. Larval cumulative mortality was then calculated as a percentage from 2 to 13 dph.

3.2. Biometry. At the beginning of each trial, ~15 larvae per family cross ($n = 3$) were randomly sampled, anesthetized with MS-222, and imaged using a digital camera (Digital Sight DS-Fi2, Nikon Corporation, Japan) attached to a zoom stereomicroscope (SMZ1270i, Nikon Corporation, Japan). Additionally, ~10 larvae from each family cross ($n = 3$), treatment ($n = 4$), and replicate ($n = 3$) were randomly sampled, anesthetized, and imaged at 4 (mouth opening), 8 (teeth formation), and 13 dph (end of the endogenous phase). NIS-Elements-D analysis software (Nikon Corporation, Japan) was used to analyse larval images, from where total body and oil drop area was measured for each larva. Larval growth and the oil-drop utilization rate were measured from the change in body and oil drop area, respectively. Growth efficiency was then measured by dividing the increase in body area by the corresponding decrease in oil drop area [3].

3.3. Gene Expression. For molecular analysis, ~30 larvae were randomly sampled at the beginning of the study (2 dph) and throughout the endogenous feeding stage (4, 8, and 13 dph) from each family cross ($n = 3$), treatment ($n = 4$), and replicate ($n = 3$). Those larvae were recorded, euthanized using MS-222, preserved in RNAlater stabilization reagent, and kept at −20°C. RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Germany) following the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometry using NanoDropTM One (ThermoFisher ScientificTM) and then transcribed using the qScriptTM cDNA synthesis kit (Quantabio, Germany) according to the manufacturer's instructions, including an additional gDNA wipe out step (PerfeCta DNase I kit (Quantabio, Germany)). The expression levels of target

Table 1: Commercial name, product description, and amount of the pre-, pro-, and synbiotics added daily to the rearing water of European eel, *Anguilla anguilla* larvae.

	Product name	Product description	Amount
Prebiotic	AgriMOS	Mannan-oligosaccharides (MOS) and β-(1,3 and 1,6)-poly-D-glucose	20 g/m^3/day
Probiotic	Bactocell	*Pediococcus acidilactici*	5 g/m^3/day
Synbiotic	Bactocell + AgriMOS		5 g/m^3/day + 20 g/m^3/day
Control	—	—	—

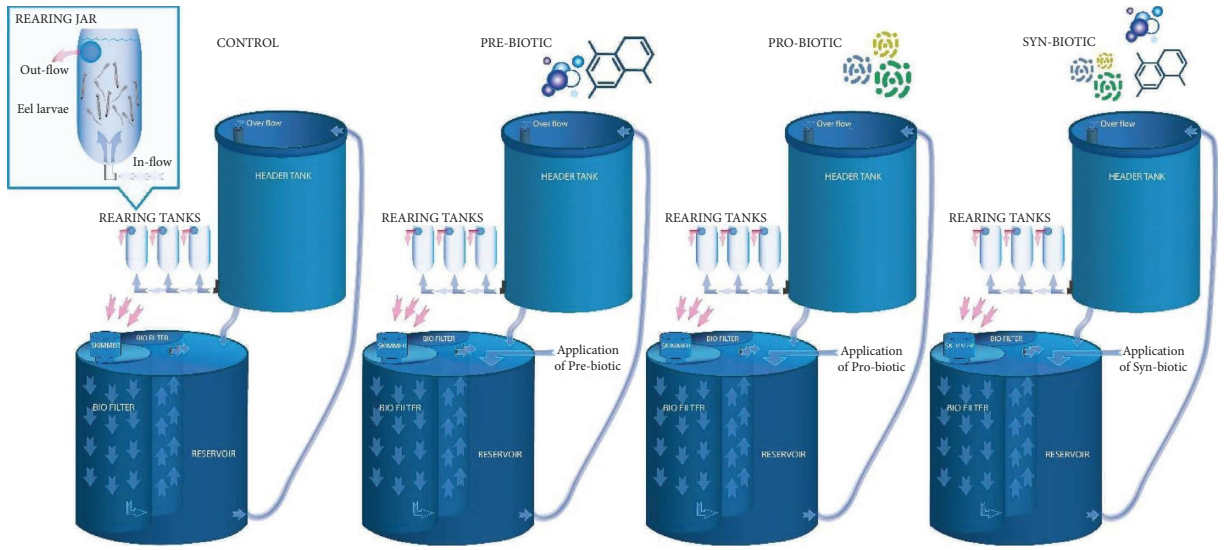

Figure 1: Schematic representation of the experimental setup, testing the application of prebiotics (AgriMOS), probiotics (Bactocell), and synbiotics (Bactocell + AgriMOS) as gut-priming agents for European eel, *Anguilla anguilla* larvae. Larvae were reared in replicated acrylic 2 L jars, featuring bottom inlets with flow rates of ~150 mL/min and a 250 μm mesh subsurface outlet. Each treatment (control, pre-, pro-, and synbiotics) was represented by three replicated jars connected to a separate recirculating aquaculture system (RAS). Each RAS unit consisted of a 50 L biofilter, protein skimmer, 100 L reservoir hosting the main pump, and 180 L header tank. Prebiotics (20 g/m^3), probiotics (5 g/m^3), and synbiotics (prebiotics (20 g/m^3) + probiotics (5 g/m^3)) were daily added according to the supplier's recommendations to the reservoir tank of the corresponding RAS. One RAS received no additives (control). Approximately, 10% of water was exchanged daily.

genes were determined by quantitative real-time PCR (RT-qPCR), using specific primers (Table 2), which were designed using primer 3 software v 0.4.0 (https://frodo.wi.mit.edu/primer3) based on cDNA sequences available in GenBank databases. All primers were designed for an amplification size ranging from 75 to 200 nucleotides and optimal Tm of ~60°C.

The expressions of genes in each larval sample from each family cross ($n = 3$), treatment ($n = 4$), replicate ($n = 2$), and larval age (2, 4, 8, and 13 dph) were analysed in two technical replicates using the qPCR BiomarkTM HD technology (Fluidigm) based on 96.96 dynamic arrays (GE chips). A preamplification step was performed with a 500 nM primer pool of all primers in TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.3 μL cDNA per sample for 10 min at 95°C and then 14 cycles of 15 sec at 95°C and 4 min at 60°C. Obtained PCR products were diluted 1 : 10 with low EDTA-TE buffer. The preamplified product was loaded onto the chip with Ssofast-EvaGreen Supermix Low Rox (Bio Rad) and DNA-binding dye sample loading reagent (Fluidigm). Primers were loaded onto the chip at a concentration of 50 μM. The chip was run according to the Fluidigm 96.96 PCR protocol with a Tm of 60°C. The relative quantity of target gene transcripts was normalized (ΔCT) to the geometric mean of the 2 most stable reference (housekeeping) genes. The *ef1a* and *rps18* genes were chosen as housekeeping genes after qBase + software revealed that these mRNA levels were stable throughout the analysed samples ($M < 0.4$); M gives the gene stability and $M < 0.5$ is typical for stably expressed reference genes [43]. The coefficient of variation (CV) of technical replicates was calculated and checked. Further analysis of gene expression was carried out according to the 2-ΔΔCt method [44].

3.4. Statistical Analyses. All data were analysed using SAS statistical analysis software (v.9.1; SAS Institute Inc., USA). Residuals were evaluated for normality (the Shapiro–Wilk test) and homoscedasticity (plot of residuals vs. predicted valves) to ensure they met model assumptions. Data were log10 or arcsine square root (percentage data) transformed to meet these assumptions when necessary. The level of significance was set at 0.05 for testing main effects and interactions. Treatment means were contrasted using Tukey's test. Body area, oil droplet area, growth rate, oil droplet utilization, growth efficiency, and survival at 13 dph as well as gene expression (14 genes) at each age (4, 8, and 13 dph) were analysed using a series of mixed model factorial

Table 2: Sequences of European eel, *Anguilla anguilla* primers used for the amplification of genes by qRT-PCR. Primers were designed based on sequences available on GenBank databases.

Full name	Function	Abbreviation	Primer sequence (5′–3′) forward	Primer sequence (5′–3′) reverse	GenBank ID
Elongation factor 1	Reference	*ef1a*	CTGAAGCCTGGTATGGTGGT	CATGGTGCATTTCCACAGAC	EU407824.1
18s ribosomal RNA		*rps18*	ACGAGGTTGAGAGAGTGGTG	TCAGCCTCTCCAGATCCTCT	XM_035428274.1
Cholecystokinin	Food intake	*cck*	CGCCAACCACAGAATAAAGG	ATTCGTATTCCTCGGCACTG	XM_035409023.1
Prepro-ghrelin	Appetite	*ghrl*	TCACCATGACTGAGGAGCTG	TGGGACGCAGGGTTTTATGA	XM_035381207.1
Amylase	Digestion	*amyl2a*	AGACCAACAGCGGTGAAATC	TGCACGTTCAAGTCCAAGAG	XM_035420193.1
Triglyceride lipase		*tgl*	CTGACTGGGACAATGAGCGT	CGTCTCGGTGTCGATGTAGG	DQ493916.1
Chemo trypsin a		*ctra*	CCAGTAACGCTTGCAGTCAG	CCAGTAACGCTTGCAGTCAG	XM_035417209.1
Chemo trypsin b		*ctrb*	ATTGTTGATGGTGCTGGGGT	ATCGTGGGAGAGCATGACAA	XM_035417164.1
Trypsin		*try*	CTGCTACAAATCCCGTGTGG	GGAGTTGTATTTGGGGTGGC	MH001533.1
Heat shock protein 70	Cellular stress/repair	*hsp70*	TCAACCCAGATGAAGCAGTG	GCAGCAGATCCTGAACATTG	XM_035380750.1
Heat shock protein 90		*hsp90*	ACCATTGCCAAGTCAGGAAC	ACTGCTCATCGTCATTGTGC	XM_035392491.1
Complement system	Immune response	*c3b*	AATATGTGCTCCCAGCCTTC	GATAACTTGCCGTGATGTCG	XM_035404253.1
Immunoglobulin M		*igm*	CCAAGGACCATTCTTTCGTC	ACTGGCTTTCAGGAAGATGC	EF062515.1
Interleukin 10		*il10*	CCTGCAAGAAACCCTTTGAG	TGAACCAGGTGTCAATGCTC	XM_035382126.1
Interleukin 1β		*il1β*	ATTGGCTGGACTTGTGTTCC	CATGTGCATTAAAGCTGACCTG	XM_035380403.1
Interferon regulatory factor 7		*irf7*	TTCCTTGGAAGCACAACTCC	TGTCGTTCGGATTCTCTCTG	KF577784.1

ANOVAs. The main model variables were gut-priming treatment (fixed effect), family (random effect), and the family × treatment interaction (random effect). Variance components (VCs) for random effects were generated using the restricted maximum-likelihood estimation method and expressed as a percentage. The mean and standard errors for each treatment and family effect were calculated. Additionally, a series of mixed effects models were run to investigate gene expression changes over time and throughout early larval ontogeny for each gut-priming regime (control, pre-, pro-, and synbiotics). These ANOVA models included the larval age (4, 8, and 13 dph) fixed effect, the random family effect, and the random age × family interaction.

4. Results

4.1. Survival and Biometry. Eel larval survival (until 13 dph) was not affected by the addition of pre- or pro-biotics and ranged from 52.7 ± 3.6 to 61.6 ± 4.6% between treatments (pre, pro, and control) but was significantly reduced ($p < 0.01$) when larvae were prefed with synbiotics (34.3 ± 4.18%). Moreover, a significant ($p < 0.01$) effect of family cross was observed, explaining 38.6% of the total variance (Figure 2(a)).

Larval body area did not differ across treatments, reaching 3.61 ± 0.02 mm^2 on 13 dph, where a significant treatment × family interaction explained 36.2% of the total variance (Figure 2(b)). Similarly, oil droplet area was not affected by treatments, reaching 0.0098 ± 0.00029 mm^2 on 13 dph, while a significant ($p < 0.01$) effect of family cross explained 95.5% of the total variance (Figure 2(c)). Moreover, the growth rate was highest when eel larvae were prefed with prebiotics (0.062 ± 0.005 mm^2/d) or reared without gut-priming agents in the control treatment (0.069 ± 0.007 mm^2/d) but significantly ($p < 0.001$) reduced when prefed with probiotics (0.037 ± 0.005 mm^2) and synbiotics (0.035 ± 0.004 mm^2/d) (Figure 2(d)). Here, the significant ($p < 0.01$) treatment × family interaction explained 38.8% of the total variance. At the same time, no statistically significant difference in the energy reserve (oil droplet) utilization rate (0.007 ± 0.0003 mm^2/d) was observed, where the VCs for family cross explained 90.4% of the observed variance (Figure 2(e)). Similarly, no statistically significant difference in growth efficiency (0.215 ± 0.013 mm^2/d) was observed among treatments, where VCs for family cross and treatment × family interaction explained 45.1% and 34.2% of the total variance, respectively (Figure 2(f)).

4.2. Appetite, Food Intake, and Digestion-Related Gene Expression. The expression of ghrelin (*ghrl*) significantly ($p < 0.01$) increased throughout development, peaking at 13 dph (Figure 3(a)), where it was driven by the significant ($p < 0.01$) family × treatment interaction, explaining 48.7% of the variability (Figure 3(b)). Similarly, the expression of cholecystokinin (*cck*) also significantly ($p < 0.01$) increased throughout the development (Figure 3(c)), while 53.9% and 38.6% of the observed variability on 4 and 8 dph, respectively, were driven by the significant ($p < 0.01$) family effect (Figure 3(d)).

All genes investigated in this study, encoding major digestive enzymes, such as trypsin (*try*) and chymotrypsin homologs (*ctr-a* and *ctr-b*) as well as triglyceride lipase (*tgl*) and amylase (*amyl*), showed similar expression patterns, where a significant ($p < 0.01$) increase throughout development was observed, peaking at 13 dph (Figures 3(e)–3(i)). The VC analysis revealed a significant ($p < 0.01$) family effect, explaining the variability observed for *try* (75.1%) and *ctr-a* (91.6%) on 4 dph; *ctr-a* (56.8%), *ctr-b* (90.9%), and *tgl* (47.6%) on 8 dph; as well as *ctr-a* (29.7%), *ctr-b* (43.1%), *tgl* (72.1%), and *amyl* (64.3%) on 13 dph (Figures 3(j)–3(n)).

4.3. Stress/Repair and Immune Response-Related Gene Expression. Regarding immune response, the complement component (*c3*), immunoglobulin (*igm*), and interleukin 10 (*il10*) expression significantly ($p < 0.01$) decreased, while interleukin 1β (*il1β*) and interferon regulating factor 7 (*irf7*) significantly ($p < 0.01$) increased throughout development (Figures 4(a)–4(e)). At the same time, the VC analysis revealed a family effect, significantly ($p < 0.01$) explaining the variability observed for *irf7* (44.5%) on 4 dph, for *igm* (41.8%) and *il1β* (36.5%) on 8 dph, as well as for *il10* (14.9%) on 13 dph (Figures 4(f)–4(j)).

Regarding stress/repair response, the expression of two genes encoding different types of heat shock proteins (*hsp70* and *hsp90*) significantly ($p < 0.01$) increased throughout development (Figures 4(k)-4(l)), while no significant effect of treatments was detected (Figures 4(m)-4(n)).

5. Discussion

The present study examined the influences of prebiotics (AgriMOS), probiotics (Bactocell), and synbiotics (Bactocell + AgriMOS) on European eel larval survival, biometrics, and gene expression, hypothesizing that they would act as gut-priming agents, promoting the maturation of the larval gut and stimulating the digestive capacity. The choice of gut-priming agents was based on positive findings in other fish species. For instance, the administration of lactic acid bacteria, similar to the ones present in Bactocell, improved growth, immunity, and health status as well as reproduction in zebrafish, *Danio rerio* [45, 46]; increased survival rates in pikeperch, *Sander lucioperca* larvae [47]; improved stress resistance in Persian sturgeon, *Acipenser* persicus larvae [48]; and induced higher activity of digestive enzymes in juvenile California halibut, *Paralichthys californicus* [49]. Moreover, the use of compounds present in AgriMOS has improved growth performance, survival, and immune status of rainbow trout, *Oncorhynchus mykiss* [50], or enhanced growth performance and feed utilization of the giant freshwater prawn, *Macrobrachium rosenbergii* juveniles [51].

Moreover, the technique chosen in the present study regarding the application of the aforementioned products was inspired by the fact that an abundance of beneficial microbiota and boosting immuno-readiness in fish can be improved by directly using gut-priming products as additives to the rearing water [21]. For instance, baths with gut-priming products can lead to improved larval size when

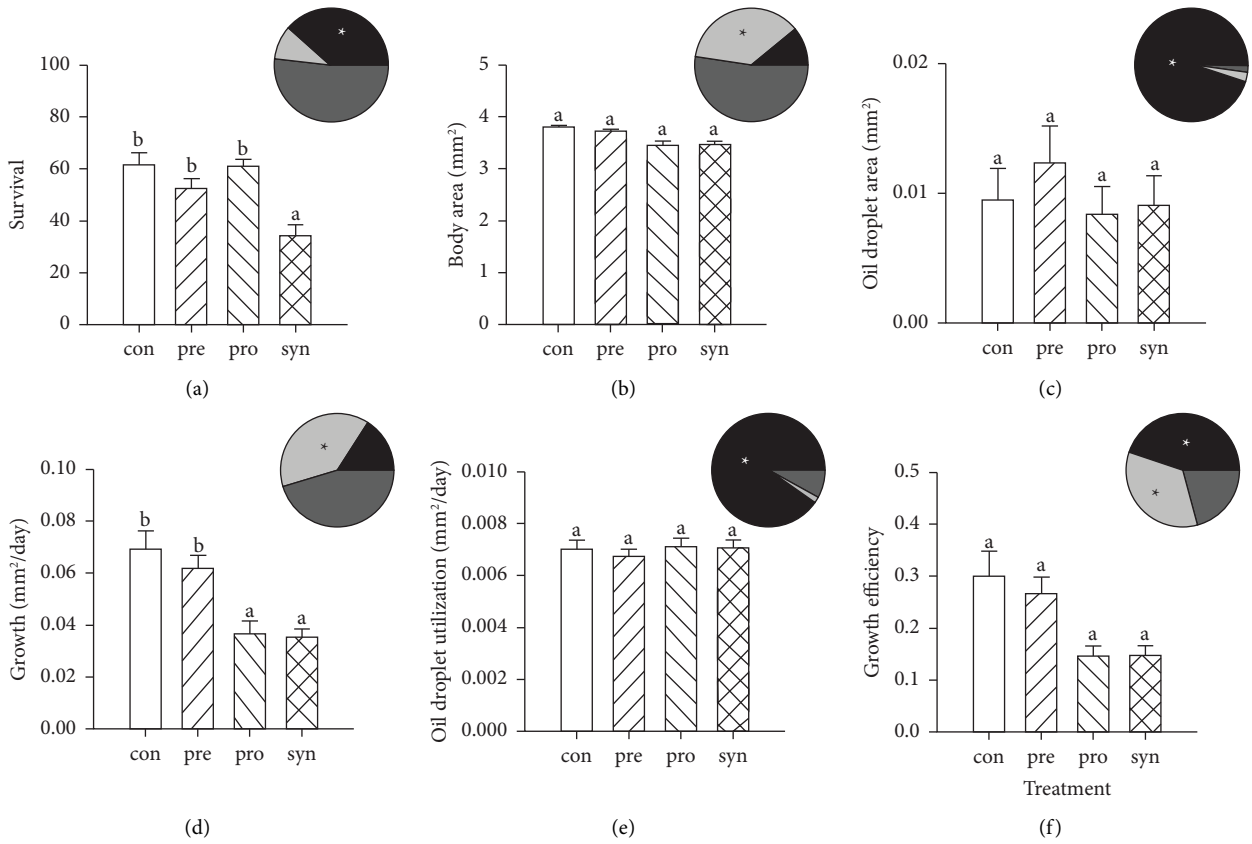

Figure 2: Survival (a), body area (b), oil droplet area (c), growth rate/day (d), oil droplet utilization/day (e), and growth efficiency (f) of European eel (*Anguilla anguilla*) larvae reared until 13 days post-hatch (dph) under four gut-priming scenarios (control, pre-, pro-, and synbiotics). The main model variables were put into gut-priming treatment (fixed effect), family (random effect), and the treatment × family interaction (random effect). Small letters represent significant differences among treatments and asterisks represent significant variance components (VCs). The VCs were generated using the restricted maximum-likelihood estimation method and expressed as a percentage. Black represents % VC family, light grey represents % VC family × treatment, and dark grey represents % VC error. The level of significance was set to 0.05.

applied during the embryonic development of Nile tilapia [22] or even to weight gain of up to 40–55% in juveniles when applied during fertilization in chum salmon, *Oncorhynchus keta* [52]. On the contrary, we observed that the presence of gut-priming agents can negatively influence eel larval survival, as evident in the case of administrating synbiotics. Moreover, the growth rate was negatively affected by the administration of synbiotics or probiotics alone, while larvae receiving only prebiotics showed similar growth rates to larvae reared without gut-priming agents (control). In this study, eel larvae were reared in tanks connected to separate RAS units for each treatment, which included a biofilter and UV treatment, targeting stability by the digestion of waste nutrients and the reduction of circulated pelagic microorganisms. However, it is worth mentioning that generally the load of bacteria in a newly started RAS is normally much higher than in flow through systems or microbially matured systems [53]. Therefore, when introducing microbiota (probiotics) and oligosaccharides (prebiotics), a stimulation of microbial growth and activity in the rearing water, but also the host, is expected. In this regard, a nonmatured RAS could potentially be negatively influenced by the excess microbial activity often driven by heterotrophic bacteria [54], resulting in compromised water quality of the entire rearing unit. Consequently, the high abundance of these bacteria may directly or indirectly affect the host organism by supporting the rise of opportunistic pathogens or potentially affecting the nitrification process of the RAS as they compete for substrate and oxygen with the autotrophic bacteria [55, 56]. Hence, the synbiotics administered to eel larvae in this study, combining pre- and pro-biotics, where prebiotics acted as a promoter for extrapolated probiotic proliferation, have likely caused deterioration of water quality, resulting in the observed impaired growth and survival.

It is also worth mentioning that part of the general mortality observed in the current study can be related to the previously described immunocompromised period between hatch and the first-feeding stage of eel larvae [57]. We observed initially upregulated mRNA levels of genes (*c3, igm*, and *il-10*) corresponding to maternally originating immune protection to decrease throughout ontogeny, following the utilization of the maternal energy resources (yolk-sac and oil-droplet) and reaching baseline levels

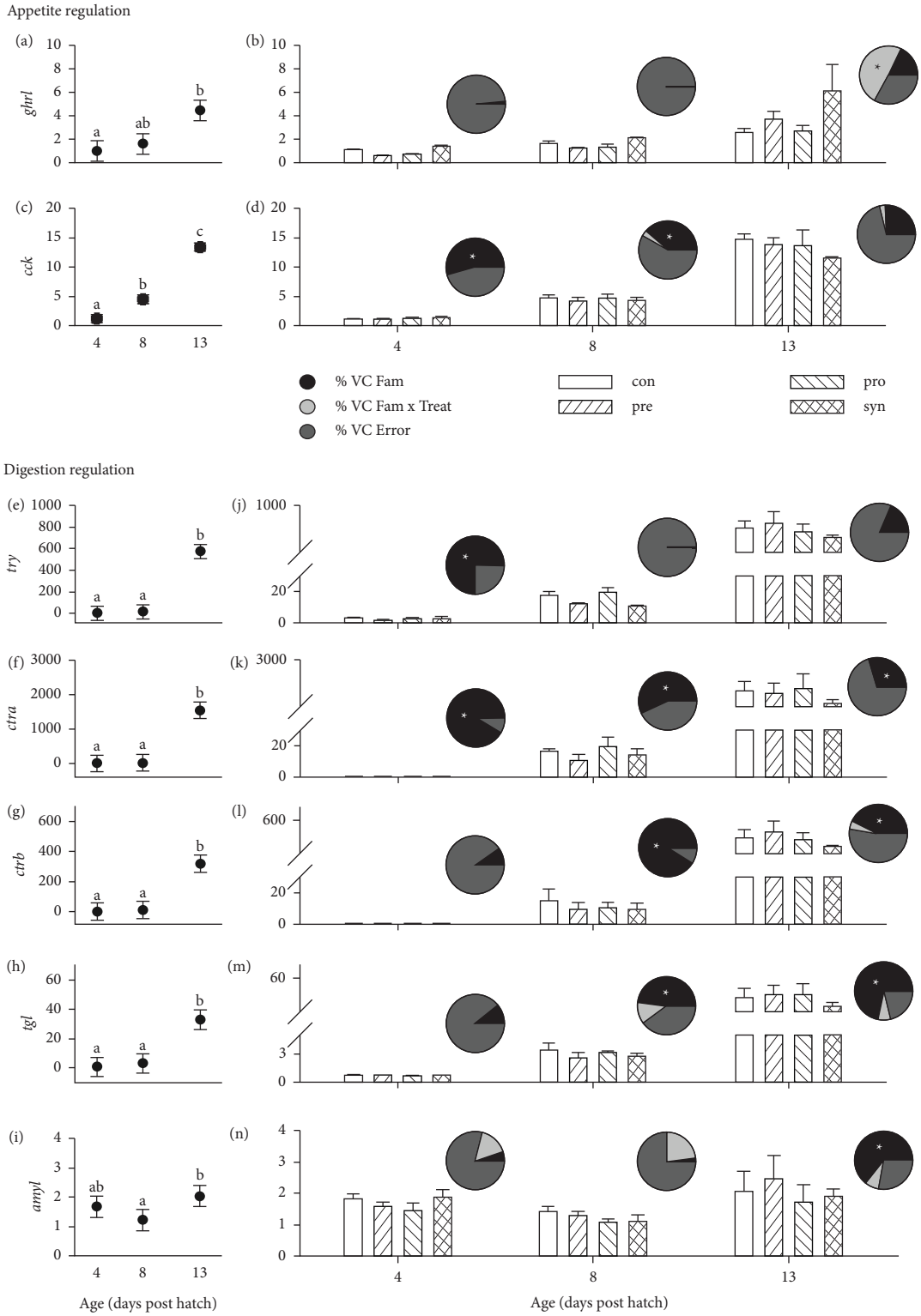

Figure 3: Expression of genes linked to appetite (ghrelin (*ghrl*)), food intake (cholecystokinin (*cck*)), and digestion (trypsin (*try*), chemotrypsin homologues (*ctra*, *ctrb*), tryglicerade lipase (*tgl*), amylase (*amyl*)) of European eel, *Anguilla anguilla*, larvae at 4, 8, and 13 days post-hatch reared under four gut-priming scenarios (control, pre-, pro-, and synbiotics). The main model variables were gut-priming treatment (fixed effect), family (random effect), and the treatment × family interaction (random effect). Small letters represent significant differences among treatments and asterisks represent significant variance components (VCs). The VCs were generated using the restricted maximum-likelihood estimation method and are expressed as a percentage. The level of significance was set to 0.05.

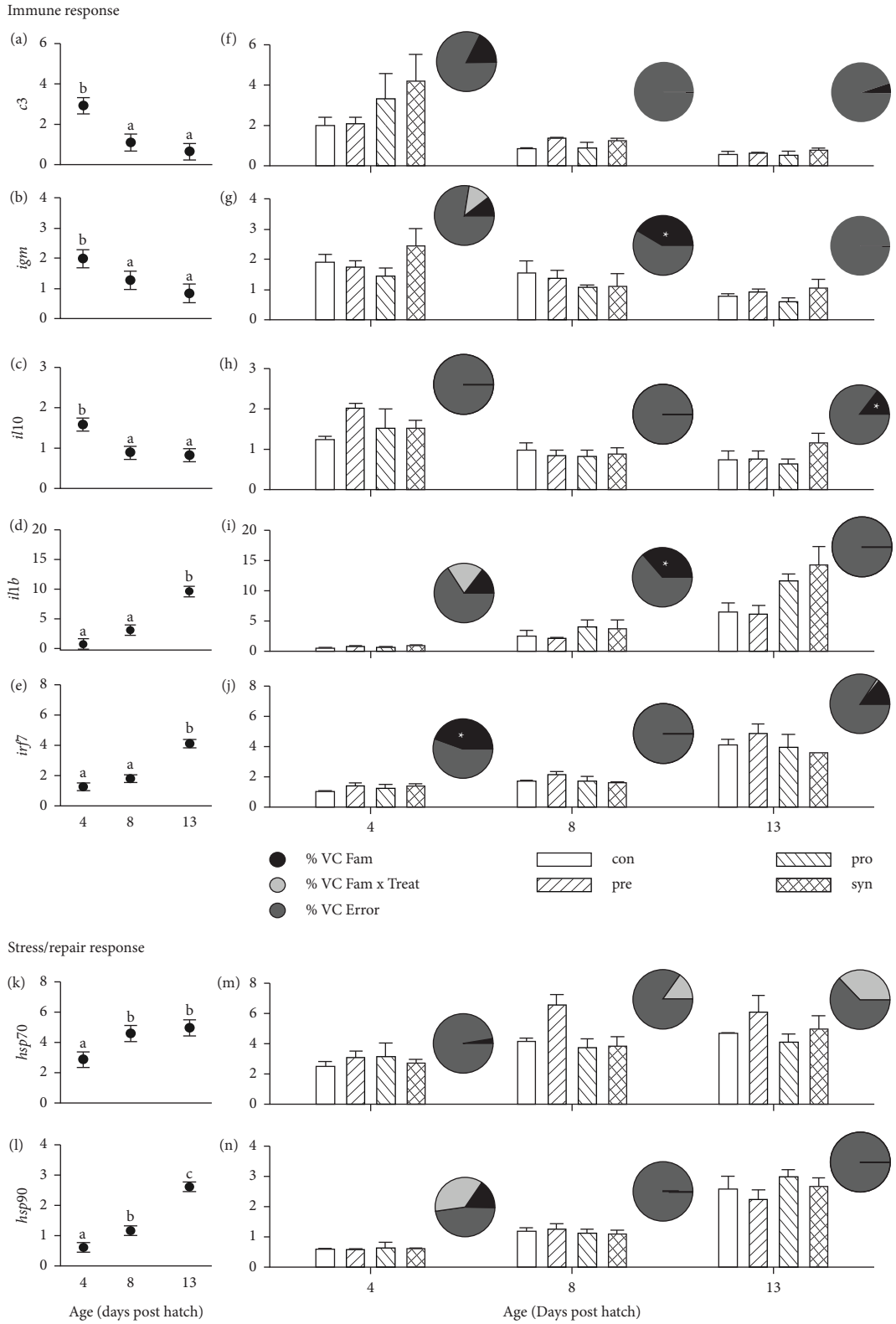

Figure 4: Expression of genes linked to stress/repair [heat shock proteins (*hsp70*, *hsp90*)] and immune response (complement component (*c3*), immunoglobulin (*igm*), interleukin 10 (*il10*), interleukin 1 β (*il1β*), interferon regulating factor 7 (*irf7*)) of European eel, *Anguilla anguilla*, larvae at 4, 8, and 13 days post-hatch, reared under four gut-priming scenarios (control, pre-, pro-, and synbiotics). The main model variables were gut-priming treatment (fixed effect), family (random effect), and the treatment × family interaction (random effect). Small letters represent significant differences among treatments and asterisks represent significant variance components (VCs). The VCs were generated using the restricted maximum-ikelihood estimation method and are expressed as a percentage. The level of significance was set to 0.05.

already at 8 dph. Similar patterns have also been observed in other fish species such as European seabass, *Dicentrarchus labrax* [58], and carp (*Cyprinus carpio*) [59]. On the other hand, the expression of interleukin 1β (*il1*β), interferon-regulating factor 7 (*irf7*), and heat shock proteins (*hsp70* and *hsp90*) increased steadily during ontogeny, indicating a maturing functionality of the larval stress/repair and immune response potential. However, we did not observe a molecular response in connection to the gut-priming agents administered in this study, probably indicating an immature immuno-readiness caused by the lag phase between the maternally inherited immune protection and the gradual build-up of the larval own immune system.

Moreover, in the present study, the expression of trypsin (*try*), chymotrypsin (*ctra* and *ctrb*), lipase (*tgl*), and amylase (*amyl*) increased throughout ontogeny and reached highest values at 13 dph, confirming the molecular ontogenetic start of the first-feeding window in European eel preleptocephalus larvae. At this point, we expected to see a molecular response in connection to the gut-priming agents, as it was previously suggested that gut-priming has the potential to trigger early development and functionality of digestive enzymes [60, 61]. Similarly, prebiotics have enhanced survival of first-feeding turbot, *Scophthalmus maximus* larvae, by altering immunity, metabolism, and microbiota [62]. However, we did not register a molecular benefit on digestive capacity through the addition of the applied gut-priming agents, indicating that the molecular ontogeny of the key players relating to the hydrolytic function of the digestive system was genetically preprogrammed and not modifiable by the applied gut-priming scenarios. Here, it needs to be mentioned that the application of gut-priming agents (β-glucans and mannan-oligosaccharides) directly into the rearing water at 5 mg·L^{-1} benefitted flounder, *Paralichthys adspersus* larvae, but higher concentrations led to contrary effects [63]. This possibly demonstrates that the product amounts used in the present study might be suboptimal or that the choice of pre- and probiotics needs to be reconsidered for eel larviculture.

In this regard, the choice of appropriate probiotic regimen depends on the probiont species, target fish species, and physiological status as well as rearing conditions and the specific goal of the application [60]. For instance, two commonly seen pathogenic *Vibrio* strains showed positive effects on scallop, *Argopecten purpuratus* larvae [64], while *Pseudomonas aeruginosa*, a member of the pathogenic skin microflora, acts as a probiotic for western king prawns, *Penaeus latisulcatus* [65]. Similarly, the fish pathogen *Shewanella putrefaciens* [66] is used as probiotic in gilthead sea bream, *Sparus aurata*, and Senegalese sole, *Solea senegalensis* [67, 68]. As such, bacterial strains that are commonly harmful to one aquatic species can be beneficial to another species when used as a probiotic [69]. Therefore, the substrains or phylogenies need to be identified and carefully considered before application as probiotics for a specific target fish species.

Interestingly, the VC analyses showed that the "family" effect drove most gene expression patterns, which could be translated into genetically preprogrammed molecular mechanisms, endowed by the parents, to control early ontogenetic processes. However, for each genotype (family), phenotypic trait, and treatment, a different reaction norm can exist [70–72]. In fact, the expression of appetite-regulating ghrelin (*ghrl*) was driven by a genetic (family) × treatment interaction, revealing differing reaction norms of each genotype (family) used in our study to the different treatments (pre-, pro-, or synbiotics) investigated. As such, this result documents phenotypic plasticity to gut-priming agents, as each genotype can produce different phenotypes (in terms of gene expression) when exposed to different additives (pre, pro-, or synbiotics). This from one side demonstrates the sensitivity for adaptation potential towards gut-priming, but from the other side indicates that the gut-priming regimens applied in this study cannot be considered a "one size fits all" solution. However, it is worth mentioning that transcripts of genes relating to digestion, food intake, and appetite were in the present study already detected at 4 dph, providing evidence that those endocrine mechanisms are present at basal levels at this early stage before progressively gaining functionality throughout ontogeny. This indicates a prospective adaptive capacity towards earlier maturation of the eel larval appetite, feeding, and digestion potential. Therefore, further investigations are encouraged to identify more suited gut-priming and/or prefeeding strategies for culturing eel larvae in the future.

To summarize, the increased mortality observed in connection with synbiotics and the impaired growth observed in connection with probiotics and synbiotics could potentially be related to the high load of organic matter in the rearing water, which probably affected water quality and increased the bacterial load in the larval tanks. At the same time, the lack of molecular responses in immune and stress/repair-related genes, indicate a still immature immuno-readiness, probably caused by the lag phase between the maternally inherited protection and the gradual build-up of the larval immune system. As such, water management strategies and rearing options need to be adapted for future gut-priming, prefeeding and feeding regimens to target optimized culture conditions and ensure the production of healthy offspring. Moreover, the early expression of genes relating to digestion, food intake, and appetite, as evidenced by their basal transcript levels already on 4 dph, as well as the phenotypic plasticity of the appetite-regulating ghrelin (*ghrl*) concerning gut-priming agents indicates a prospective adaptive capacity towards earlier maturation of the larval digestive capacity. However, despite the valuable knowledge gained by this study, the gut-priming products and/or the application regimens did not seem optimally adapted for eel larval culture. Therefore, further investigations are encouraged to identify more suited gut-priming and/or prefeeding strategies for culturing eel larvae in the future. Specifically, the elucidation of the eel larval intestinal microbiota and host-microbiota interactions could lead to the development of more refined and efficacious microbiota-intervention strategies to improve the health and performance of cultured eel offspring.

Data Availability

The data that support the results in this study are available from the corresponding author upon reasonable request.

Ethical Approval

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, and Agriculture and Fisheries (permit number: 2020-15-0201–00768). In brief, adult eels were anesthetized using ethyl p-aminobenzoate (benzocaine) before tagging and handling. European eel larvae were anesthetized prior to handling and euthanized prior to sampling by using tricaine methane sulfonate (MS-222).

Disclosure

An earlier version of this manuscript was included in a PhD thesis [73].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

JT, SE, EB, and SP conceptualized the study. JT, SS, EB, JM, and SP was responsible for the methodology. IB and SP performed formal analysis. EB, ES, and SP performed investigation for the study. JT, JM, and SP was responsible for the resources. EB, IB, JM, and SP performed data curation. EB, ES, and SP wrote the original draft. SS, IB, EB, and SP performed visualization. JT, SE, and SP supervised the study. JT, SS, and SP was responsible for project administration and funding acquisition. All authors reviewed, edited, and validated the study.

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