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Oleaginous *Mortierella alpina* biomass enhances embryogenesis, hatching success, and lipid accumulation in zebrafish (*Danio rerio*) with implications for sustainable aquaculture intensification

Sourik Mukherjee[1], Aman Kumar Mohanty[1], Suresh Kumar Saravanan[1], Uma Rao[2], Sashi Bhushan Rao[2], Raj Kumar Chinnadurai[1], Abhijit Poddar[1]*

[1]Mahatma Gandhi Medical Advanced Research Institute (MGMARI), Sri Balaji Vidyapeeth (Deemed-to-be-University), Pondicherry, India.
[2]Convergent Biosciences Pvt. Ltd., Hyderabad, India.

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ABSTRACT

Microorganisms play a major role in sustainable aquaculture intensification by improving fish health and nutrition. However, their specific roles in embryogenesis, hatching regulation, and early development remain poorly understood. In this study, we investigated the effects of oleaginous *Mortierella alpina* EI-9 biomass on zebrafish (*Danio rerio*) embryotoxicity, embryogenesis, hatching success, and lipid accumulation. A sodium polyacrylate-based biomass formulation was added to E3 medium at varying concentrations. A concentration-dependent toxicity response identified an LC_{50} of 2.47 mg/ml. At 1.0 mg/ml, the formulation produced the most favorable developmental outcomes, with accelerated development and significantly higher hatching rates (71% at 72 hpf) than the controls (13%), without any visible morphological abnormalities. Nile red staining showed significantly higher lipid accumulation in treated embryos at the prim-6 stage (159.50 ± 5.53 a.u.) than in controls (134.93 ± 5.12 a.u.), and this difference persisted into the larval phase. The larvae also showed significant upregulation of lipid metabolism-related genes, including *apoA-Ia* (6.15 ± 0.7-fold), *fads* (2.6 ± 0.4-fold), and *elovl5* (5.63 ± 1.2-fold), indicating enhanced lipid utilization. As such, this study demonstrates the valorization potential of an oleaginous *M. alpina* biomass-based formulation as a sustainable bioresource that can support aquaculture intensification by benefiting early life stages.

1. INTRODUCTION

As the aquatic equivalent of agriculture, aquaculture is one of the fastest-growing food-producing sectors and plays a key role in global food security and economic development in developing countries [1,2]. To meet these expanding demands, hatcheries face increasing pressure to increase productivity while reducing production costs. This has driven efforts toward holistic approaches to optimize embryogenesis, hatching, larval development, and survival [3,4].

Embryogenesis and hatching are tightly coordinated physiological events that involve rapid cell growth, organ formation, membrane development, and metabolic processes. Fatty acids, primarily derived from the lipids stored in the yolk sac, are indispensable molecules in fish embryogenesis. They fuel the high-energy demands of rapid cell division, differentiation, and morphogenesis [5]. Importantly, essential fatty acids (EFAs) cannot be synthesized by fish in sufficient quantities, either due to a lack of or a limited capacity in key lipid metabolic pathways to synthesize them *de novo* [6,7]. Therefore, externally supplied lipids, specifically EFAs and phospholipids, are crucial for optimizing fish hatching rates, larval survival rates, and overall egg quality.

Among microbial resources, *Mortierella alpina*, a saprophytic, oleaginous, and non-pathogenic soil fungus, stands out due to its significant valorization potential. With more than 50% of its dry weight comprising lipids and a natural ability to produce high-value long-chain polyunsaturated fatty acids (LC-PUFAs), particularly arachidonic acid (ARA), *M. alpina* has been classically used as a model organism for lipid metabolism research and is regarded as a potential species for the industrial-scale production of various types of PUFAs [8,9]. With its high oil yield and diverse bioactive compounds, *M. alpina* is being explored for applications in microbial oil production, nutraceuticals, and aquaculture biotechnology [10].

More recently, we demonstrated the potential of *M. alpina* EI-9 whole biomass as a sustainable aquafeed ingredient using both juvenile and adult zebrafish in a 60-day feeding trial [11]. The fatty acid profile of the strain revealed a higher abundance of ω-6 PUFAs such as ARA (20.18% ± 0.96%), gamma-linolenic acid (6.76% ± 0.36%), and

*Corresponding Author
Abhijit Poddar, Mahatma Gandhi Medical Advanced Research Institute (MGMARI), Sri Balaji Vidyapeeth (Deemed-to-be-University), Pondicherry, India. E-mail: abhijitp @ mgmari.sbvu.ac.in

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linoleic acid (2.03% ± 0.07%). Zebrafish fed an experimental diet containing 2% dried fungal biomass resulted in improved growth performance while maintaining stable gut ecology and normal tissue histology. In continuation of these findings, we sought to determine whether the biological effects of *M. alpina* EI-9 extend beyond post-hatch nutritional benefits to the earliest stages of zebrafish development. Therefore, in the present study, we exposed zebrafish embryos to different concentrations of whole *M. alpina* EI-9 biomass-based formulation in E3 medium to evaluate its concentration-dependent developmental effects, toxicity, and influence on embryogenesis and hatching success. To the best of our knowledge, this is the first study to demonstrate the valorization potential of *M. alpina* biomass in embryonic development, demonstrating its potential as a biologically active microbial resource beyond its established applications in lipid production and aquaculture nutrition.

2. MATERIALS AND METHODS

2.1. Organism and Experimental Formulation

Mortierella alpina strain EI-9 was used in the present study (Fig. 1). The growth, harvesting, drying, and preparation of the fungal biomass, as well as the experimental formulation, were performed as described previously by us [11]. Briefly, the strain was cultured in Potato Dextrose Broth, and the resulting mycelial biomass was harvested and vacuum-dried at 70°C for 24 hours. The dried biomass was stored at 4°C until further use. The biomass was subsequently blended with the aquaculture feed binder sodium polyacrylate (SPA; Eastchem Inc., Canada) at a 1:1 ratio to improve dispersion in aqueous media. This resulting powdered mixture was referred to as the "*M. alpina* formulation (MAF)" to distinguish it from the fungal dried biomass.

A stock solution of MAF was prepared by dissolving it in E3 medium at room temperature to obtain a final concentration of 10 mg/ml. The samples were stored at 4°C for short-term preservation and used in the experiments. For the experiment, the stock was serially diluted in E3 embryo medium to obtain seven test concentrations: T1 (0.01 mg/ml), T2 (0.05 mg/ml), T3 (0.1 mg/ml), T4 (0.5 mg/ml), T5 (1.0 mg/ml), T6 (5.0 mg/ml), and T7 (10.0 mg/ml). The respective dilutions were prepared directly in 96-well plates (Sigma-Aldrich, USA), with a final volume of 200 µl per well. The concentration gradients were adapted from [10] for calculation of the median lethal concentration (LC_{50}) [12].



Fig. 1. The rosette-shaped colony morphology of *Mortierella alpina* EI-9.

Furthermore, to rule out the embryotoxicity of SPA, we added a single SPA concentration (0.5 mg/ml) to the E3 medium, as described previously. This concentration matched the SPA present in the T5 MAF, which we identified as the most suitable for further application (presented later).

2.2. Zebrafish Strain, Husbandry, and Breeding

Adult zebrafish (10 months of age) of the wild-type AB strain were purchased from the Tarun Fish Farm, Chennai [13]. The fish were maintained in our laboratory following the rearing and husbandry practices as described previously [11].

For spawning, male and female zebrafish were paired at a 1:2 (male: female) ratio and transferred to a breeding tank equipped with a bottom net partition to retain the eggs and a central partition to separate the males and females. The fish were maintained overnight with the partitions in place. In the morning, with the first light, the central partition was removed, allowing natural spawning for 1 hour. Adults were then removed, and embryos were collected from the bottom [14,15].

The collected embryos were then washed and kept in a Petri dish containing E3 medium [16]. An initial quality check was performed under a stereomicroscope (Stemi 2000-C, Zeiss, 50x magnification), where unfertilized and coagulated embryos were discarded. For experimental consistency, healthy embryos were manually selected to synchronize with the 12th blastula stage, corresponding to 2.5–3 hour post-fertilization (hpf). Only embryos of the highest uniform quality, confirmed to exhibit no spontaneous developmental defects beyond this critical stage, were chosen for the experiments [17].

2.3. Embryo and Larval Toxicity Assay of the Formulation

Embryotoxicity was assessed following the OECD Fish Embryo Acute Toxicity Test guideline 236, with minor modifications as previously described by Usuldin *et al.* [18]. For each concentration, 30 embryos were individually exposed (with one embryo per well) per replicate. Three independent biological replicates were performed using separate biological pairs, yielding a total sample size of 90 embryos per treatment group ($N = 90$). The control group similarly consisted of 90 embryos in total (30 embryos per replicate across three biological replicates) maintained in E3 medium without MAF. Both control and treated embryos were incubated at 28°C ± 1.0°C under the same photoperiod, with observations taken at defined intervals (24, 48, 72, and 96 hpf) [15]. The primary parameters evaluated included mortality, survival, and hatching rates, which were calculated using the following formulas:

$$\text{Mortality rate (\%)} = \frac{\text{Number of coagulated embryos}}{\text{Total number of embryos used in the experiment}} \times 100$$

$$\text{Survival rate (\%)} = 100 - \text{Mortality rate}$$

$$\text{Hatching rate (\%)} = \frac{\text{Number of embryos hatched}}{\text{Total number of surviving embryos}} \times 100$$

Teratogenic effects in embryos, such as pericardial and/or yolk sac edema, spinal curvature (scoliotic or kyphotic), craniofacial and tail malformations, and visible growth retardation, were recorded under a stereomicroscope (Stemi 2000-C, Zeiss, 50x magnification) [19,20]. Physiological endpoints, such as cardiac function (heart rate and arrhythmia) and the presence of spontaneous embryonic movement, were also monitored to identify potential cardiotoxic or neurotoxic effects. Heart rates were measured for three embryos from each group for one minute using a stopwatch [12]. Moreover, qualitative assessment of arrhythmia, which involved visual inspection for deviations from a regular rhythm, such as bradycardia (a slow heart rate) and tachycardia (a fast heart rate), was also monitored [21].

Even after hatching at 96 hpf, the toxicity study continued for 7 days post-fertilization (dpf) to assess the effects of both SPA and MAF treatments on larval development. The larvae were examined daily for the presence or absence of developmental abnormalities, including pericardial edema, yolk sac edema, tail malformations, spinal deformities, craniofacial abnormalities, and irregular body curvature [22].

2.4. Determination of LC_{50}

The LC_{50} of MAF at 96 hpf was calculated according to the OECD 236 test guidelines and their modifications [18]. Initially, seven test concentrations (T1–T7) were evaluated to determine the dose-dependent mortality, as described in the previous section. Subsequently, four additional geometric progression series (1.5, 2.0, 2.5, and 3.0 mg/ml) were used to narrow the mortality range as part of a range-finding exercise [23].

2.5. Embryo Staging Experiment

Beginning at 12 hpf, live embryos in the control and T5-treated groups were monitored microscopically to assess the impact of treatment on the progression through the developmental stages until hatching was complete [24]. For this specific stage, stage-dependent developmental milestones were recorded as described by Danilova *et al.* [25]. During the early stages of zebrafish embryonic development, up to the 17-somite stage, the embryos were staged by counting the somites. At 24 hpf, the presence or absence of a visible heart rate was used as a developmental marker of cardiac development. For embryos beyond 23 hpf, developmental progression was assessed by determining the number of muscle segments (myotomes) reached by the posterior end of the migrating lateral line primordium, a measure referred to as the prim number. The number of embryos that successfully hatched was recorded and used as a criterion for tracking developmental progress.

All images were captured using an EVOS FL Auto 2 Imaging System (Invitrogen) with a 10x objective, white-light illumination, and integrated EVOS FL Auto 2 software.

2.6. RNA Isolation, Reverse Transcription, and Quantitative Polymerase Chain Reaction Assays

The expression of three major lipid metabolism genes, namely *fads*, *elovl5*, and *apoA-Ia,* in larvae was quantified by quantitative polymerase chain reaction (qPCR) using *18S* as a reference gene. Post-hatching, 60 control and T5-treated larvae were anesthetized using 1.5 mM Tricaine (Sigma-Aldrich, USA) and homogenized separately using DEPC-treated micropestles (Tarsons, India). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Purity and concentration were determined using a NanoDrop™ Spectrophotometer (Thermo Scientific™, USA). cDNA was synthesized from the extracted RNA using the PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan), following the manufacturer's instructions. The qPCR was performed using TB Green Premix ExTaq II (Takara, Japan). The qPCR analyses were performed in duplicate for each group using the Bio-Rad CFX96 Real-Time System. Thermal cycling conditions were as follows: an initial step of 95°C for 1 minute, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing for 30 seconds at 55°C (for genes *fads*, *elovl5*, *apoA-Ia)* and at 59°C for *18S*, and extension at 55°C for 30 seconds. The primer sequences for the genes used in this study are presented in Table 1. Relative expression levels of target genes were normalized against *18S* and determined using the $2^{-\Delta\Delta CT}$ method [26].

2.7. Lipid Accumulation Test

To understand the extent of lipid accumulation through external supplementation, the control and T5-treated embryos and larvae were stained with Nile Red, following a protocol described by Tingaud-Sequeira *et al.* [27]. Briefly, Nile red powder (Sigma-Aldrich, USA) was dissolved in acetone to prepare a 1 mg/ml stock solution. Both control and treated embryos at similar developmental stages, namely, the 6-somite, prim-6, and pec fin stages (20 per well in a 24-well microtiter plate), were separately stained with a Nile Red working solution prepared from stock by diluting it in E3 medium to a final concentration of 0.5 µg/ml. The plates were incubated at room temperature in the dark. The embryos were washed thrice with freshly

Table 1. The primer sequences used in this study.

Gene	Direction	Sequences	Length	Tm	Amplicon length	References
fads	Forward	CATCACGCTAAACCCAACA	19	60	158	[41]
	Reverse	GGGAGGACCAATGAAGAAGA	20	60		
elovl5	Forward	TGGATGGGACCGAAATACAT	20	60	173	
	Reverse	GTCTCCTCCACTGTGGGTGT	20	60		
apoA-Ia	Forward	TCGTGGCTCTTGCACTGAC	19	58	232	[38]
	Reverse	TCCATGTAGGGCTCCATGC	19	58		
18S	Forward	GGTGAAATTCTTGGACCGGC	20	59.47	232	This study
	Reverse	GCTTTGCAACCATACTCCCC	20	59.18		

prepared E3 medium and observed under a microscope. Similarly, 20 larvae (7 dpf) hatched from the control and treated embryos were euthanized with Tricaine and stained in a microtiter plate. These were finally washed thrice in freshly prepared E3 medium. Lipid accumulation was analyzed using intensity and integrated density analysis as described by Qiao *et al.* [28]. Briefly, images were captured using an EVOS FL Auto 2 (Invitrogen, USA) imaging system with a 10X objective and the integrated EVOS FL Auto 2 software. White light and an RFP filter covering the excitation and emission wavelengths of Nile Red (531/40 and 593/40, respectively) were used [29]. The images were then stacked into a single file using ImageJ (National Institutes of Health, USA) software to produce an overall view of the fluorescent larvae. Finally, these images were converted to 8-bit grayscale to measure the mean gray value using ImageJ [28].

2.8. Statistical Analysis

Data were expressed as mean ± standard deviation. All graphs were created using GraphPad Prism version 8.0 (GraphPad Software, Inc.). Probit regression analysis of cumulative mortality was performed using IBM SPSS Statistics software (v. 27) to identify the LC_{50} value [30]. Survival rates were depicted using Kaplan–Meier survival curves [31]. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison post hoc test [17]. For data involving two independent variables, a two-way ANOVA with Tukey's HSD post hoc test was used for pairwise comparisons [32]. A two-tailed *t*-test was performed to compare the fluorescence intensity and integrated density of 7-dpf (treated and control) larvae. To compare the expression levels of genes between the control and treatment groups, a two-tailed *t*-test was performed [33]. Differences between the means of the treated groups were deemed statistically significant if $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ compared to the control group.

3. RESULTS AND DISCUSSION

3.1. Zebrafish Embryo and Larval Toxicity

An apparent concentration-dependent increase in embryo mortality at 96 hpf followed a sigmoidal dose-response mortality curve (Fig. 2A), with a calculated LC_{50} value of 2.47 mg/ml. Embryos in the control, T1, and T2 groups maintained a high survival rate of 98.88% at 96 hpf (Fig. 2B). Embryos in the T3 group showed a non-significant decline in the survival rate to 97.78%. A statistically significant concentration-dependent decline was observed in the T4 and T5 groups, with survival rates of 96.66% and 95.55%, respectively. However, severe toxic effects were observed in the T6 and T7 groups, with survival decreasing to 0% within 24 hpf. The observed increase in mortality across the tested concentrations was statistically significant ($p < 0.001$), indicating a robust toxicological effect of MAF on zebrafish embryos within the 96 hpf exposure period.

In zebrafish embryo toxicity studies involving fungi or their products, variations in LC_{50} values are common. For example, the LC_{50} values of the cultivated biomass and exopolysaccharides (EPS) of *Lignosus rhinocerus* were 0.77 mg/ml and 0.41 mg/ml, respectively [18]. Similarly, the LC_{50} values for the biomass and EPS of Malaysian *Ganoderma lucidum* were reported to be 1.65 mg/ml and 2.64 mg/ml, respectively [17]. Another study determined the LC_{50} values for EPS and endopolysaccharides of European *Ganoderma applanatum* as 1.41 mg/ml and 0.87 mg/ml, respectively [12]. As such, compared to the LC_{50} values of these microbial biomasses, MAF exhibited a higher LC_{50}, suggesting a comparatively safer profile.

3.2. Comprehensive Endpoint Analysis

Endpoint analysis of embryos from the control, SPA, and MAF (T1-T5) groups showed no morphological abnormalities up to 96 hpf (Table 2). Specifically, no observable pericardial edema, yolk sac edema, spine malformations, or irregular body curvature were observed. All monitored cardiac functions, including heart rate and rhythm, were normal, and no signs of arrhythmia were noted in any of the embryos. Specifically, embryos treated with SPA and MAF (T1, T2, T4, and T5) exhibited heart rates comparable to the control group (158 beats/min). Although a slight reduction in heart rate to approximately 135–140 beats/minute was observed in the T3 and T4 groups, these changes were not statistically significant (Fig. 2C). Similarly, at seven dpf, no observable pericardial edema, yolk sac edema, spine malformations, or irregular body curvature were found (Fig. 3A). No morphological

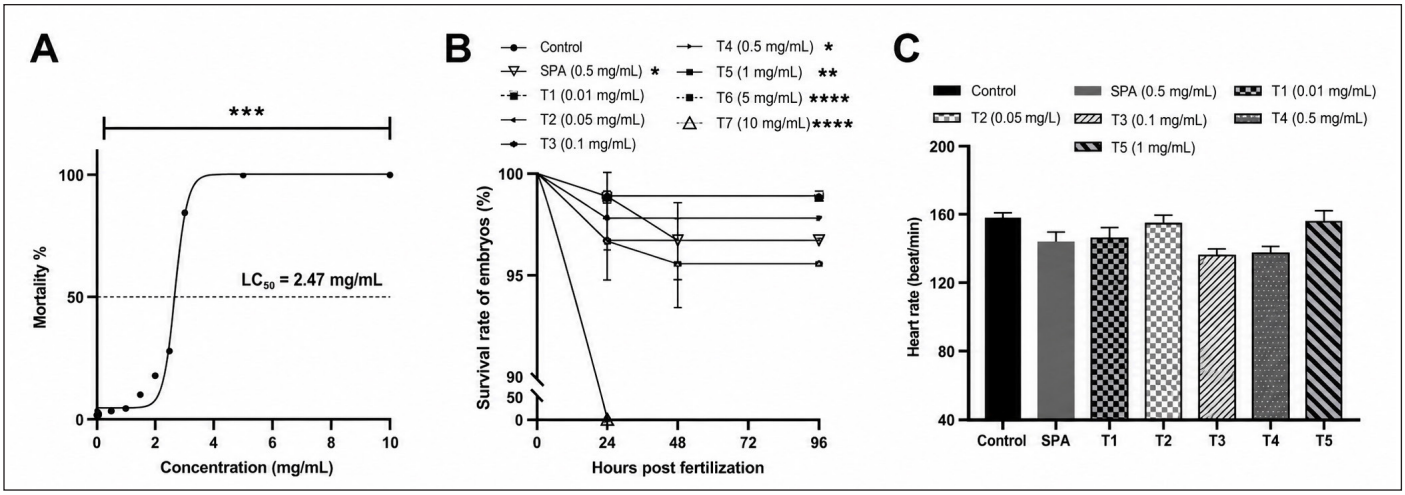

Fig. 2. Effect of *M. alpina* formulation (MAF) on A. Mortality rate and LC_{50} value. B. Survival rate. C. Heart rate of zebrafish embryo post-treatment with sodium polyacrylate (SPA) and MAF (0.01 – 1 mg/ml) after 96 hpf. The LC_{50} was determined to be 2.47 mg/ml. Mortality rate and survival rate data are presented as mean ± SD ($n = 90$ embryos, with 30 embryos per biological replicate). Heart rate data are presented as mean ± SD ($n = 3$). Symbols: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Table 2. Endpoint analysis of zebrafish embryos at 96 hours post-fertilization (hpf).

Characteristics	Control	SPA (mg/ml)	Treatment: MAF concentration (mg/ml)				
		0.5	T1 (0.01)	T2 (0.05)	T3 (0.1)	T4 (0.5)	T5 (1.0)
Coagulation (% Mortality)	1.11%	4.44 %	1.11%	1.11%	2.22%	3.33%	4.44%
Motility	+++	+++	+++	+++	+++	+++	+++
Hatching (%)	23.73 ± 3.41	25.00 ± 5.00	85.66 ± 2.08	89.0 ± 1.00	95.33 ± 0.57	99.66 ± 0.57	99.66 ± 0.57
Somite formation	+++	+++	+++	+++	+++	+++	+++
Tail detachment	+++	+++	+++	+++	+++	+++	+++

+ Low, ++ Moderate, +++ Complete/High. SPA, Sodium Polyacrylate. MAF, *Mortierella alpina* Formulation.

Data were expressed as mean ± SD from three independent biological replicates ($N = 90$). Coagulation (% mortality) was determined using 30 embryos per replicate ($N = 90$ embryos per group). Hatching rate was calculated based on the surviving embryos from this 90-embryo cohort.

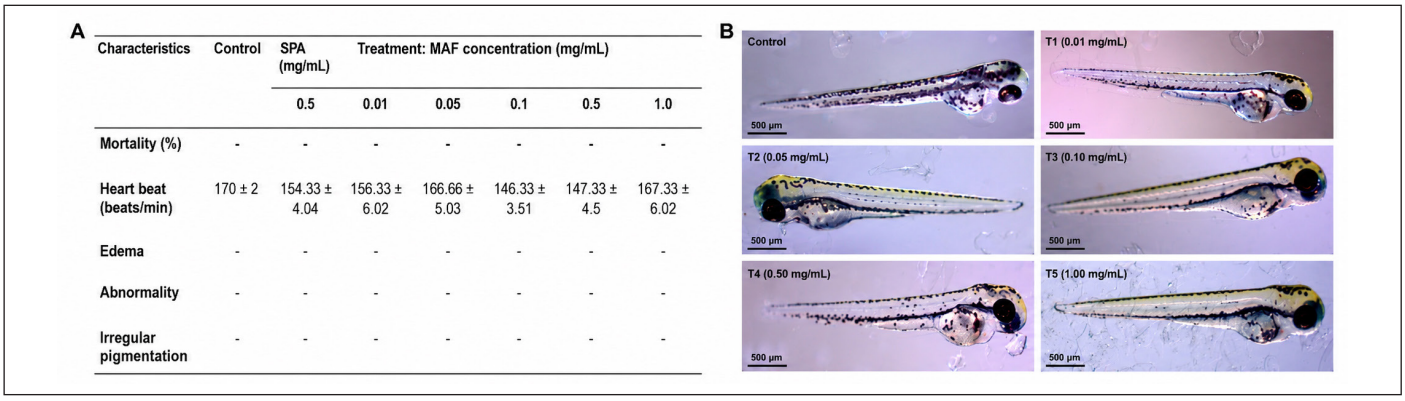

Fig. 3. Effect of sodium polyacrylate (SPA) and *M. alpina* formulation (MAF) on the morphology of zebrafish larvae at 7 days post-fertilization. A. Endpoint analysis of the larvae. Symbol: - Absent. Edema includes pericardial and yolk-sac edema. Abnormalities include tail, spine, craniofacial, and organ malformations, as well as irregular body curvature. B. Representative images of the larval morphology in control and MAF-treated groups. Images were taken using a Stemi 2000-C Zeiss stereomicroscope at 50X magnification (Bar 500 µm).

Table 3. Comparison of zebrafish embryo toxicity across various microbial biomass, EPS, ENS, and ethanol extracts.

Microorganism	Product	Impact								References
		Mortality	Edema	Irregular body curvature	Irregular heart rate	Irregular pigmentation	Delayed Hatching	Malformation	LC_{50} (mg/ml)	
Mortierella alpina	Biomass and sodium polyacrylate (1:1)	+	-	-	-	-	-	-	2.47	*Current study*
Ganoderma lucidum	Biomass	+	-	+	-	NR	+	+	1.65	[17]
Ganoderma lucidum	EPS	+	-	+	-	NR	+	+	2.64	[17]
Lignosus rhinocerus	Biomass	+	-	+	-	NR	+	+	0.77	[18]
Lignosus rhinocerus	EPS	+	-	+	-	NR	+	+	0.41	[18]
Ganoderma applanatum	EPS	+	-	+	-	NR	+	+	1.41	[12]
Ganoderma applanatum	ENS	+	-	+	-	NR	+	+	0.87	[12]

+, present; -, absent; NR, Not Reported.

EPS: Exopolysaccharide, ENS: Endopolysaccharide, LC_{50}: Concentration at which 50% lethality is observed, PUFAs: Polyunsaturated Fatty Acids.

abnormalities were observed in the hatched larvae of the control, SPA, and MAF groups (Fig. 3B).

These favorable outcomes contrast with the findings of studies on other fungal products (Table 3). Biomass exopolysaccharides and endopolysaccharides from *G. lucidum*, *L. rhinocerus*, and *G. applanatum* consistently demonstrated concentration-dependent effects, showing irregular body curvature, delayed hatching, and malformations [12,17,18]. However, in this study, no developmental abnormalities were observed in the surviving embryos or larvae.

3.3. Embryo Hatching and Staging

Treatment with SPA had a comparable effect on embryo hatching to that of the control group. The embryos started to hatch at 72 hpf in both the control and SPA groups, with a hatching rate below 50% (Fig. 4A). In contrast, the varying concentrations of MAF exhibited a concentration-dependent effect on embryo hatching over 96 hours. At 24 hpf, no hatching was observed in any group, which was expected as hatching typically begins later in zebrafish development [24]. By 48 hpf, a very low percentage of embryos, approximately 5% in the T4

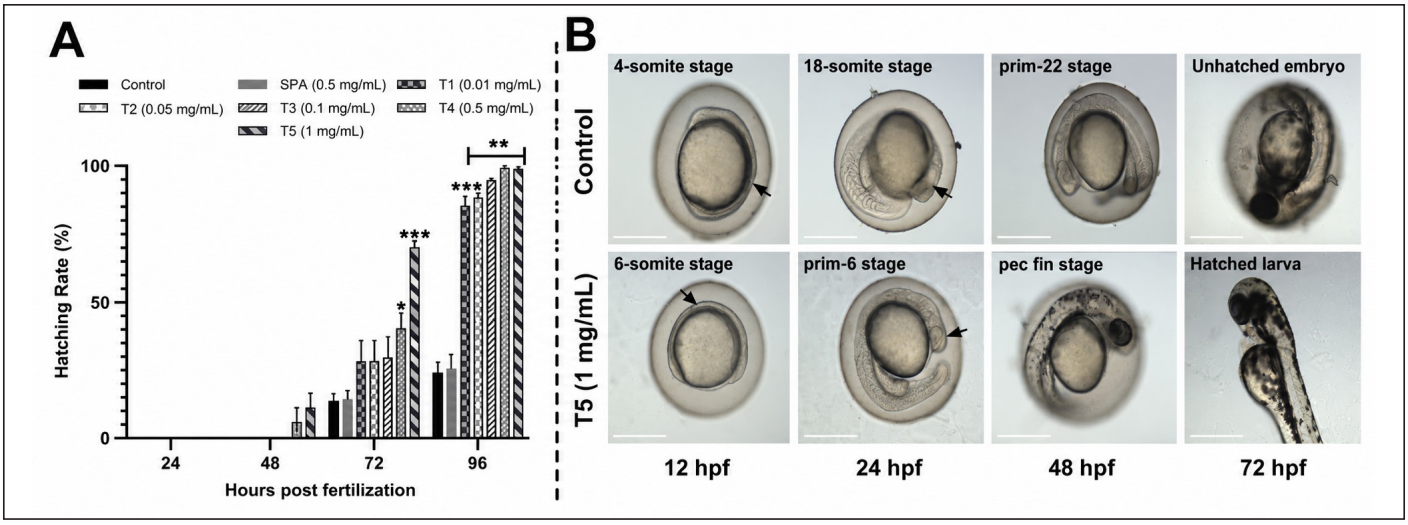

Fig. 4. Effect of sodium polyacrylate (SPA) and *M. alpina* formulation (MAF) on the hatching rate and development of zebrafish embryos. A. Hatching rate in SPA- and MAF-treated (0.01-1 mg/ml) embryos. Data are presented as mean ± SD ($n = 3$). Symbols: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. B. Comparative stage-based imaging showing the effect of T5 MAF (1.0 mg/ml) on the early development of treated embryos compared to control. Observations were made using the EVOS FL Auto 2 Imaging System (Invitrogen) (Bar 200 µm). At 12 hpf, arrows indicate the formation of four somites in the control group and six somites in the treated group. At 24 hpf, the arrow in the control group highlights the absence of the eye primordium, whereas in the treated group, the arrow indicates the presence of the eye primordium. At 48 hpf, higher pigmentation of the embryo is evident in the MAF-treated group compared to the control group. At 72 hpf, control group embryos remained unhatched compared to the treated group, where hatching was evident.

group and 10% in the T5 group, began to hatch, whereas the control and other treatment groups showed no hatching. The impact of T5 on advancing the hatching process became more apparent at 72 hpf, with a 71% hatching rate. By 96 hpf, the hatching rates of all MAF groups substantially increased. In contrast, the control and SPA groups had comparatively low hatching rates.

The staging experiment explained the significantly higher hatching rate observed in T5-treated embryos. Exposure to this concentration of MAF significantly accelerated the embryonic development of zebrafish, resulting in more advanced morphological stages at earlier time points and promoting earlier hatching compared to untreated control embryos (Fig. 4B). At 12 hpf, control embryos reached the 4-somite stage, whereas T5-treated embryos exhibited a more advanced 6-somite stage, indicating a slight acceleration in early somite formation. This accelerated development became more pronounced by 24 hpf, with control embryos at the 18-somite stage and T5-treated embryos at the prim-6 stage. The control group lacked a lens primordium and had a shorter tail, whereas the treated embryos had a well-defined lens primordium and a comparatively larger tail. By 48 hpf, control embryos were at the prim-22 stage, whereas treated embryos had developed to the pec fin stage. In addition, at 48 hpf, the treated embryos showed higher pigmentation than the controls. Consistent with accelerated development, at 72 hpf, the control group still contained unhatched embryos, whereas the T5-treated group showed 71% hatched larvae.

Overall, the delayed development and hatching observed in the control embryos up to 96 hpf can be attributed to the static 96-well culture conditions, which restricted oxygen availability and induced developmental hypoxia [34,35]. In contrast, MAF treatment conferred a protective effect against hypoxic stress and promoted timely embryonic development and hatching.

3.4. Gene Expression

Significant upregulation of three lipid metabolism genes was observed in T5-treated zebrafish larvae (Fig. 5). Compared to the control, the treatment group exhibited a 2.6 ± 0.4-fold increase in *fads* expression ($p < 0.05$), a 5.63 ± 1.2-fold increase in *elovl5* expression ($p < 0.001$), and a 6.15 ± 0.7-fold increase in *apoA-Ia* expression ($p < 0.01$).

Expression analysis of these genes is essential to assess the impact of supplements on lipid metabolism and associated dysregulation, which may inform developmental defects [36,37]. In this study, we tested selected lipid transport and metabolism genes that have been well studied in lipidomic research [38]. Among the transport genes, *apoA-Ia* is associated with high-density lipoprotein production and plays a crucial role in lipid transport. Its expression is particularly prominent in the yolk syncytial layer after hatching, as reported by Babin et al. [39]. In contrast, genes such as *fads* and *elovl5* are fundamental to the biosynthesis of LC-PUFAs in zebrafish. Notably, *fads* encodes fatty acyl desaturases with both Δ6/Δ5 activities that are unique to vertebrates [40,41]. In contrast, *elovl5* is responsible for elongating fatty acid chains. Together, these genes represent the primary desaturase and elongase required for LC-PUFA synthesis in zebrafish embryos [41]. As such, the upregulation of these genes in the T5 group indicates an active yet regular lipid metabolic pathway [42,43]. This also explains the lipid transport and accumulation, as well as the absence of morphological and developmental abnormalities in embryos and larvae.

3.5. Lipid Accumulation

To confirm the entry of externally supplied lipids, Nile Red staining, a lipophilic dye widely used to visualize intracellular lipid droplets in zebrafish embryos, was performed [44]. Given that MAF induces early embryogenesis, we performed Nile red staining on control and T5-treated embryos at specific developmental stages to ensure uniformity rather than relying on time-dependent staining.

At the 6-somite stage, treated embryos exhibited fluorescence intensities comparable to those of the controls (247.53 ± 5.4 a.u. and 246 ± 4.75 a.u., respectively). However, by the prim-6 stage, treated embryos exhibited significantly higher fluorescence intensity (159.50 ± 5.53 a.u., $p < 0.0001$) than the controls (134.93 ± 5.12 a.u.). The

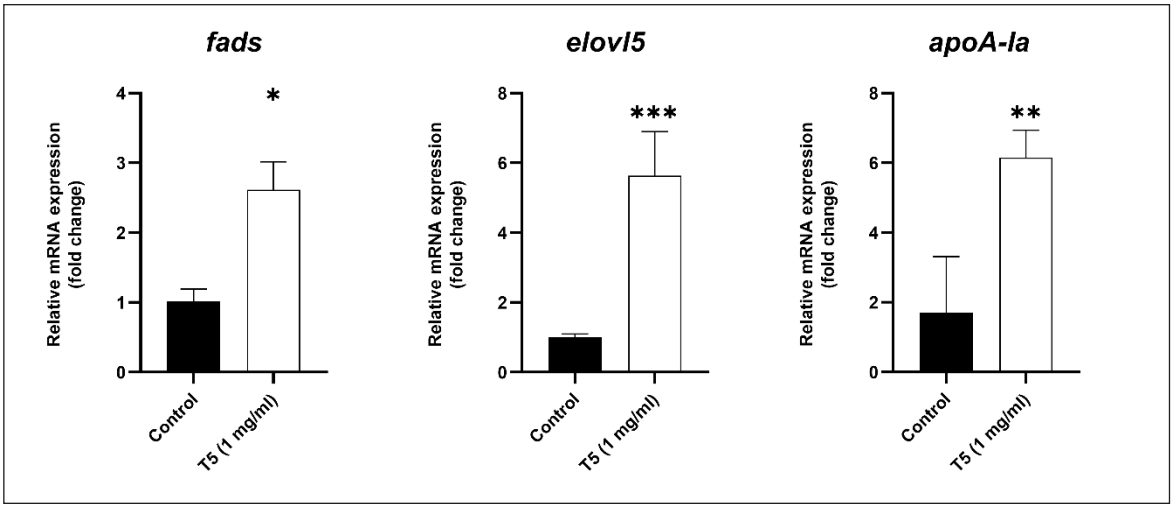

Fig. 5. Relative expression of lipid metabolism-related genes in T5 MAF (1.0 mg/ml). Treated zebrafish larvae post-hatching. Data are presented as mean ± SD (n = 3). Symbols: *p < 0.05, **p < 0.01, and ***p < 0.001.

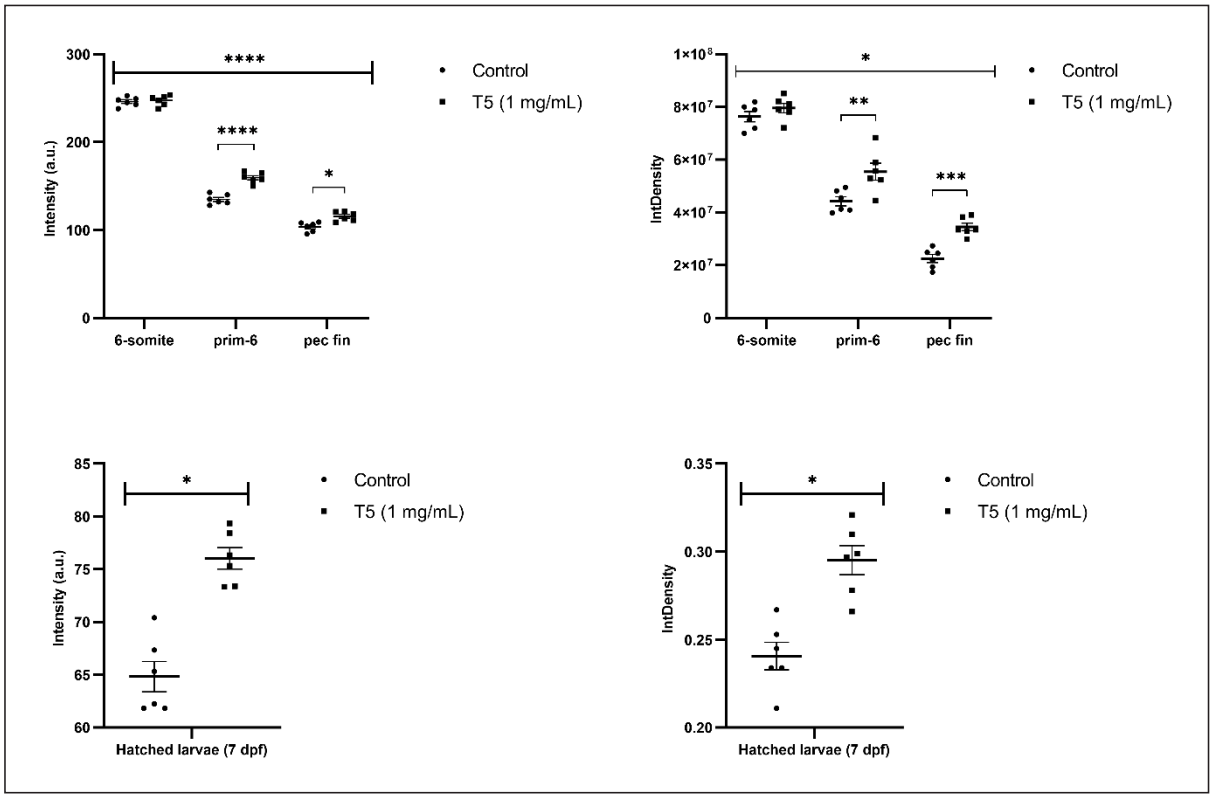

Fig. 6. Quantitative evaluation of fluorescence intensity (A) and integrated density (B) of control and T5 MAF (1.0 mg/ml) treated embryos. Quantitative evaluation of fluorescence intensity (C) and integrated density (D) of control and T5 MAF (1 mg/ml) group larvae. Data are presented as mean ± SD (n = 3). Symbols: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

integrated density similarly increased from 0.21 ± 0.01 in the controls to 0.27 ± 0.01 (p < 0.001) in the treated embryos. At the pec fin stage, treated embryos maintained higher fluorescence intensity (115.65 ± 4.75 a.u., p < 0.05) than the controls (103.65 ± 5.0 a.u.). The integrated density was also significantly greater in the treatment group (0.33 ± 0.03) than in the control group (0.25 ± 0.01, p < 0.01) (Fig. 6A and B).

This increased lipid accumulation in T5-treated embryos persisted during the larval stage. Quantitative analyses confirmed increased fluorescent intensity in treated larvae (76.03 ± 2.2 a.u., p < 0.05) compared to controls (64.82 ± 3.2 a.u.) (Fig. 6C). The integrated density also significantly increased from 0.24 ± 0.01 in the control group to 0.29 ± 0.01 (p < 0.05) in the treated group (Fig. 6D).

Two key findings were obtained. First, in both the control and T5 groups, lipids were concentrated in the yolk sac and continuously consumed by the growing embryos, as indicated by a reduction in fluorescence intensity and integrated density throughout embryogenesis. This

aligns with findings that a progressive decline in lipid species within the embryo yolk sac occurs over time and is predominantly utilized by 5 dpf [42].

Second, externally supplied lipids accumulated in the yolk sac, as evidenced by increased fluorescence in the T5-treated group, relative to the control. In the early stages (6 somites), when the yolk sac was already saturated with lipids, external supplementation resulted in a slight increase in fluorescence intensity compared to the control. However, at the prim-6 stage, as the metabolic activity increased in the living embryos, the lipids in the control embryos began to deplete rapidly. In T5-treated embryos, MAF replenishment of the lipid reservoir via external supplementation resulted in significantly higher lipid levels than in the control group. A similar trend continued at the pec fin stage, where the treated embryos benefited from the additional available yolk sac lipids. Consequently, larvae hatched from the treated embryos exhibited significantly greater lipid distribution across the craniofacial region than the control larvae. These additional lipids may represent an increased energy reserve that could support the energetic demands of early larval development. However, whether this increased lipid reserve improves tolerance to nutritional or environmental stress remains to be experimentally determined. At the same time, this reserve had no adverse impact on morphological and developmental endpoints (Fig. 2).

4. CONCLUSION

In conclusion, the present study demonstrates that an externally supplied oleaginous *M. alpina* EI-9 biomass-based formulation in embryonic media enhances embryogenesis, accelerates hatching, and increases embryonic lipid accumulation relative to the concurrent control under the experimental conditions employed. However, these benefits are concentration-dependent, with higher concentrations exhibiting toxic effects, likely due to nutritional overdose and disruption of the fatty acid balance required for embryogenesis. To the best of our knowledge, existing studies involving zebrafish embryos have predominantly focused on the developmental toxicity and embryotoxicological assessment of microbial products and bioactive compounds, whereas the beneficial developmental effects of externally supplemented oleaginous fungal biomass remain largely unexplored. Therefore, the present findings provide new evidence that *M. alpina* EI-9 biomass may serve as a positive developmental modulator during early vertebrate embryogenesis.

The use of whole *M. alpina* biomass rather than purified microbial oils may offer additional nutritional and industrial advantages. In addition to PUFAs, whole fungal biomass contains proteins, sterols, vitamins, antioxidants, and other potentially bioactive constituents that may collectively contribute to its biological effects. From a bioprocessing perspective, utilization of the whole biomass may also reduce the requirement for extensive lipid extraction and purification, potentially improving resource utilization and process efficiency. Nevertheless, these proposed nutritional, economic, and environmental advantages require quantitative validation through comparative compositional, techno-economic, and life-cycle assessments.

Moreover, whether these effects translate into improved hatchery performance, larval survival, and production efficiency remains to be established. Accordingly, the present study should be viewed as an initial proof-of-concept rather than evidence of sustainable aquaculture intensification or direct alignment with production-scale outcomes envisaged under the FAO Blue Transformation framework.

However, before being used in commercial aquaculture applications, it must be tested and optimized across different aquatic species in real-time aquaculture systems that account for environmental variability and fish developmental stages. Moreover, future studies should evaluate MAF formulations containing varying SPA concentrations, testing each concentration in parallel with an SPA-only control. This approach would more clearly distinguish effects attributable to the fungal biomass from potential effects associated with the SPA component. Mechanistic investigations examining lipid uptake and incorporation, fatty acid metabolism, lipidomic profiles, and relevant developmental signaling pathways would further help elucidate the biological basis of the observed responses. Such studies will be essential for determining whether the effects identified under the present experimental conditions can be translated into practical aquaculture applications.

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. DECLARATION OF COMPETING INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript. Although two of the authors, SBR and UR, are affiliated with an industry organisation (Convergent Biosciences Pvt. Ltd., Hyderabad, India), which provided the organism used in this study, their contributions were limited to supplying the biological material and describing the associated methodology. The company provided no financial support and had no influence on the experimental work, data analysis, interpretation of results, or decision to publish. All other authors declare no competing interests.

8. ETHICAL APPROVAL

The handling and use of adult zebrafish and larvae for experiments were approved by the Institutional Animal Ethics Committee (IAEC), Mahatma Gandhi Medical Advanced Research Institute, Sri Balaji Vidyapeeth, Pondicherry, India (03/IAEC/MGMPRC/14/2024-I, dated 27.04.2024). Experiments involving embryos up to 96 hpf were exempted from IAEC approval requirements as these were considered not fish and outside animal-experiment regulations.

9. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

Following preparation of the initial manuscript draft, the authors used Grammarly (Grammarly, Inc.) to assist with grammatical corrections, word choice, sentence structure, and readability. All suggested revisions were reviewed and verified by the authors, who take full

responsibility for the accuracy, integrity, and final content of the published article.

10. DATA AVAILABILITY

All data have been shared here and are also available to the authors. These can be made available upon request.

11. PUBLISHER'S NOTE

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