



Oxidative Stress–Mediated Metabolic and Mitochondrial Dysregulation by Tartrazine, Sunset Yellow, Aspartame, and Sodium Benzoate in Zebrafish Embryos: Implications for Diabetes Risk

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Abstract

The growing global prevalence of diabetes underscores the importance of understanding dietary contributors to metabolic disorders. This study investigates the impact of four commonly used food additives—tartrazine (TTZ), sunset yellow (SY), aspartame (AST), and sodium benzoate (SB)—on diabetes related mechanisms using zebrafish embryos. These compounds were chosen for their frequent dietary consumption, distinct chemical classes, and documented associations with oxidative stress and metabolic disturbances. Exposure to the additives induced dose- and stage-dependent oxidative stress, evidenced by elevated malondialdehyde (MDA) levels and decreased glutathione (GSH) activity, with SB producing the most pronounced effects. Metabolomic profiling via nuclear magnetic resonance (¹H-NMR) revealed additive-specific disruptions in glucose, triglyceride, cholesterol, branched-chain amino acid, and aromatic amino acid metabolism, with SB and AST causing the most extensive metabolic perturbations indicative of mitochondrial dysfunction and impaired energy homeostasis. Gene expression analyses demonstrated upregulation of *ptf1a*, *glrx*, and *cox*, linking additive exposure to pancreatic dysregulation, glutathione metabolism disruption, and variable mitochondrial effects. These findings suggest that early-life exposure to TTZ, SY, AST, and particularly SB may predispose organisms to metabolic dysregulation and diabetes risk through oxidative stress, altered energy metabolism, and mitochondrial impairment. The results underscore the importance of re-evaluating the dietary safety of commonly consumed food additives.

Introduction

Building on our previous investigations into the neurodevelopmental effects of food additives, particularly

their modulation of circadian clock genes and the dopamine system in zebrafish (*Danio rerio*) (Christy et al., 2024), this study shifts its focus to metabolic health, with an emphasis on diabetes. Diabetes has become a

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significant global health challenge, projected to affect 642 million people by 2040 (Ong et al., 2023). The parallel rise in diabetes and obesity underscores the potential role of environmental and dietary factors, particularly food additives, in the development of this disease. Despite regulatory agencies deeming many of these additives safe, growing concerns about their long-term effects on chronic diseases like diabetes persist (Salame et al., 2024; Lyu & Liu, 2024).

Recent research has provided clues about the potential impact of food additives on metabolic health. Emulsifiers, such as polysorbate-80 (P80), have been shown to disrupt gut microbiota, induce low-grade inflammation, and promote insulin resistance and glucose intolerance in animal models (Delaroque & Chassaing, 2024). Similarly, a systematic review by Angelin et al. (2024) found significant associations between artificial sweeteners such as aspartame and sucralose and an increased risk of type 2 diabetes. These studies highlight the need for further investigation into the mechanisms by which food additives contribute to metabolic dysregulation.

This study focuses on four commonly used food additives: tartrazine (TTZ), sunset yellow (SY), aspartame (AST), and sodium benzoate (SB), and their potential role in disrupting metabolic health, specifically through the modulation of the *ptfla* gene and mitochondrial function. The *ptfla* gene, essential for pancreatic development and differentiation, has recently been implicated in mitochondrial function (Alsagheir et al., 2023). Mitochondria are central to energy production, and any disruption in their function can lead to insulin resistance, impaired glucose metabolism, and eventually, diabetes (Rivera Nieves et al., 2024; Sangwung et al., 2020). Moreover, *ptfla* expression is not limited to the pancreas but also occurs in other tissues such as the liver, brain, and skeletal muscles, where mitochondria play essential roles in energy homeostasis (Jin & Xiang, 2019). Impaired mitochondrial function has been linked to reduced ATP production, elevated levels of reactive oxygen species (ROS), and disrupted insulin signaling, all of which contribute to the development of type 2 diabetes (Zong et al., 2024).

To investigate these effects, we employ zebrafish embryos as a model system, a well established model in developmental and metabolic research. Zebrafish offer unique advantages such as optical transparency, rapid development, and strong genetic homology with humans, making them ideal for studying the early impacts of

environmental exposures on metabolic processes (Bondue et al., 2023). Through gene expression analysis, mitochondrial function assessment, and NMR based metabolomics, we aim to identify metabolic changes resulting from food additive exposure.

NMR metabolomics provides a high-resolution platform for profiling metabolic pathways and identifying disease related biomarkers. Prior research has demonstrated the utility of this approach in assessing metabolic disturbances. For instance, Panyod et al. (2024) showed that emulsifier exposure altered lipid metabolism and inflammation-related pathways in mice. Similarly, Junot et al. (2023) highlighted the utility of NMR metabolomics in identifying metabolic disturbances linked to dietary exposures.

By combining these techniques, this study aims to elucidate how early developmental exposure to food additives may influence diabetes susceptibility through metabolic disruption. Ultimately, this research builds on our previous work on the neurodevelopmental effects of food additives and addresses a critical gap in understanding how such exposures may contribute to chronic diseases, specifically type 2 diabetes.

Materials and methods

1. Chemicals

Tartrazine (TTZ, CAS No. 1934-21-0, purity $\geq 99\%$), sunset yellow FCF (SY, CAS No. 2783-94-0, purity $\geq 98\%$), aspartame (AST, CAS No. 22839-47-0, purity $\geq 98\%$), and sodium benzoate (SB, CAS No. 532-32-1, purity $\geq 99\%$) were purchased from Sigma-Aldrich (Sigma-Aldrich Co., Ltd., USA). All reagents used for biochemical assays, including thiobarbituric acid (TBA, CAS No. 504-17-6), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, CAS No. 69-78-3), and reduced glutathione (GSH, CAS No. 70-18-8), were also obtained from Sigma-Aldrich with analytical grade purity.

TRIzol reagent (CAS No. 155960-60-8) and the High-Capacity cDNA Reverse Transcription Kit were procured from Thermo Fisher Scientific (Thermo Fisher Scientific Inc., USA). SYBR Green Master Mix for qPCR was purchased from Applied Biosystems (Applied Biosystems, USA).

2. Zebrafish rearing and embryo collection

Wild-type zebrafish (*Danio rerio*) were maintained in a controlled aquatic facility under standard laboratory conditions at $28 \pm 1^\circ\text{C}$ with a 14:10 h light-dark cycle. Adult zebrafish were fed a balanced diet and embryos

were obtained by natural spawning (Aleström et al., 2020). Embryos were collected within 1 h of fertilization and examined under a stereomicroscope. Fertilized embryos at 18 h post-fertilization (hpf) with normal development were selected for experiments.

In this study, we use the term “embryos” consistently for 18–96 hpf stages, including those undergoing hatching, since sampling was restricted to the embryonic window prior to the onset of free-swimming larval development. All experiments using zebrafish embryos were performed in accordance with institutional guidelines. As the experiments were conducted on embryos prior to 5 days post-fertilization (≤ 120 hpf), ethical approval was not required under CPCSEA regulations

3. Experimental design and protocol

This study evaluated the effects of selected food additives at concentrations based on prior zebrafish toxicity and neurodevelopmental assessments (Christy et al., 2024). The maximum concentrations were chosen to fall within a sublethal effect window—doses high enough to produce measurable biochemical and transcriptional responses but below thresholds that cause gross malformations or significant mortality. This design ensured that oxidative stress, gene-expression, and metabolomic endpoints could be interpreted without confounding from overt developmental toxicity.

For context, the maximum test concentrations and corresponding human Acceptable Daily Intake (ADI) values established by Joint FAO/WHO Expert Committee on Food Additives (JECFA)/ European Food Safety Authority (EFSA) were: tartrazine (TTZ, 5.5 ppm; ADI: 0–7.5 mg/kg bw/day), sunset yellow (SY, 2.5 ppm; ADI: 0–4 mg/kg bw/day), aspartame (AST, 20 ppm; ADI: 0–40 mg/kg bw/day), and sodium benzoate (SB, 200 ppm; ADI: 0–5 mg/kg bw/day). Because ADIs are expressed as mg/kg body weight/day for oral human exposure, they are not directly comparable to zebrafish waterborne concentrations; therefore, ADIs are provided here only for public-health context. Cross-chemical comparisons in this study rely on equitoxic metrics (e.g., EC_x values) rather than nominal concentrations.

Zebrafish embryos ($n=30$ /group) were randomly assigned to be exposed to 4 food additives as follows:

1. Control: 1 group exposed to embryo medium without additives.
2. TTZ Group: 3 groups, each group exposed to 1.5, 3.5 and 5.5 ppm of TTZ.
3. SY Group: 3 groups, each group exposed to 0.5, 1.0 and 2.5 ppm of SY.

4. AST Group: 3 groups, each group exposed to 5, 10 and 20 ppm of AST.

5. SB Group: 3 groups, each group exposed to 50, 100 and 200 ppm of SB.

Test solutions were prepared using embryo medium and replaced daily to maintain consistent exposure. Treatments were administered from 18 to 96 h post-fertilization (hpf), with control embryos maintained under identical conditions without additives.

Embryos of each group were sampled at 24, 48, 72 and 96 hpf for oxidative stress analysis, including lipid peroxidation (LPO) and reduced glutathione (GSH) activity. Gene expression of *ptfla*, *glrx*, and *cox2* was also assessed. At 96 hpf, pooled embryos from each group underwent biochemical index determination and metabolomic profiling. For these assays, zebrafish embryos ($n=30$ /group, ~ 100 mg wet mass) at the designated developmental stages were homogenized in 60 μ L of ice-cold phosphate-buffered saline (PBS, pH 7.4) using a mechanical homogenizer, followed by centrifugation at $5000\times g$ for 10 min at 4°C. The resulting supernatant was aliquoted and used for all downstream assays, including LPO, GSH, and biochemical index determinations (glucose, triglyceride, and cholesterol). For metabolomics, a separate extraction using methanol:water:chloroform (2.0:1.85:2.0) was performed as described in Section 7.

4. Lipid peroxidation LPO assay

Lipid peroxidation levels were assessed using the thiobarbituric acid reactive substances (TBARS) method, as described by Li et al. (2023). Briefly, 200 μ L of tissue homogenates were mixed with 1.5 mL of 20% (w/v) trichloroacetic acid (TCA) and 1.5 mL of 0.8% (w/v) thiobarbituric acid (TBA) solution. The mixture was vortexed and incubated at 90°C for 60 min (Sachett et al., 2020). Absorbance was measured at 535 nm, and malondialdehyde (MDA) levels were expressed as μ mol/g protein.

5. Quantification of reduced glutathione (GSH) activity

GSH levels were quantified using Elman’s reagent (DTNB) (Massarsky et al., 2017). Briefly, 500 μ L of embryo homogenate was mixed with 500 μ L of 5% TCA to precipitate proteins. The mixture was centrifuged at $10,000\times g$ for 10 min, and the supernatant was collected for analysis. For the assay, 100 μ L of the supernatant was added to reacted with 100 μ L of DTNB solution (10 mM) in phosphate buffer (pH 7.4). The reaction mixture was incubated at room temperature for 5 min, and absorbance

was measured at 412 nm. The GSH activity was calculated using the following equation

$$\text{GSH } (\mu\text{mole/g tissue}) = \frac{\Delta \text{O.D} \times \text{Vol total}}{\epsilon \times l \times W}$$

where $\epsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$ (the molar extinction coefficient of DTNB), $l = 1 \text{ cm}$, $W = \text{tissue weight (g)}$.

6. Biochemical indexes determination

Aliquots of homogenates prepared at 96 hpf (Section 2) were used for enzymatic determination of glucose, triglyceride, and total cholesterol. Briefly, 2.5 μL of supernatant from each sample was analyzed using the glucose oxidase–phenol aminoantipyrine (COD-PAP) method for glucose, the glycerol-3-phosphate oxidase–phenol aminoantipyrine (GPO-PAP) method for triglycerides, and the glucose oxidase (GOD) method for total cholesterol, as described by Miao et al. (2022).

7. Metabolomics

At 96 hpf, zebrafish embryos from control and treatment groups were collected for metabolomic analysis to evaluate food additive–induced metabolic perturbations (Ganesan et al., 2021; Fujita et al., 2021). Briefly, zebrafish embryos (100 mg wet mass) were homogenized in a solution of methanol, Milli-Q water, and chloroform at a ratio of 2.0:1.85:2.0. The homogenized samples were vortexed, incubated on ice for 10 min, and centrifuged at $2,000 \times g$ for 5 min at 4°C to remove precipitated proteins and debris. The polar phase was carefully collected, lyophilized to remove solvents, and reconstituted in deuterium oxide (D_2O) containing 0.1% trimethylsilyl propionic acid (TSP) as an internal chemical shift reference (0.00 ppm).

Spectra were acquired using a Bruker Avance III 500 MHz NMR spectrometer (Bruker BioSpin GmbH, Germany) equipped with a 5 mm Quadrupole Inverse Probe (QXI). One-dimensional (1D) proton (^1H) NMR spectra were recorded using a standard presaturation pulse sequence for water suppression. Spectra were Fourier transformed, phase- and baseline-corrected using Bruker TOPSPIN 2.1 software (Bruker BioSpin GmbH, Germany), referenced to TSP at 0.00 ppm, and the residual water region (4.60–4.80 ppm) was excluded from analysis. Spectra were normalized to total area to minimize concentration-dependent variations between samples.

To identify metabolic perturbations associated with food additive exposure, key differentiating metabolites

were assigned by matching chemical shift values, multiplicity patterns, and known coupling characteristics with reference spectra from established databases, including the Human Metabolome Database (HMDB) and the Biological Magnetic Resonance Data Bank (BMRB). Metabolite assignments were considered valid when chemical shift deviations were within $\pm 0.02 \text{ ppm}$ and were consistently observed across biological replicates.

Although untargeted ^1H -NMR metabolomics was employed, the putatively identified metabolites were subsequently mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for *Danio rerio*, and associated Gene Ontology (GO) biological processes were used to contextualize the observed metabolic perturbations within established metabolic and functional frameworks, enabling interpretation of potential disruptions in energy metabolism, amino acid metabolism, carbohydrate utilization, and redox-related pathways.

8. RNA isolation and reverse transcription real-time PCR (RT-qPCR)

RNA isolation was performed using TRIzol reagent following established protocols (Peterson & Freeman, 2009). One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) using the Applied Biosystems High-Capacity cDNA Synthesis Kit. RT-qPCR was then conducted on an Applied Biosystems Step One instrument (Applied Biosystems, USA) with SYBR Green chemistry to quantify the gene expression of *ptfla*, *glrx*, and *cox2*. Primer sequences were designed using NCBI Primer-BLAST and verified for specificity via BLAST alignment. Primer sequences and annealing temperatures are detailed in Table 1. β -actin was used as the internal reference gene. All reactions were performed in biological triplicates, each with technical duplicates. Relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak & Schmittgen, 2001).

Table 1 Primer Sequences and annealing temperatures.

Primer name	Sequence	Primer length (bp)	Ta ($^\circ\text{C}$)
<i>ptfla</i> F	ACCGAGGAACAAGATCCCCAT	156	60
<i>ptfla</i> R	GCAACTTTTCGTGGGTCTCT		
<i>glrx</i> F	GTAAGATGGCAAAGGGTGTC	135	60
<i>glrx</i> R	GTCAGTTCGCCTAAAGTCTC		
<i>cox2</i> F	GATACTGTCTTCGTGTTAGCC	169	62
<i>cox2</i> R	GATGCGGAGGTGTGGGTAA		

Remark: F: Forward, R: Reverse and Ta: Annealing temperature

9. Statistical analysis

Data were analyzed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., USA). Group differences were assessed using ANOVA with post hoc tests for multiple comparisons. Results were expressed as mean±SEM, with statistical significance set at $p < 0.05$, with the following notation used throughout: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Results and discussion

The present study investigates the metabolic, oxidative, and genetic effects of food additives—TTZ, SY, AST, and SB—on zebrafish embryos, highlighting their potential to augment diabetes risk (Wu, 2021). By integrating oxidative stress biomarkers, transcriptional profiling, and metabolomic analyses, we demonstrate that these additives disrupt core metabolic pathways, such as glucose homeostasis (Alsunni, 2020; Puttegowda et al., 2024) and fatty acid oxidation (Simmons et al., 2014), and induce mitochondrial dysfunction (Gribsch et al., 2023). These perturbations align with established

mechanisms of metabolic dysregulation, suggesting a plausible role for dietary additives in priming diabetogenic pathways (Salame et al., 2024).

1. Lipid peroxidation and antioxidant defense in zebrafish embryos

Exposure to food additives led to a significant increase in malondialdehyde (MDA) levels, indicating enhanced lipid peroxidation, and a concurrent decrease in reduced glutathione (GSH) activity, reflecting impaired antioxidant defence (Fig. 1–2). These effects were generally dose-dependent and varied across developmental stages, with early and late embryonic stages showing the greatest sensitivity.

Overall, these findings demonstrate that food additives simultaneously induce oxidative damage (positive effect) and reduce antioxidant capacity (negative effect) in developing embryos. The results indicate that embryos are particularly vulnerable during critical windows of development and suggest that additive exposure may act as a metabolic disruptor. Detailed stage- and concentration-specific data are provided in Tables 2 and 3.

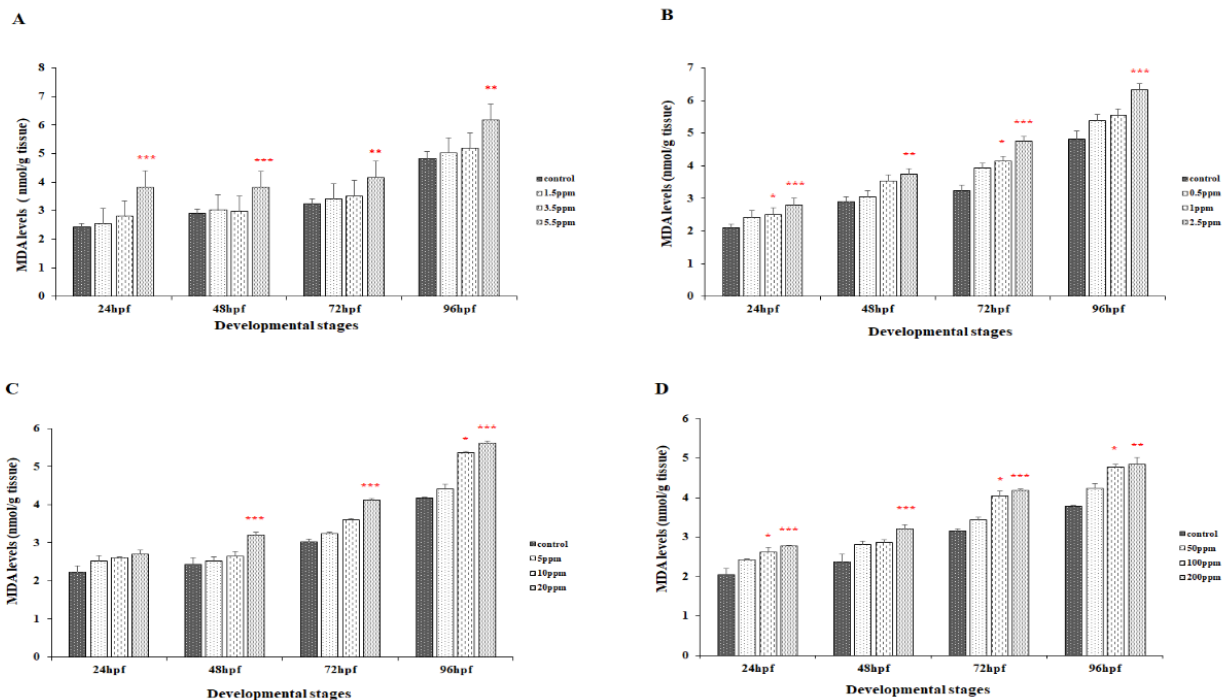


Fig. 1 Effects of food additives on lipid peroxidation (MDA levels) in zebrafish embryos. Embryos were exposure from 18 hpf during the critical development window to A) tartrazine (TTZ), B) sunset yellow (SY), C) aspartame (AST), or D) sodium benzoate (SB). Malondialdehyde (MDA) levels, a marker of lipid peroxidation (LPO), were quantified at 24, 48, 72, and 96 hpf. Values are expressed as nmol/g tissue. Data are presented as mean±SEM (n=30/group). Statistical significance was determined relative to the time-matched control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

These observations are consistent with previous studies linking oxidative stress to metabolic dysregulation (Yaribeygi et al., 2020; Caturano et al., 2023; Pitocco et al., 2013) and highlight the need to carefully consider

the safety of commonly used food additives. Notably, SB appears to pose the greatest oxidative risk, underscoring the need to reassess the safety of this widely used preservative (Walker, 2009).

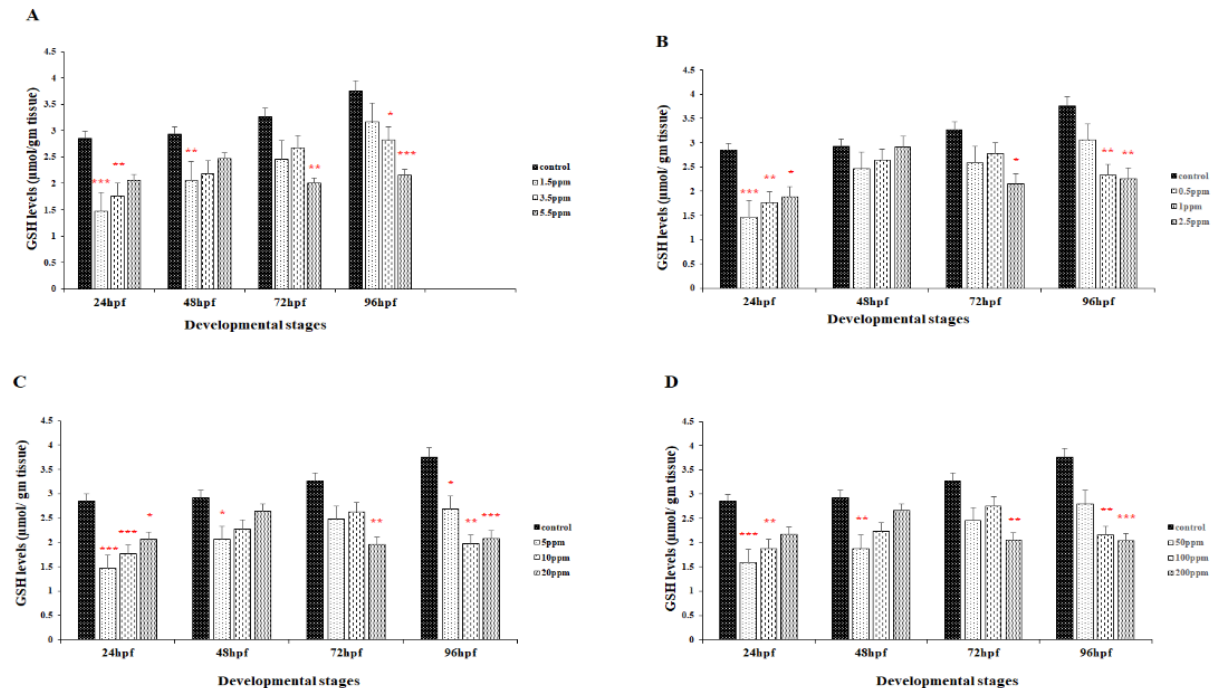


Fig. 2 Effects of Food Additives on glutathione (GSH) activity in zebrafish embryos. A) Tartrazine (TTZ), B) Sunset Yellow (SY), C) Aspartame (AST), and D) Sodium Benzoate (SB). Embryos were exposed from 18 h post-fertilization (hpf) to the respective additives. GSH activity was quantified at 24, 48, 72, and 96 hpf and expressed as μmol DTNB conjugate formed/g tissue. Data are presented as mean±SEM (n=30 p/group). Significant reductions in GSH activity were observed in a dose- and time-dependent manner, with higher concentrations inducing greater oxidative stress. Statistical significance was determined relative to the time-matched control group (*p<0.05, **p<0.01, ***p<0.001).

Table 2 Percentage change and statistical significance (p-values) of MDA levels in zebrafish embryos exposed to food additives at different developmental stages

Additive	Concentration	24 hpf (% change, p)	48 hpf (% change, p)	72 hpf (% change, p)	96 hpf (% change, p)
TTZ	1.5 ppm	+4.53%, p = 0.231 (ns)	+3.78%, p = 0.227 (ns)	+5.25%, p = 0.262 (ns)	+4.15%, p = 0.173 (ns)
	3.5 ppm	+15.64%, ns	+2.41%, p = 0.331 (ns)	+8.33%, p = 0.278 (ns)	+7.47%, p = 0.202 (ns)
	5.5 ppm	+57.61%, p = 0.001 ***	+30.93%, p = 0.002 **	+28.70%, p = 0.028 *	+28.01%, p = 0.032 *
SY	0.5 ppm	+15.71%, p = 0.110 (ns)	+4.81%, p = 0.237 (ns)	+21.60%, p = 0.062 (ns)	+11.62%, p = 0.173 (ns)
	1.0 ppm	+19.52%, p = 0.065 (ns)	+21.65%, p = 0.071 (ns)	+27.78%, p = 0.038 *	+15.15%, p = 0.082 (ns)
	2.5 ppm	+33.33%, p = 0.005 **	+28.52%, p = 0.011 *	+46.60%, p = 0.001 ***	+31.33%, p = 0.005 **
AST	5 ppm	+12.95%, p = 0.101 (ns)	+4.12%, p = 0.217 (ns)	+6.93%, p = 0.262 (ns)	+5.74%, p = 0.173 (ns)
	10 ppm	+16.52%, p = 0.090 (ns)	+9.05%, p = 0.171 (ns)	+19.14%, p = 0.078 (ns)	+28.23%, p = 0.020 *
	20 ppm	+20.98%, p = 0.073 (ns)	+31.69%, p = 0.001 ***	+36.30%, p = 0.001 ***	+34.45%, p = 0.001 ***
SB	50 ppm	+18.54%, p = 0.091 (ns)	+18.57%, p = 0.097 (ns)	+9.21%, p = 0.242 (ns)	+11.90%, p = 0.173 (ns)
	100 ppm	+27.80%, p = 0.025 *	+20.68%, p = 0.061 (ns)	+28.57%, p = 0.020 *	+26.46%, p = 0.032 *
	200 ppm	+35.61%, p = 0.005 **	+35.44%, p = 0.005 **	+32.70%, p = 0.004 **	+28.31%, p = 0.011 *

Remark: Values represent % change relative to control at each developmental stage. ns=not significant (p>0.05); * p<0.05; ** p<0.01; *** p<0.001.

Table 3 Percentage change and statistical significance (p-values) of GSH activity in zebrafish embryos exposed to food additives at different developmental stages

Additive	Concentration	24 hpf (% change, p)	48 hpf (% change, p)	72 hpf (% change, p)	96 hpf (% change, p)
TTZ	1.5 ppm	-48.42%, p = 0.001 ***	-29.69%, p = 0.027 *	-24.77%, p = 0.072 (ns)	-15.69%, p = 0.030 *
	3.5 ppm	-38.25%, p = 0.005 **	-25.60%, p = 0.051 (ns)	-18.35%, p = 0.078 (ns)	-24.73%, p = 0.070 (ns)
	5.5 ppm	-27.72%, p = 0.053 (ns)	-15.70%, p = 0.110 (ns)	-38.84%, p = 0.002 **	-42.55%, p = 0.001 ***
SY	0.5 ppm	-48.42%, p = 0.001 ***	-15.70%, p = 0.077 (ns)	-20.80%, p = 0.062 (ns)	-18.62%, p = 0.073 (ns)
	1.0 ppm	-38.25%, p = 0.005 **	-9.90%, p = 0.151 (ns)	-14.98%, p = 0.088 (ns)	-38.03%, p = 0.005 **
	2.5 ppm	-34.04%, p = 0.025 *	-0.68%, p = 0.211 (ns)	-34.25%, p = 0.044 *	-39.89%, p = 0.008 **
AST	5 ppm	-48.42%, p = 0.001 ***	-29.69%, p = 0.027 *	-24.16%, p = 0.061 (ns)	-28.72%, p = 0.035 *
	10 ppm	-38.25%, p = 0.001 ***	-22.53%, p = 0.055 (ns)	-19.57%, p = 0.088 (ns)	-47.61%, p = 0.006 **
	20 ppm	-27.72%, p = 0.042 *	-9.90%, p = 0.103 (ns)	-40.06%, p = 0.002 **	-44.68%, p = 0.005 **
SB	50 ppm	-44.21%, p = 0.001 ***	-35.84%, p = 0.007 **	-25.08%, p = 0.062 (ns)	-25.53%, p = 0.073 (ns)
	100 ppm	-34.04%, p = 0.005 **	-23.89%, p = 0.071 (ns)	-15.60%, p = 0.078 (ns)	-42.55%, p = 0.002 **
	200 ppm	-23.51%, p = 0.053 (ns)	-9.22%, p = 0.110 (ns)	-37.00%, p = 0.008 **	-45.74%, p = 0.001 ***

Remark: Values represent % change relative to control at each developmental stage. ns=not significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

2. Effects of food additives on glucose, triglyceride, and cholesterol levels in zebrafish embryos

Exposure to TTZ, SY, AST, and SB markedly disrupted carbohydrate and lipid metabolism in zebrafish embryos (Fig. 3). Detailed quantitative values, including percentage changes and p-values for each treatment group, are presented in Table 4, supporting the trends illustrated in Fig. 3

A clear compound-specific pattern emerged. TTZ and SY triggered strong hyperglycemia, with glucose levels nearly doubling relative to controls, whereas AST and SB caused modest but significant hypoglycemia, pointing to divergent effects of food additives on carbohydrate regulation. Lipid metabolism was also significantly altered. Triglyceride levels declined across all additive groups, SY and AST producing the largest reductions, TTZ and SB causing modest but still significant decreases.

Total cholesterol levels showed the most consistent response, with significant decreases in all additive-exposed groups. The strongest effect was observed in SB, followed by TTZ, while SY and AST induced moderate but significant reductions. This uniform suppression of cholesterol highlights its sensitivity as a biomarker of additive-induced metabolic stress.

Taken together, these results indicate that food additives induce metabolic imbalance through distinct but overlapping mechanisms. While TTZ and SY predominantly elevated glucose levels, AST and SB suppress them; however, all four converged in lowering triglyceride, and cholesterol levels. These findings align with prior studies linking food additive exposure to dysregulated glucose and lipid homeostasis in aquatic and mammalian systems, and suggest that early developmental exposure may predispose organisms to

long-term metabolic disorders (Paula Neto et al., 2017; Heindel et al., 2017; Warner, 2024).

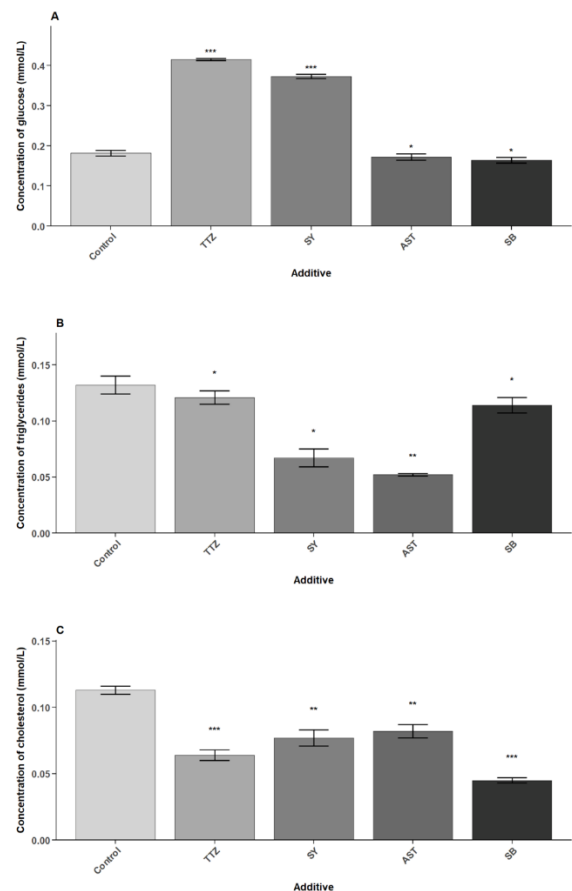


Fig. 3 Effects of TTZ, SY, AST, and SB on metabolic parameters in zebrafish embryos at 96 hpf. A) glucose levels, B) triglyceride levels, C) total cholesterol levels. Data are expressed as mean $\pm$ SEM (n=30/group). Statistical significance relative to control: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Y-axis units are shown as concentration (mmol/L).

Table 4 Effects of food additives on glucose, triglyceride, and cholesterol levels in zebrafish embryos

Additive	Concentration	Glucose (mmol/L, % change, p)	Triglycerides (mmol/L, % change, p)	Total cholesterol (mmol/L, % change, p)
Control	—	0.182±0.007 (–, –)	0.132±0.008 (–, –)	0.101±0.006 (–, –)
TTZ	5.5 ppm	0.415±0.003 (+128.0%, p<0.001 ***)	0.121±0.006 (–8.3%, p<0.05 *)	0.064±0.004 (–36.6%, p<0.001 ***)
SY	2.5 ppm	0.373±0.005 (+105.0%, p<0.001 ***)	0.067±0.008 (–49.2%, p<0.01 **)	0.077±0.006 (–23.8%, p<0.01 **)
AST	20 ppm	0.172±0.008 (–5.5%, p<0.05 *)	0.052±0.001 (–60.6%, p<0.01 **)	0.082±0.005 (–18.8%, p<0.01 **)
SB	200 ppm	0.164±0.007 (–9.9%, p<0.05 *)	0.114±0.007 (–13.6%, p<0.05 *)	0.045±0.002 (–55.4%, p<0.001 ***)

Remark: Glucose, triglyceride, and total cholesterol levels were quantified and expressed as mean ± SEM relative to untreated controls. Percentage change values indicate deviations from the control group, and statistical significance was calculated using one-way ANOVA followed by Tukey’s post hoc test. *p<0.05, **p<0.01, ***p<0.001 compared to control.

3. Gene expression analysis of *ptfla*, *glrx*, and *cox* genes in zebrafish embryos exposed to food additives

The expression levels of *ptfla*, *glrx*, and *cox* genes were analyzed in zebrafish embryos exposed to TTZ, AST, and SB, at different developmental stages (24, 48, 72, and 96 hpf) (Fig. 4). Gene expression analysis for embryos exposed to SY was not performed due to poor RNA yield and quality, likely reflecting reduced embryo viability.

ptfla and *glrx* showed significant upregulation in response to all three additives, particularly at later stages (72–96 hpf) (Fig. 4A, B), whereas *cox* displayed variable, additive responses (Fig. 4C).

TTZ and SB showed consistent increases in *ptfla* across all time points, with SB showing the most pronounced effects. AST caused a delayed but strong upregulation at 96 hpf.

glrx expression followed a similar pattern, with significant increases at 72 and 96 hpf for all additives,

indicating disruption of glutathione metabolism and potential oxidative stress.

cox expression was more variable. TTZ significantly increased *cox* at 48 and 72 hpf, AST had minimal effects, and SB caused significant upregulation at 24 and 96 hpf, suggesting additive-specific impacts on mitochondrial respiration.

The pronounced upregulation of *ptfla* in SB-exposed embryos implicates this gene in additive-induced pancreatic and mitochondrial dysfunction. Given *ptfla*’s role in pancreatic development and insulin regulation (Duque et al., 2022), its dysregulation may contribute to glucose intolerance and diabetes. Similarly, altered *glrx* expression indicates perturbations in glutathione metabolism, potentially aggravating oxidative damage (Zhou et al., 2024). The variable *cox* expression highlights that food additive differentially effects mitochondrial energy metabolism (Salame et al., 2024).

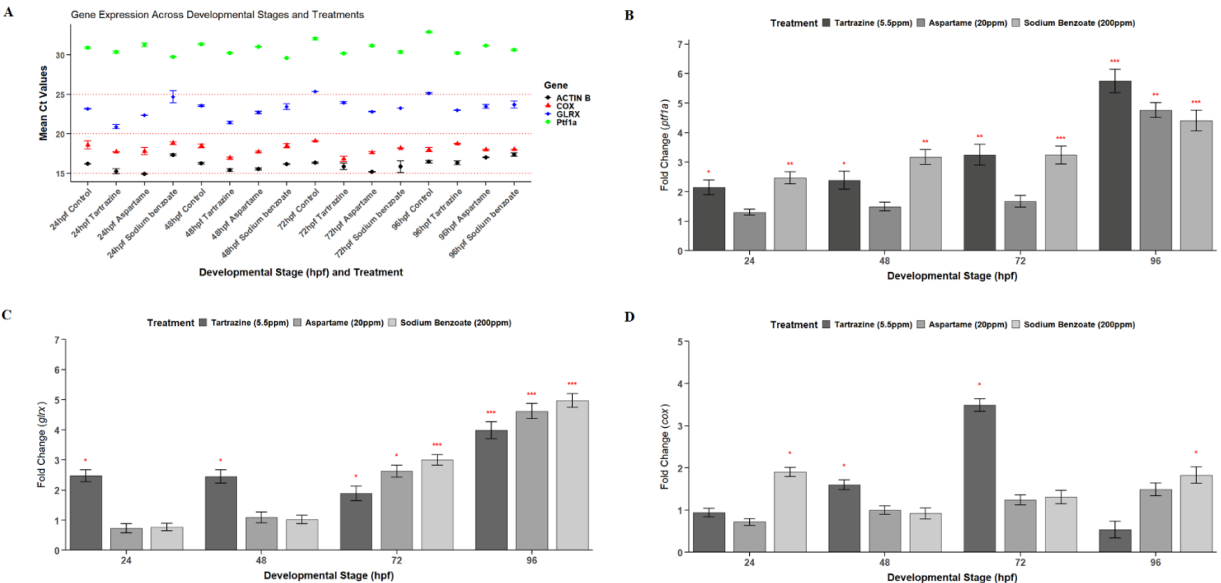


Fig. 4 Gene expression of *ptfla*, *glrx*, and *cox* in zebrafish embryos exposed to food additives. (A) Mean expression levels of β -actin, *cox*, *glrx*, and *ptfla* across developmental stages. (B–D) Fold change of *ptfla* (B), *glrx* (C), and *cox* (D) at 24, 48, 72, and 96 hpf following exposure to TTZ (5.5 ppm), AST (20 ppm), and SB (200 ppm). Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta Ct = Ct(\text{target}) - Ct(\beta\text{-actin})$, $\Delta\Delta Ct = \Delta Ct(\text{treated}) - \Delta Ct(\text{control})$, and fold change = $2^{-\Delta\Delta Ct}$. Statistical significance (*p<0.05, **p<0.01, ***p<0.001) vs control (n=3 replicates).

4. Metabolic alterations in zebrafish embryos induced by food additives: NMR spectral analysis

Untargeted ¹H-NMR metabolomic profiling was conducted in 96 hpf embryos of zebrafish following exposure to tartrazine (TTZ), sunset yellow (SY), aspartame (AST), and sodium benzoate (SB) (Fig. 5). Metabolic peaks were assigned based on their chemical shifts (ppm) using established metabolite databases and

published zebrafish NMR metabolomics studies (Zuberi et al., 2019; Fujita et al., 2021). Table 5 summarizes peak positions and their presence or absence across treatment groups.

The predominant spectral responses demonstrated distinct compound-specific patterns: tartrazine (TTZ) and sunset yellow (SY) largely preserved overall metabolite detectability with only minor intensity

Table 5 Proton NMR (¹H-NMR) chemical shifts (ppm) of zebrafish embryos at 96 hpf after exposure to different food additives

Peak	Control	Tartrazine	Sunset yellow	Aspartame	Sodium benzoate	Putative metabolite
1	0.905	0.904	0.903	—	—	Valine (CH ₃)
2	0.922	0.921	0.920	—	—	Leucine (CH ₃)
3	0.936	0.935	0.935	—	—	Isoleucine (CH ₃)
4	1.086	1.069	1.069	—	—	Lipid CH ₂
5	1.098	1.083	1.094	—	—	Lipid CH ₂
6	—	1.104	1.107	—	—	Lipid CH ₃
7	1.207	1.206	1.205	1.199	1.199	Alanine (CH ₃)
8	—	—	1.212	1.213	—	Lipid CH ₂
9	1.371	1.371	1.370	—	1.350	Lipid CH ₂
10	—	—	1.364	—	—	—
11	1.597	1.597	1.595	—	—	Lactate (CH ₃)
12	—	—	1.794	1.795	—	—
13	1.904	1.903	1.903	—	1.896	Lipid CH ₂ /Glutamate
14	—	—	1.935	—	—	—
15	2.001	2.001	2.001	—	—	Glutamate (CH ₂)
16	2.013	2.020	2.015	—	—	Glutamine (CH ₂)
17	2.020	2.031	2.029	—	—	Glutamine (CH ₂)
18	2.027	2.116	2.222	—	—	Glutamate/Glutamine
19	2.041	2.637	—	—	—	Lactate/Acetate
20	2.168	—	—	—	—	Lipid CH ₂
21	2.184	—	—	—	—	Lipid CH ₂
22	2.198	—	—	—	—	Lipid CH ₂
23	2.513	—	2.289	—	—	Lipid CH ₂ /Alanine
24	3.089	3.087	3.087	3.081	3.082	Glucose H2-H5
25	3.108	3.107	3.106	3.101	3.101	Glucose H2-H5
26	—	—	3.109	—	—	—
27	3.153	3.152	3.151	3.145	3.146	Glucose H2-H5
28	3.290	3.312	3.296	—	—	Glycerol/CH ₂ OH
29	3.304	3.394	3.309	3.233	—	CH ₂ OH/Serine
30	3.317	3.441	3.322	—	—	CH ₂ OH/Serine
31	3.395	—	3.394	—	3.387	Glycine/Serine
32	3.440	—	3.440	—	3.434	Glycine/Serine
33	3.622	3.628	3.627	—	—	Glucose H6
34	3.627	3.642	3.637	—	—	Glucose H6
35	3.637	3.658	3.642	—	—	Glucose H6
36	3.641	3.672	3.651	—	—	Glucose H6
37	3.652	—	—	—	—	—
38	3.657	—	—	—	—	—
39	3.790	3.789	3.671	—	—	CH ₂ OH/Lipids
40	3.815	3.814	3.789	3.784	3.784	Glycine/Serine
41	—	3.814	3.808	3.808	—	—
42	4.002	4.000	4.000	—	—	Glucose H1
43	—	4.700	—	4.700	4.700	α/β-Glucose H1
44	6.896	6.789	6.948	—	—	Phenylalanine
45	—	7.069	6.964	—	—	Tyrosine
46	7.299	7.298	7.296	—	—	Aromatic AA
47	7.315	7.311	7.311	—	—	Aromatic AA
48	7.800	7.634	7.626	—	—	Phenylalanine/Tyrosine
49	8.225	8.232	8.228	—	—	Nucleotides/DNA
50	8.341	8.340	8.339	8.333	—	Nucleotides/DNA
51	8.479	8.442	8.462	—	—	Nucleotides/DNA

modulations compared to control, whereas TTZ additionally exhibited selective chemical shift displacement of specific resonances, suggesting localized alterations in the metabolic microenvironment; in contrast, aspartame (AST) and sodium benzoate (SB) exposure resulted in marked attenuation or complete loss of detectable metabolite peaks, indicative of substantial metabolic disruption and possible depletion of key metabolite pools.

Data are shown for control, tartrazine (TTZ), sunset yellow (SY), aspartame (AST), and sodium benzoate (SB). Dashes (–) indicate peaks not detected or below the limit of reliable quantification. Putative metabolite assignments were based on literature and databases (HMDB, BMRB) and include amino acids, glucose derivatives, lipids, and nucleotides. Peaks in 0–2 ppm correspond to lipids and amino acids, 2–4 ppm to small organic molecules and glucose derivatives, and 6–9 ppm to aromatic compounds and nucleotides.

4.1 Lipid and Branched-Chain Amino Acid Perturbations

Control embryos displayed characteristic Branched-Chain Amino Acid (BCAA) methyl resonances

(0.905–0.936 ppm) and lipid methylene signals (~1.08–1.10 ppm). These peaks remained detectable in TTZ and SY groups but were absent in both AST and SB groups.

BCAA (valine, leucine, isoleucine) are central substrates for mitochondrial anaplerosis, feeding acetyl-CoA and succinyl-CoA into the tricarboxylic acid (TCA) cycle. Loss of these signals suggests perturbation in KEGG pathway map00280 (valine, leucine and isoleucine degradation).

Mitochondrial bioenergetic impairment is a common downstream consequence of xenobiotic-induced oxidative stress (Zuo et al., 2024). Concurrent absence of lipid-associated peaks further supports potential alterations in fatty acid metabolism and membrane lipid turnover. Oxidative stress-mediated lipid peroxidation has been shown to modify lipid detectability in NMR-based metabolomics (Halliwell & Gutteridge, 2015; Zuberi et al., 2019).

Together, these findings indicate that AST and SB exposures are associated with coordinated perturbations in amino acid and lipid metabolism linked to mitochondrial function.

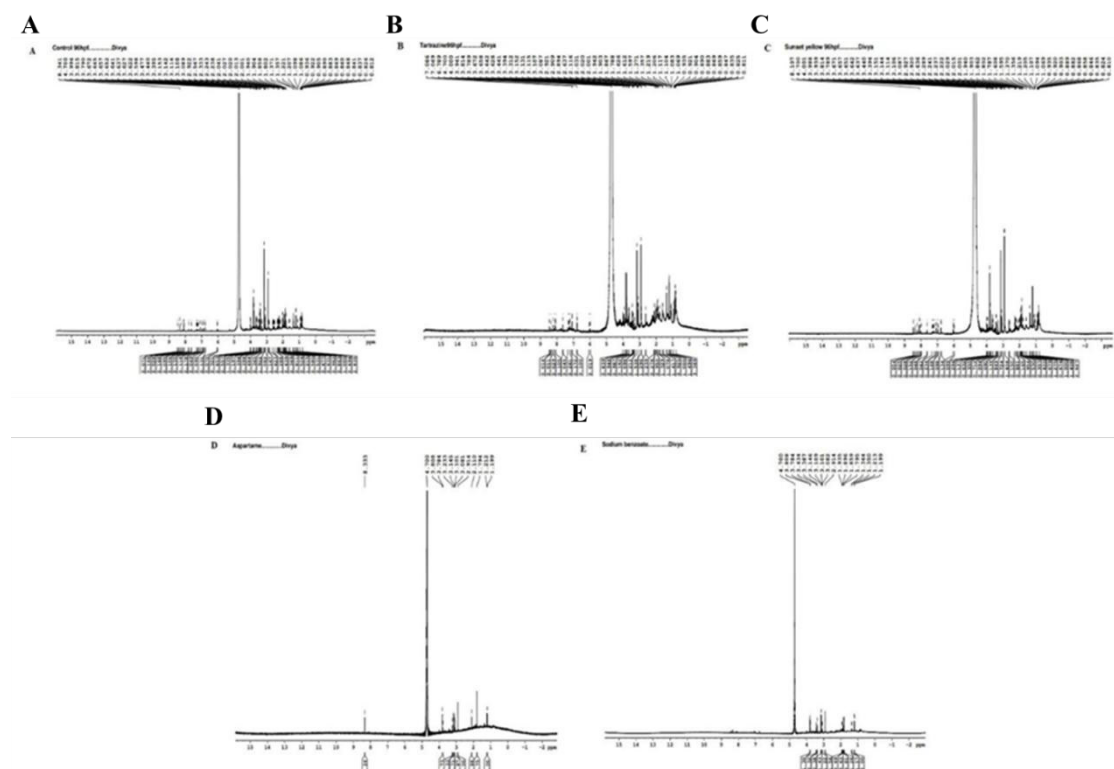


Fig. 5 Representative proton NMR spectral profiles of zebrafish embryos exposed to different food additives at 96 hpf, showing variations in metabolite signals compared to (A) Control. Additives include (B) Tartrazine, (C) Sunset Yellow, (D) Aspartame, and (E) Sodium Benzoate.

4.2 Disruption of Pyruvate and Lactate-Associated Metabolism

Lactate-associated peaks (1.597 ppm) were present in control, TTZ, and SY groups but absent in AST and SB groups. This disappearance suggests disturbance of glycolytic end-product equilibrium and pyruvate metabolism (KEGG map00010 and map00620).

Under mitochondrial stress, metabolic systems often undergo carbon flux redistribution to compensate for impaired oxidative phosphorylation (Vander Heiden et al., 2009; Liberti & Locasale, 2016). While direct flux measurements were not performed, simultaneous loss of BCAA and lactate signals in AST and SB groups supports altered integration between cytosolic glycolysis and mitochondrial respiration.

In contrast, TTZ exposure resulted in a discrete chemical shift displacement (2.041→2.637 ppm), indicating modification of metabolite microenvironment without complete loss of detectability.

4.3 Carbohydrate Metabolism and Redox Regulation

Glucose-associated multiplets (3–4 ppm), including H2–H6 and H1 resonances, were consistently detected in control, TTZ, and SY embryos. In contrast, AST-treated embryos exhibited loss of multiple glucose peaks, particularly within the H6 region (3.622–3.641 ppm) and the H1 signal (4.002 ppm). SB-treated embryos retained several glucose H2–H5 peaks but similarly lacked H6 and H1 signals.

Thus, both AST and SB altered carbohydrate-associated spectral regions; however, AST demonstrated comparatively broader loss of glucose-related resonances.

Glucose metabolism is directly linked to glycolysis (KEGG map00010) and indirectly connected to the pentose phosphate pathway, which generates NADPH required for antioxidant defense (Duy-Thanh et al., 2022). Although PPP intermediates were not directly measured, the observed alterations in glucose-associated peaks suggest perturbation of carbohydrate metabolism that may influence cellular redox balance.

Given the high energetic demand of embryonic development in *Danio rerio*, disruption of glucose-related metabolites may have downstream effects on bioenergetic homeostasis.

4.4 Aromatic Amino Acid and Nitrogen Metabolism

Distinct aromatic-region peaks (6–8 ppm), corresponding to phenylalanine and tyrosine, were present in control, TTZ, and SY groups but absent in both AST and SB groups.

These metabolites map to KEGG pathways

map00360 (phenylalanine metabolism) and map00350 (tyrosine metabolism). Aromatic amino acids are precursors for catecholamines and thyroid hormones and are particularly susceptible to oxidative modification, including tyrosine nitration under reactive oxygen species (ROS) stress (Souza et al., 2008).

Aspartame metabolism releases phenylalanine, and disturbances in phenylalanine homeostasis have been linked to metabolic and neurological alterations (Magnuson et al., 2007). The absence of aromatic signals in AST and SB groups therefore suggests disruption of amino acid equilibrium potentially associated with oxidative or metabolic stress.

4.5 Nucleotide-Associated Metabolites

Nucleotide-associated peaks (8.3–8.4 ppm) were largely preserved in control, TTZ, and SY groups. SB exhibited selective absence of specific peaks (e.g., 8.341 ppm), while AST retained nucleotide signals.

These metabolites correspond to purine metabolism (KEGG map00230), which is tightly coupled to ATP turnover and cellular energy status. Although nucleotide perturbations were less extensive than amino acid changes, selective alterations in SB embryos may reflect modified cellular energy balance.

Collectively, the metabolomic profile distinguishes two response categories, with TTZ and SY exhibiting minimal loss of metabolite detectability accompanied primarily by modest chemical shift variations and relative preservation of amino acid and lipid signals, albeit with indications of altered glucose-associated resonances suggestive of subtle modulation of carbohydrate handling (Fujita et al., 2021; Lee et al., 2024), whereas AST and particularly SB demonstrated coordinated attenuation of BCAA, lipid, lactate, glucose, and aromatic amino acid signals, consistent with systemic metabolic disruption characterized by mitochondrial dysfunction, impaired anaplerotic flux, altered glycolytic integration, and compromised redox homeostasis (Zong et al., 2024; Zuo et al., 2024). Notably, sodium benzoate elicited the most pronounced and widespread spectral attenuation across lipid, carbohydrate, and aromatic amino acid regions, indicating broad perturbation of energy-regulating metabolic networks (Alsunni, 2020; Lennerz et al., 2015).

The metabolic signature observed in AST- and SB-exposed embryos aligns with interconnected perturbations across valine, leucine, and isoleucine degradation (KEGG map00280), the tricarboxylic acid cycle (map00020), glycolysis/gluconeogenesis (map00010), pyruvate metabolism (map00620), the

pentose phosphate pathway (map00030), phenylalanine and tyrosine metabolism (map00360 and map00350), and purine metabolism (map00230), collectively indicating disruption of tightly integrated carbon, nitrogen, and energy metabolic pathways that sustain embryonic growth and bioenergetic balance (Fujita et al., 2021; Duy-Thanh et al., 2022). Concomitant alterations in glucose-associated signals in TTZ and SY embryos and reductions in lipid-related resonances across multiple exposure groups further reinforce the hypothesis of impaired systemic energy homeostasis and metabolic stress (Angelin et al., 2024; Lee et al., 2024).

Such multi-pathway interference is mechanistically consistent with oxidative stress-associated mitochondrial impairment during early vertebrate development, wherein altered redox balance, diminished NADPH-generating capacity, disrupted substrate oxidation, and reduced oxidative phosphorylation efficiency converge to compromise bioenergetic stability (Zuo et al., 2024; Zong et al., 2024). Given the centrality of coordinated carbohydrate and lipid metabolism in maintaining glycemic regulation and metabolic equilibrium, these findings raise concern that early-life exposure to certain food additives—particularly sodium benzoate—may predispose organisms to long-term metabolic dysregulation, warranting further longitudinal and translational investigation (Salame et al., 2024; Duy-Thanh et al., 2022).

Conclusion

This study demonstrates that early life exposure to common food additives (TTZ, SY, AST and SB) induces oxidative stress, disrupt metabolic homeostasis, and alter gene expression in zebrafish embryos. All additives elevated malondialdehyde (MDA) levels and reduced glutathione (GSH) activity, confirming compound- and stage-specific oxidative damage, with SB posing the most pronounced oxidative risk. Metabolic profiling revealed distinct additive-specific effects; TTZ and SY largely preserved overall metabolite detectability but modulated glucose and lipid-associated signals, whereas AST and SB caused coordinated attenuation of BCAA, lipid, lactate, glucose, and aromatic amino acid peaks, reflecting broad disruption of mitochondrial bioenergetics, glycolytic integration, and redox homeostasis.

Gene expression analyses corroborated these findings: *pf1a* and *glrx* were consistently upregulated,

implicating perturbations in pancreatic function and glutathione metabolism, while *cox* expression varied in an additive- and stage- specific manner, suggesting differential effects on mitochondrial respiration.

Collectively, these results indicate that SB and AST elicit the most systemic metabolic disturbances, whereas TTZ and SY primarily affect carbohydrate and lipid regulation with subtler metabolic impacts.

Taken together, these findings highlight that early developmental exposure to these additives may compromise antioxidant defenses, impair energy and lipid metabolism, and disrupt critical molecular pathways, potentially predisposing organisms to long-term metabolic disorders, including glucose intolerance and diabetes. The zebrafish embryo model underscores the need to reevaluate the safety of widely used food additives, particularly sodium benzoate, and encourages further longitudinal and mammalian studies to assess persistent and transgenerational health effects.

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Conflicts of interest

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used ChatGPT to improve language clarity and grammar. The generated suggestions were carefully reviewed, revised, and verified by the authors. The authors take full responsibility for the accuracy, originality, and integrity of the final manuscript.

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