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Research Article
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Roles of *try* and *amy* in feeding, digestion, growth, and development of the Japanese medaka (*Oryzias latipes*): insight from a comparative gene knockout study

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Abstract: Digestive enzymes of fish are critical to food digestion at the larval stage, but convincing evidence proving the function and necessity of the associated digestive enzymes remains lacking. In this study, we generated the trypsin (*try*) gene and amylase (*amy*) gene in the Japanese medaka (*Oryzias latipes*) using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) for the first time. *try* deletion significantly decreased the expression of *try* and digestive capacity in $try^{-/-}$ medaka larvae; after 8.5 h of digestion, incompletely digested brine shrimp was observed in the digestive tract at 4 and 15 d post-hatching (dph) of $try^{-/-}$ medaka larvae. Furthermore, the height of intestinal villi and total body length decreased significantly within 15-dph $try^{-/-}$ medaka larvae. However, *amy* deletion did not influence the digestion of medaka larvae at 4 dph. Only a small amount of incompletely digested brine shrimp was observed in 15-dph $amy^{-/-}$ medaka larvae. Further analysis of the growth, nitrogen metabolism, and intestinal microbes of $try^{-/-}$ adult medaka showed that the body length and weight of adult medaka decreased significantly, while the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the blood significantly increased. Pathological observation of the liver and intestinal tissues showed that *try* knockout resulted in vacuolar degeneration of liver cells, thinning of the intestinal wall, sparse arrangement of villi, and lower villi height. High-throughput 16S ribosomal RNA (rRNA) sequencing revealed that *try* knockout reduced the diversity of intestinal microbes. These findings demonstrated that *try* was indispensable for medaka larvae because it continuously affects their growth, nitrogen metabolism, and intestinal development.

Key words: *try*; *amy*; Japanese medaka; Knockout; Feeding; Digestion

1 Introduction

Digestion is an important physiological process that directly influences the availability of nutrients required for biological activity in animals; thus, the study of digestive physiology has attracted global attention (Gisbert et al., 2009; Ma et al., 2014; Navarro-Guillén et al., 2015; Nazemroaya et al., 2015). Digestion processes in fish are poorly understood in comparison to those in mammals, but digestive physiology is critical in fish (Rønnestad et al., 2013; Gisbert et al., 2014). Unlike infant mammals, most fish larvae have a very rudimentary digestive system when they hatch, so their digestive capacity is limited. The types and functions of digestive enzymes largely determine the efficiency of the whole digestive process (Rønnestad et al., 2013; Ueberschär et al., 2018; Yúfera et al., 2018; Aakanchhaa et al., 2020).

Fish larvae are extremely sensitive to environmental factors when they shift from endogenous to exogenous feeding, and the type and activity of digestive enzymes are crucial in determining digestive capacity, which ultimately affects larval growth performance (Yang et al., 2010; Anderson et al., 2018; Castro-Ruiz et al., 2019). Therefore, fish larvae require suitable, highly efficient digestive enzymes that are able to process the amount of ingested food needed to support rapid growth and development (Yúfera et al., 2018).

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Knowledge of the digestive enzymes and related genes of fish during the early stages is an invaluable tool for optimizing feeding protocols and increasing seed production while enhancing the larval survival rate and growth performance (Lazo et al., 2011; Tong et al., 2012; Moguel-Hernández et al., 2016; Khoa et al., 2019, 2020).

For agastric fish and fish larvae whose functional stomachs are not fully developed, the main enzymes for protein digestion are alkaline proteases in the pancreas and intestine, including trypsin and chymotrypsin (Infante and Cahu, 2001; Chong et al., 2002; Cara et al., 2003; Cahu et al., 2004). Trypsin can not only decompose protein components in food, but also rapidly activate chymotrypsinogen secreted by tissues such as the pancreas, thus playing an important role in fish protein digestion (Ahsan et al., 2001; Halangk et al., 2002; Darias et al., 2007; Rønnestad et al., 2013; Srichanun et al., 2013; Mata-Sotres et al., 2016b; Shen et al., 2018; Hernández-López et al., 2021). Amylase is another important digestive enzyme that can catalyze the hydrolysis of starch into smaller carbohydrate molecules, but the function of amylase during early development stages is not completely understood in fish (Srichanun et al., 2013; Mata-Sotres et al., 2016a; Mir et al., 2019). Up to now, studies on trypsin and amylase have focused on the ontogeny of development and compared gene expression and activity among several enzymes (Lazo et al., 2007; Ma et al., 2012; Rønnestad et al., 2013; Mata-Sotres et al., 2016b; Yúfera et al., 2018; Hernández-López et al., 2021; Khoa et al., 2021a). However, these traditional approaches lack convincing evidence to illustrate the full function of trypsin and amylase (Srichanun et al., 2013; Khoa et al., 2019).

Recently, molecular biological tools have provided complementary and essential insights into the structure and function of digestive enzyme-encoding genes (Moguel-Hernández et al., 2016; Yúfera et al., 2018; Khoa et al., 2019). Their results suggest that synthesis of digestive enzymes is genetically pre-programmed and appears before exogenous feeding in fish larvae (Lazo et al., 2011; Nazemroaya et al., 2015; Pérez-Sirkin et al., 2020). Therefore, in this study, we used the Japanese medaka (*Oryzias latipes*), a useful model organism which has a well-described genome, to explore the function and necessity of trypsin and amylase in fish after the trypsin (*try*) gene and amylase (*amy*) gene were knocked out with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) gene editing.

2 Materials and methods

2.1 Larval rearing

Japanese medaka were obtained from the Institute of Hydrobiology of the Chinese Academy of Sciences (Wuhan, China). All medaka were kept in 5-L cuboid tanks at (28.0±1.0) ℃ and the photoperiod of the fish room was controlled, with 14 h of light and 10 h of dark. Embryos were obtained by natural spawning and incubated with aerated oxygen water. The medaka were fed with brine shrimp (*Artemia nauplii*) from 4 d post-hatching (dph), at least twice a day.

2.2 CRISPR/Cas9 gene editing and generation of mutant medaka

The method of CRISPR/Cas9 gene editing was determined based on that described by Cai et al. (2021). We designed single-guide RNAs (sgRNAs) for the Japanese medaka *try* gene (ENSORLG00000013582) and *amy* gene (ENSORLG00000020006) using a CRISPR design tool (https://chopchop.cbu.uib.no). The *try* gene is also called the *prss1* gene. In humans, protease serine 1 (PRSS1) is the main digestive enzyme secreted by the pancreas (Athwal et al., 2014; Németh and Sahin-Tóth, 2014; Zou et al., 2022). In Japanese medaka, we found that the *prss1* gene was the only orthologous gene with human *PRSS1*. Two targets located in the second exon were designed for the *try* gene, while one target located in the fourth exon was designed for the *amy* gene. The corresponding sgRNAs and detection primers are displayed in Table S1. We predicted protein domains using the Conserved Domain Database in National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/cdd). Amino-acid sequence was aligned using CLC Sequence Viewer 6 (CLC bio, Aarhus, Denmark) (Jesus et al., 2017).

2.3 Feeding experiments on medaka larvae

Twenty wild-type (WT), $try^{-/-}$, and $amy^{-/-}$ medaka larvae (4 dph) were kept in separate 300-mL tanks. Larvae were fed abundant brine shrimp, and 0.5 h after feeding, they were anesthetized with MS-222 (Argent Chemical Laboratories, Redmond, WA, USA) and fixed

in 4% (0.04 g/mL) paraformaldehyde overnight at 4 °C. The specimens were then observed with a stereomicroscope (MshOt, China). The same experiment was performed three times, and the proportions of feeding and food intake were tracked.

2.4 Growth performance and survival rate

Twenty WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae were randomly selected and kept in separate 300-mL tanks. Larvae were fed abundant brine shrimp twice daily from 4 dph. The total length and survival rate of larvae of different genotypes were determined at 15 dph.

2.5 Enzyme assay

The activities of trypsin and amylase in the medaka larvae were determined using kits (trypsin kit, No. A080-2 and amylase kit, No. C016; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the liver and serum were measured using assay kits (ALT kit, No. C009-2-1 and AST assay kit, No. C010-2-1; Nanjing Jiancheng Bioengineering Institute). The protein content in the liver tissue was determined using a protein quantitative assay kit (No. A045-2; Nanjing Jiancheng Bioengineering Institute). All enzyme activity measurements were completed within 24 h after sacrifice.

2.6 Histological analysis

The medaka larvae, liver, and intestinal tissues preserved in 4% paraformaldehyde were dehydrated, cleared, immersed in wax, and embedded to make paraffin blocks. Next, paraffin sections were prepared using a microtome. The sections were stained with hematoxylin and eosin (H&E) at Wuhan Servicebio Technology Co., Ltd. (Wuhan, China) and then examined under a light microscope (MshOt); the images were analyzed and annotated with CaseViewer software (Servicebio) (Zhang et al., 2013).

2.7 Gene expression analysis

According to the manufacturer's instructions, we used TRIzol reagent (TaKaRa, Japan) to extract the total RNA from the brain and intestinal tissues of medaka. Then 1 μg of RNA was reverse-transcribed into complementary DNA (cDNA) using the Evo Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) Mix Kit with genomic DNA (gDNA) clean for quantitative polymerase chain reaction (qPCR) Ver. 2, AG11728 (Accurate Biotechnology Co., Ltd., Hunan, China) according to the manufacturer's instructions. Reverse transcription-qPCR (RT-qPCR) was performed with the SYBR Green Premix Pro Taq HS qPCR Kit II AG11702 (Accurate Biotechnology Co., Ltd.). The primers used for RT-qPCR are presented in Table S2. We quantified the messenger RNA (mRNA) expression levels of target genes relative to the expression of *β-actin* using the optimized comparative C_T ($2^{-\Delta\Delta C_T}$) value method (Livak and Schmittgen, 2001).

2.8 Gut microbiota analysis

The QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) was used to extract the total DNA of medaka entire gut tissues. Based on the Illumina NovaSeq 6000 sequencing platform (Illumina, San Diego, USA) at Tsingke Biotechnology Co., Ltd. (Tianjin, China), we constructed paired-end libraries for sequencing. Raw reads were deposited into the NCBI Sequence Read Archive (SRA) database. A total of 97% of sequence similarity was identified as the same operational taxonomic units (OTUs). We performed alpha diversity analysis based on Ace, Chao1, PD_whole_tree, and Simpson indexes to reflect the richness and species diversity of gut microbiota. QIIME software was used to generate species abundance tables at different taxonomic levels, and then R language tools were used to draw community structure maps at each taxonomic level of a sample. Linear discriminant analysis (LDA) effect size (LefSe) analysis was used to analyze significant differences in gut microbiota.

2.9 Statistical analysis

All data are reported as mean±standard error of the mean (SEM). All analyses were conducted using IBM SPSS Statistics 25.0 (IBM, Armonk, NY, USA). We tested statistical differences between two groups using the independent samples *t*-test, and considered a value of $P<0.05$ to indicate statistical significance. The data from three medaka larva genotypes were analyzed by one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test at $P=0.05$ significance level. All statistical analyses were performed using GraphPad Prism version 8.02 for Windows (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Generation of homozygous *try* and *amy* knockout medaka

The genomic target sites of *try* and *amy* were located in exon 2 and exon 4, respectively (Figs. 1a1 and 1b1). After microinjection and generation, we successfully obtained *try* and *amy* mutants. We found that in the *try* mutant line with a fragment of 21-base-pair (bp) deletion in the first target site, 3-bp insertion and 1-bp deletion in the second target site, the second target site resulted in a pre-stop codon (Fig. 1a1). The *amy* mutant

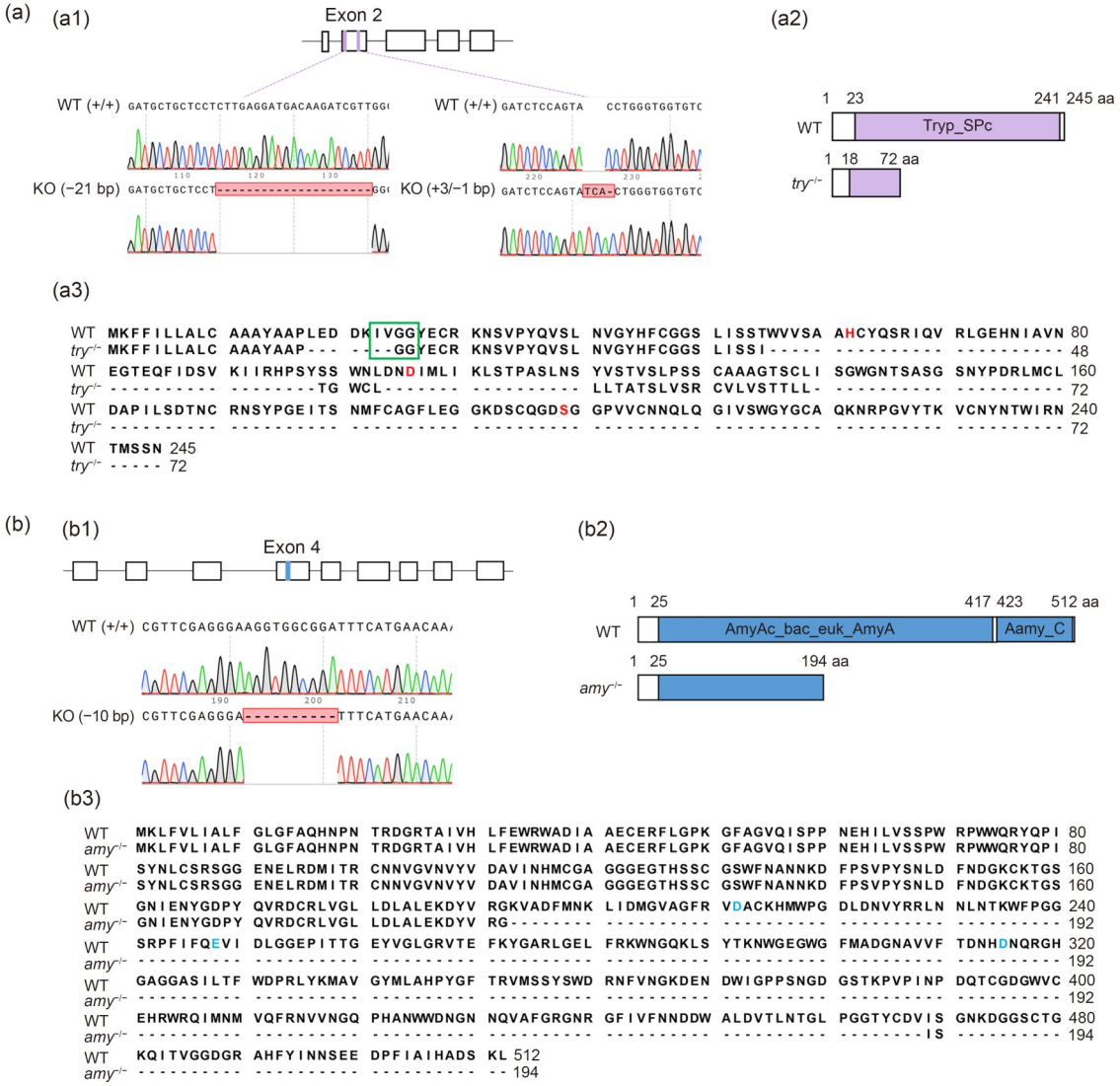

Fig. 1 Generation of homozygous mutants with the clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system. (a) Establishment of medaka *try* mutant line. (a1) The two genomic target sites of CRISPR were located in exon 2, and the sequencing results of wild-type (WT) medaka and homozygous mutants at two targets showed 21-base-pair (bp) deletion in the first target site, 3-bp insertion and 1-bp deletion in the second target site. (a2) The trypsin-like serine protease region (Tryp_SPc) was disrupted in the predicted truncated trypsin mutant protein. (a3) Amino acid sequence alignments of trypsin for WT and *try*$^{-/-}$ medaka. Active-site triad residues His62, Asp106, and Ser199 (marked with red) were absent in *try*$^{-/-}$ medaka, and the putative start of the mature peptide (residues 23–26: IVGG) (marked with a green box) was disrupted. (b) Establishment of medaka *amy* mutant line. (b1) The genomic target site of CRISPR was located in exon 4, and the sequencing results of WT medaka and homozygous mutants at this target showed 10-bp deletion in the target site. (b2) The α-amylase catalytic domain (AmyAc_bac_euk_AmyA) was disrupted in the predicted truncated mutant amylase protein. (b3) Amino acid sequence alignments of amyase for WT and *amy*$^{-/-}$ medaka. Active-site triad residues Asp212, Glu248, and Asp315 (marked with blue) were absent. KO: knockout.

line with a fragment of 10-bp deletion in the target site resulted in a pre-stop codon (Fig. 1b1).

Compared with WT medaka, in *try*$^{-/-}$ medaka, insertion and deletion induced truncated protein that had lost a greater part of the trypsin-like serine protease region (Tryp_SPc) (Fig. 1a2). The trypsin of *try*$^{-/-}$ medaka comprised only 72 amino acids and the amino acids of the catalytic triad (His62, Asp106, and Ser199); the putative start of the mature peptide (amino acid residues 23–26: IVGG) was absent in *try*$^{-/-}$ medaka (Figs. 1a2 and 1a3). In addition, the deletion in *amy*$^{-/-}$ medaka induced truncated protein that had lost the part of α-amylase catalytic domain (AmyAc_bac_euk_AmyA) (Fig. 1b2). The amylase of *amy*$^{-/-}$ medaka comprised only 194 amino acids. Amino acids of the catalytic triad (Asp212, Glu248, and Asp315) were also absent in *amy*$^{-/-}$ medaka (Figs. 1b2 and 1b3).

3.2 First feeding and digestion of medaka larvae

At 4 dph, the larvae were fed brine shrimp for the first time. Compared with WT medaka larvae, the *try*$^{-/-}$ medaka larvae performed a low proportion of feeding; after 8.5 h of digestion, the incompletely digested brine shrimp was observed in the digestive tracts of *try*$^{-/-}$ medaka larvae, and the expression of *npy* had significantly decreased (Figs. 2a, 2c, and 2e). The proportion of feeding, digestive capacity, and expression of *npy* in *amy*$^{-/-}$ medaka larvae showed no significant differences from WT medaka larvae (Figs. 2a–2c and 2e). Moreover, the expression of *pomc* was not significantly different among the three genotypes (Fig. 2d).

3.3 Histological analysis of the digestive tract in the three genotypes of medaka larvae

Microphotographs of the gut of the 15-dph medaka larvae showed significant alterations of the intestinal villi between the different genotypes (Fig. 3). Compared with WT medaka larvae (Fig. 3a), *try*$^{-/-}$ medaka larvae had a much lower height of the intestinal villi (Figs. 3b and 3d), but no significant change was observed in *amy*$^{-/-}$ medaka larvae (Figs. 3c and 3d).

3.4 Digestive capacity, growth performance, and survival rate of 15-dph medaka larvae

There was a large quantity of incompletely digested brine shrimp in 15-dph *try*$^{-/-}$ medaka larval digestive tracts after 8.5 h of digestion. In contrast, only a small amount was observed in 15-dph *amy*$^{-/-}$ medaka larvae (Fig. 4a). Compared with WT medaka larvae, *try*$^{-/-}$ medaka larvae had a much shorter total length. The *amy*$^{-/-}$ medaka larvae showed a decreasing trend at 15 dph but the differences were not statistically significant (Fig. 4b). In addition, the expression of *gh* and *igf* significantly decreased in *try*$^{-/-}$ medaka larvae but increased significantly in *amy*$^{-/-}$ medaka larvae (Figs. 4d and 4e). However, the survival rates were not significantly different among the three genotypes (Fig. 4c).

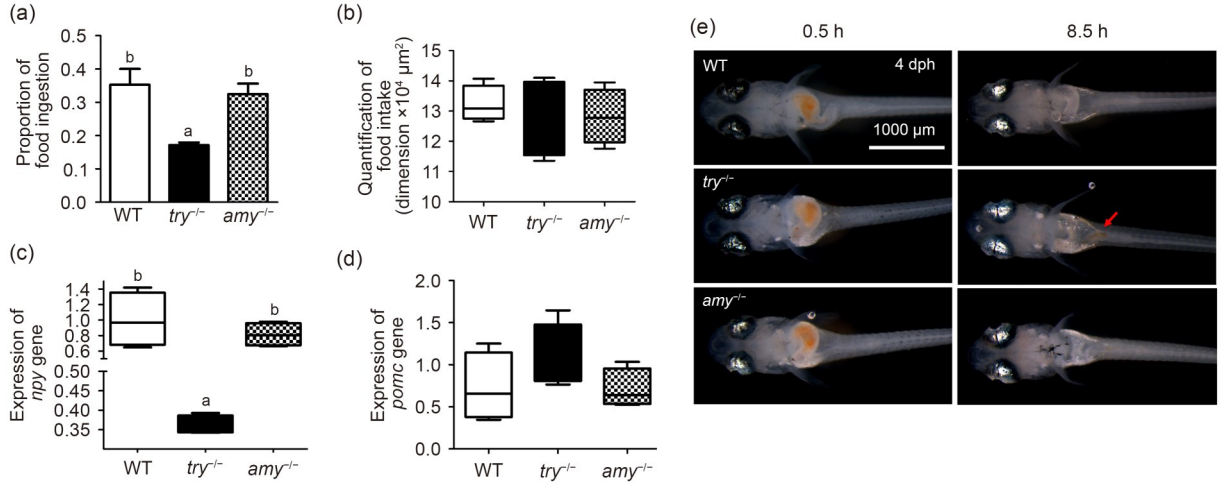

Fig. 2 First feeding and digestion of the three genotypes of medaka larvae at 4 dph. (a) Comparison of the proportion of feeding among the three genotypes. (b) Quantification of food intake of the three genotypes. (c, d) Expression of *npy* (c) and *pomc* (d) in the three genotypes. (e) Images of 4-dph larvae fed brine shrimp after 0.5 h and then starved for 8.5 h. The red arrow indicates incompletely digested brine shrimp. The results are expressed as mean±standard error of the mean (SEM) (n=6). Different superscript letters indicate significant differences between the genotypes (P<0.05).

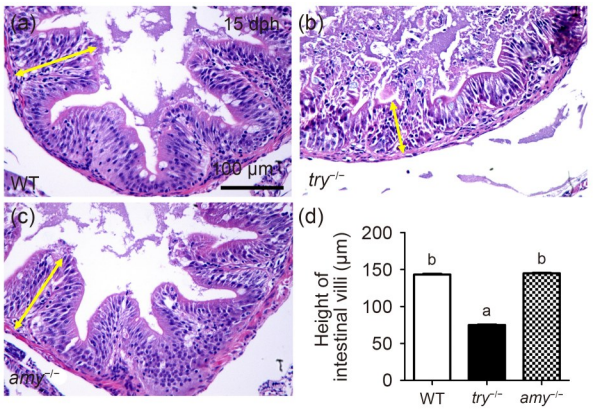

Fig. 3 Histological analysis of the digestive tract in 15-dph medaka larvae. (a–c) Microphotographs of the digestive tract and intestinal villi in wild-type (WT) (a), *try*$^{-/-}$ (b), and *amy*$^{-/-}$ (c) medaka larvae. The height of intestinal villi is marked with double yellow arrows. (d) Statistical analysis of the height of intestinal villi in WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae. The results are expressed as mean±standard error of the mean (SEM) (*n*=6). Different superscript letters indicate significant differences between the genotypes (*P*<0.05).

3.5 Growth performance of *try*$^{-/-}$ adult medaka

Our previous description showed that the digestive capacity of *try*$^{-/-}$ medaka larvae for brine shrimp was significantly reduced, as were the total length and intestinal villi height of the larvae. Although 15-dph *amy*$^{-/-}$ medaka larvae showed a slight decrease in their ability to digest brine shrimp, there was no significant difference in digestive tract development or total length between *amy*$^{-/-}$ medaka larvae and WT medaka larvae. Therefore, we next focused on the effects of *try* knockout on the growth, nitrogen metabolism, and gut microbiota of adult medaka.

We found that the total length (Fig. 5a) and body weight (Fig. 5b) of *try*$^{-/-}$ adult medaka decreased significantly compared with the WT.

3.6 Gene expression levels of *try*$^{-/-}$ adult medaka

We detected the expression levels of the appetite-stimulating genes *agrp* and *npy* in the brain and of *ctra*, *ctrb*, and *mtor* in the intestine, using RT-qPCR. The results showed that the expression levels of *agrp* and *npy* were much lower in *try*$^{-/-}$ adult medaka compared with the WT (Figs. 6a and 6b). Similarly, the expression levels of *ctra*, *ctrb*, and *mtor* in the intestine of *try*$^{-/-}$ adult medaka were significantly lower than those of the WT (Figs. 6c–6e).

3.7 Enzyme activity of *try*$^{-/-}$ adult medaka

The results of AST and ALT enzyme activity in the blood and liver are shown in Fig. 7. In liver tissue, there was no significant difference in AST or ALT activity between WT and *try*$^{-/-}$ adult medaka (Figs. 7a and 7b). The enzyme activities of AST and ALT in the blood of *try*$^{-/-}$ adult medaka were significantly increased compared with the WT (Figs. 7c and 7d).

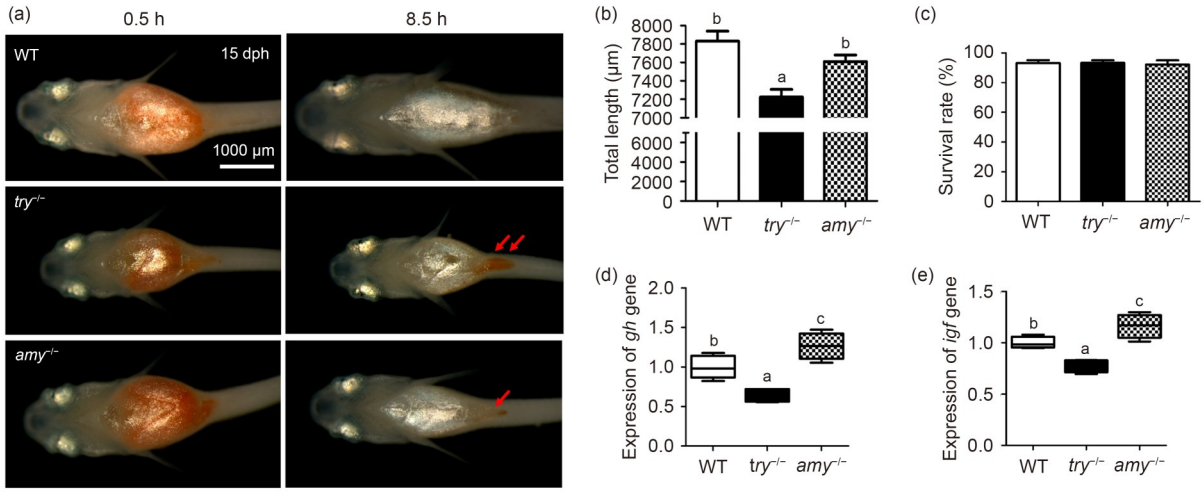

Fig. 4 Analyses of digestive capacity, total length, survival rate, and expression of growth performance-related genes in 15-dph medaka larvae. (a) The 15-dph larvae fed brine shrimp after 0.5 h and then starved for 8.5 h. The red arrows indicate incompletely digested brine shrimp. (b) Total lengths of wild-type (WT), *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae. (c) Survival rates of WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae. (d, e) Expression of *gh* (d) and *igf* (e) in WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae. The results are expressed as mean±standard error of the mean (SEM) (*n*=6). Different superscript letters indicate significant differences between the genotypes (*P*<0.05).

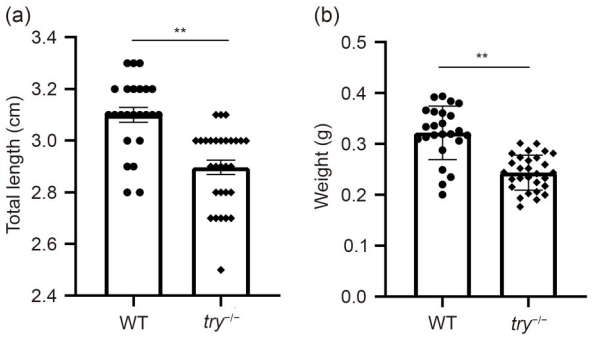

Fig. 5 Total length and body weight of medaka at four months of age. (a) Total length; (b) Body weight. The results are expressed as mean±standard error of the mean (SEM) (*n*=24 for wild-type (WT) medaka and *n*=29 for *try*$^{-/-}$ medaka). ** *P*< 0.01.

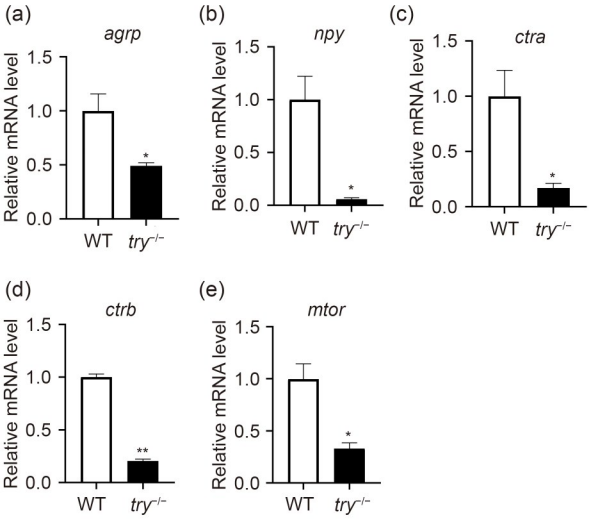

Fig. 6 Gene expression levels of *try*$^{-/-}$ adult medaka. Quantitative analysis of *agrp* (a), *npy* (b), *ctra* (c), *ctrb* (d), and *mtor* (e) gene messenger RNA (mRNA) expression levels. The mRNA levels of *try*$^{-/-}$ adult medaka are expressed as values relative to those of the wild-type (WT) medaka in each gene analysis. The results are shown as mean± standard error of the mean (SEM) (*n*=6). * *P*<0.05, ** *P*< 0.01, vs. WT.

3.8 Analysis of intestinal and liver tissue histology in *try*$^{-/-}$ adult medaka

Compared with the WT, the intestinal walls of *try*$^{-/-}$ adult medaka were thinner, and the villi were sparsely arranged and shorter. The red arrows in Fig. 8 show where the striatum was damaged and imperfect (Fig. 8a). However, in the *try*$^{-/-}$ group, the cells showed vacuolar degeneration (as indicated by the arrow). The cells were also significantly enlarged, the cytoplasm was blank, and the nucleus was squeezed to one side (Fig. 8b).

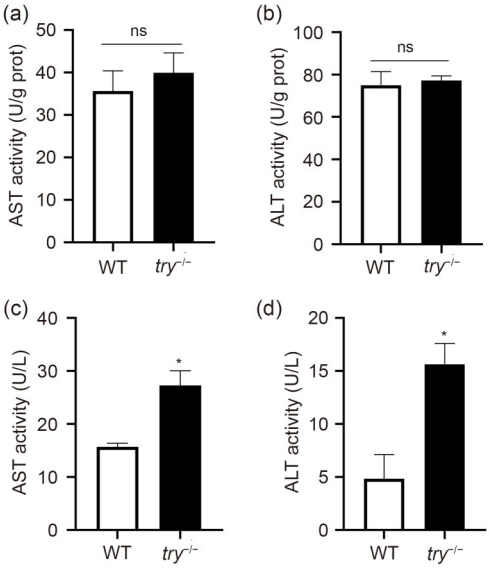

Fig. 7 Nitrogen-metabolizing enzyme activity in the liver and blood of *try*$^{-/-}$ adult medaka. (a) Aspartate aminotransferase (AST) enzyme activity in the liver. (b) Alanine aminotransferase (ALT) enzyme activity in the liver. (c) AST enzyme activity in blood. (d) ALT enzyme activity in blood. The results are shown as mean±standard error of the mean (SEM) (*n*=6). * *P*<0.05, vs. WT. ns: not significant; prot: protein; WT: wild-type.

3.9 Gut microbiota diversity in *try*$^{-/-}$ adult medaka

High-throughput 16S ribosomal RNA (rRNA) sequencing was performed on the intestinal tissues of *try*$^{-/-}$ and WT adult medaka. A Venn diagram of OTUs shows that 316 OTUs were shared by the two groups: 3575 OTUs specific to the knockout group and 2042 OTUs specific to the WT group (Fig. 9a). The species richness and species diversity of the gut microbiota in the medaka were analyzed in terms of alpha diversity. WT and *try*$^{-/-}$ adult medaka, as assessed by the accumulated cyclone energy (ACE) index, Chao1 index, and phylogenetic diversity (PD)_whole_tree index, had no significant differences (Figs. 9b–9d). However, compared with the WT Simpson index, the knockout medaka index clearly increased (Fig. 9e), indicating that knockout had no significant effect on the species richness of intestinal flora but led to an increase in species diversity.

3.10 Intestinal microbial composition in *try*$^{-/-}$ adult medaka

Based on relative abundance, *try* knockout significantly affected the dominant flora in medaka intestine. At the phylum level (Fig. 10a), the dominant flora for

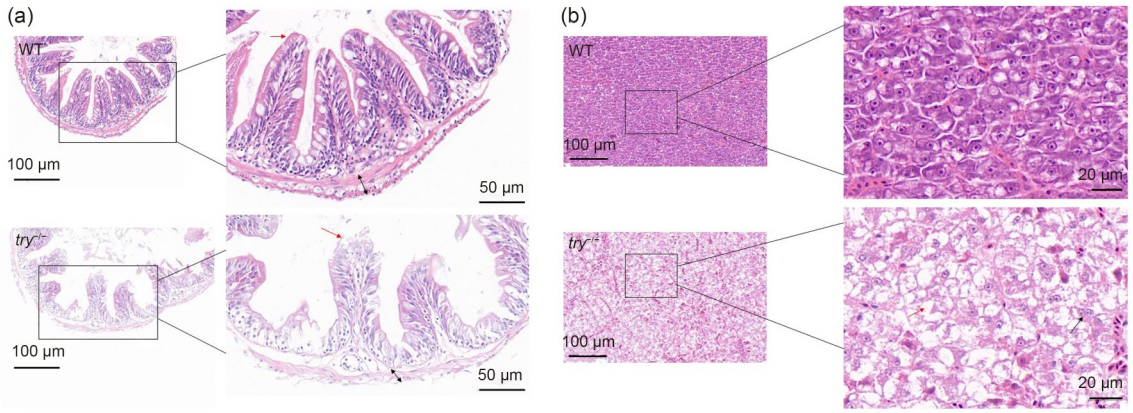

Fig. 8 Analysis of intestinal and liver tissue histology in *try*$^{-/-}$ adult medaka. (a) Hematoxylin and eosin (H&E) staining of intestine. The black double arrow indicates intestinal-wall thickness and the red arrow indicates striatum. (b) H&E staining of the liver. WT: wild-type.

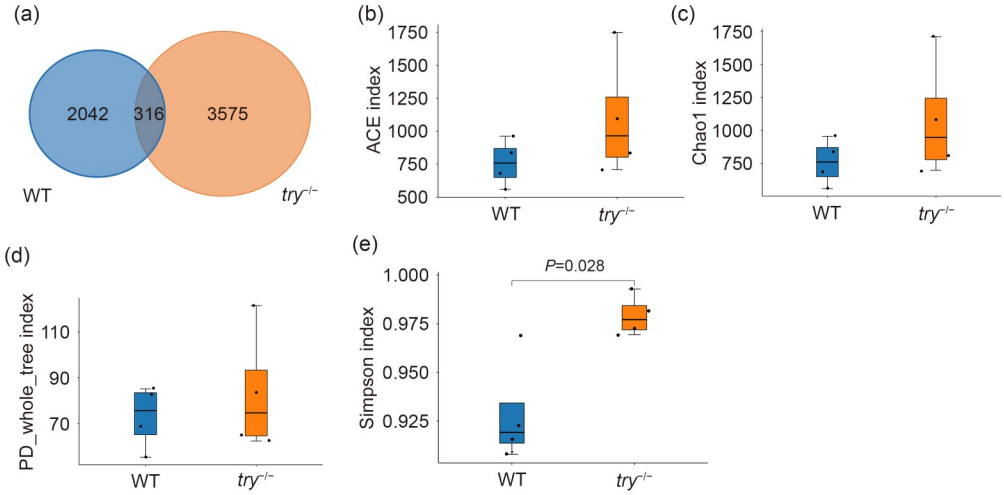

Fig. 9 Effects of *try* knockout on the intestinal microbial diversity of medaka. (a) OTU Venn diagram. (b) Accumulated cyclone energy (ACE) index. (c) Chao1 index. (d) Phylogenetic diversity (PD)_whole_tree index. (e) Simpson index. The results are shown as mean±standard error of the mean (SEM) (*n*=4). WT: wild-type.

try$^{-/-}$ adult medaka was Firmicutes, followed by Proteobacteria. At the genus level (Fig. 10b), *Thauera* and *Calorithrix* were the dominant intestinal flora in both WT and adult *try*$^{-/-}$ medaka. However, the relative abundances of these two genera were significantly lower in the *try*$^{-/-}$ medaka. LDA analysis identified 25 differentially abundant bacterial species across ten taxonomic groups, including p_Firmicutes, c_Clostridia, o_Oscillospirales, and seven others that showed marked changes in *try*$^{-/-}$ medaka (Fig. 10c). An evolutionary branching diagram showed that Proteobacteria and Calditrichota played a major role in the WT, while Firmicutes played a major role in *try*$^{-/-}$ adult medaka (Fig. 10d).

4 Discussion

The early stage is an important period in animal development, and one of the principal factors influencing the survival of larvae is the effective digestion of their diet. The efficiency of digestion depends on the type and function of the digestive enzymes (Kolkovski, 2001; Cahu et al., 2004; Lazo et al., 2007; Ma et al., 2014; Ghasemi et al., 2020). Trypsin and amylase are two important digestive enzymes, and reports on these two enzymes are focused on the ontogeny of development and comparison (Rønnestad et al., 2013; Khoa et al., 2019; Hernández-López et al., 2021). Convincing evidence to support the importance of trypsin and

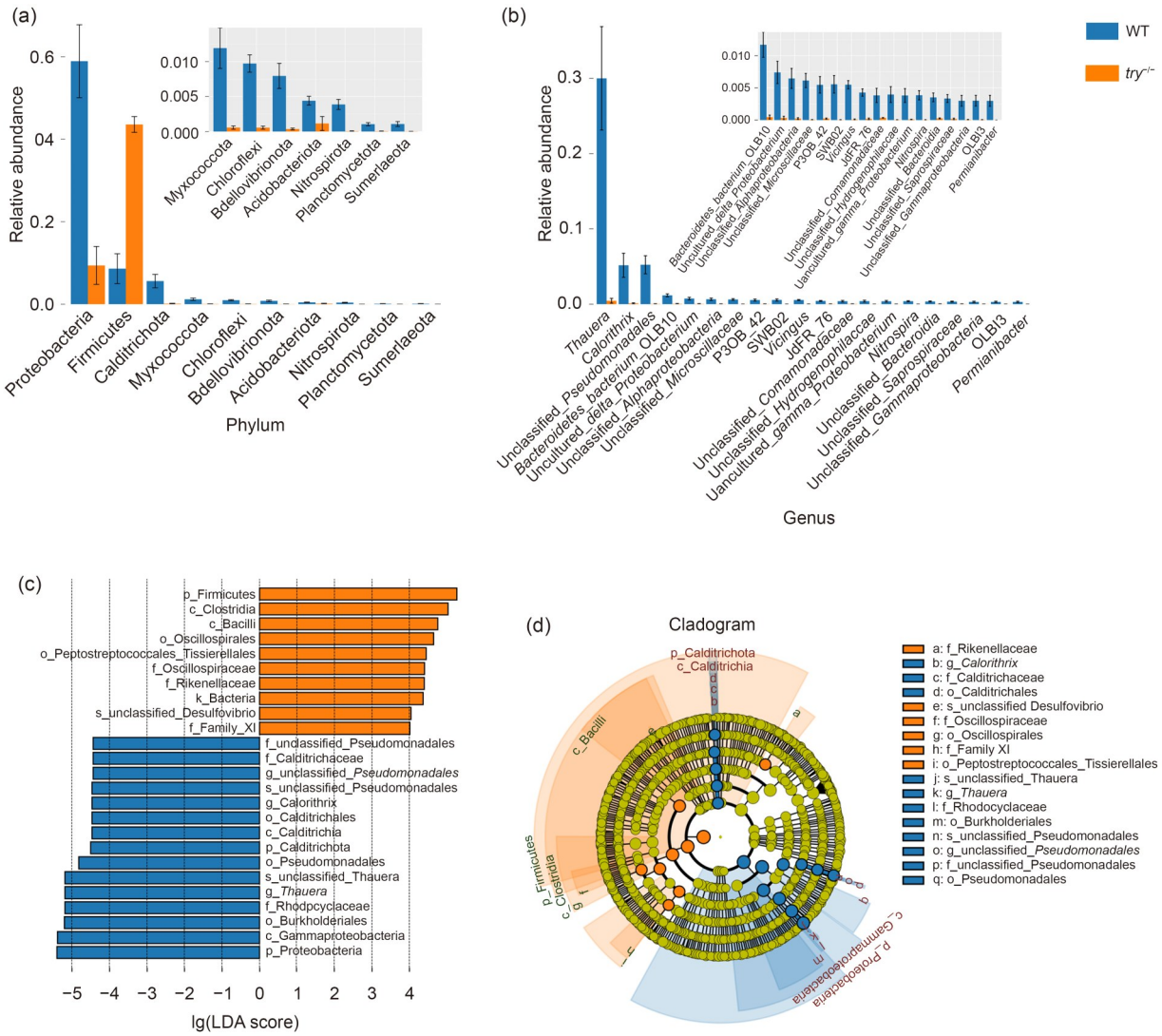

Fig. 10 Relative abundance of intestinal microorganisms in medaka at the phylum and genus levels. (a) Relative abundance at phylum level. (b) Relative abundance at genus level. (c) Linear discriminant analysis (LDA) analysis columnar graph. (d) Evolutionary branching diagram. (a, b) The results are shown as mean±standard error of the mean (SEM) (*n*=4). WT: wild-type.

amylase in fish is still lacking. This is the first study to generate digestive enzymes in knockout fish. Using the genome-editing system CRISPR/Cas9, we disrupted *try* and *amy* in the medaka.

In this study, *try*$^{-/-}$ medaka larvae showed significantly low digestive capacity at 4 and 15 dph. We observed incompletely digested brine shrimp in *try*$^{-/-}$ medaka larval digestive tracts after 8.5 h of digestion (Figs. 2 and 4). This result demonstrates that *try* really plays the key role in food digestion. Other studies also suggest that trypsin is the main digestive enzyme during the early development of the piscine digestive system, so it can be used as an excellent indicator of protein digestion in larvae before the development of the functional stomach and pepsin activity (Nazemroaya et al., 2015; Mir et al., 2018; Hernández-López et al., 2021; Khoa et al., 2021a). Amylase is another important digestive enzyme in fish that participates in the first stages of starch digestion (Kortner et al., 2011; Murashita et al., 2014; Mata-Sotres et al., 2016a; Mir et al., 2019; Pérez-Sirkin et al., 2020). Unexpectedly, compared with the WT medaka larvae, *amy*$^{-/-}$ medaka larvae show normal digestive capacity at 4 dph. Previous research has proved that trypsin has a strong capacity to digest brine shrimp (Arndt et al., 2015), and our study suggests that *amy*$^{-/-}$ medaka may digest brine shrimp by trypsin at 4 dph.

The height of the intestinal villi significantly decreased in 15-dph *try*$^{-/-}$ medaka larvae (Fig. 3), which might be related to low trypsin activity. A previous study also demonstrated that low digestive capacity could decrease the height of intestinal villi in longsnout seahorse *Hippocampus reidi* larvae (de Souza et al., 2020). Furthermore, low digestive capacity is also reflected in larval growth (Wang and Gu, 2010; Zokaeifar et al., 2012; Karasov and Douglas, 2013; de Souza et al., 2020). The results of this study show that the total body length of 15-dph *try*$^{-/-}$ larvae was significantly shorter, which might be related to the lack of nutrition due to low digestive capacity. Fish larvae obviously need sufficient nutrition to support their growth (Hamre et al., 2013; Gisbert et al., 2022). This study indicates that low digestive capacity and incomplete intestinal villi might influence the obtainment of essential nutrients by *try*$^{-/-}$ medaka larvae, limiting larval development and growth. *gh* and *igf1* have been considered possible markers for evaluating the growth rate of fish (Pérez-Sánchez et al., 2018; Khoa et al., 2021b). The decrease in the expression of *gh* and *igf* in fish might also be due to low digestive capacity (Zhou et al., 2015; Butt and Volkoff, 2019; Khoa et al., 2020). In addition, our study shows that the expression of *gh* and *igf* markedly decreased in 15-dph *try*$^{-/-}$ larvae. This indicates that the absence of *try* influences not only larval digestive capacity but also whole-larva development. However, in 15-dph *amy*$^{-/-}$ larvae, the intestinal villi and total length are not different from those of WT larvae, while the expression of *gh* and *igf* significantly increased. It could be that larvae increase the expression of *gh* and *igf* to support faster growth.

Based on our study of medaka larvae, *try* plays a more important role in food digestion than *amy*, and a lack of *try* leads to more food digestion problems. Therefore, we went on to investigate the effects of *try* knockout on the growth, nitrogen metabolism, and gut microbes of adult medaka. In 4-month-old *try*$^{-/-}$ medaka, the total length is shorter and the body weight is lower compared with the WT, and the expression levels of *npy* and *agrp* genes are significantly reduced in the brain tissue of these larvae, suggesting that a deficiency of *try* may continuously reduce the feeding intake of *try*$^{-/-}$ medaka and thus affect their growth and development.

Chymotrypsin, a digestive enzyme, is present in the pancreatic tissue and intestine of vertebrates. There are only two types of chymotrypsin in fish, namely, *ctra* and *ctrb* (Zhou et al., 2011). Chymotrypsin activity is influenced by many factors, including trypsin concentration, growth and development stage, feeding, and nutritional status of fish (Zhou et al., 2011; Seo et al., 2022). In the present study, the mRNA expression of *ctra* and *ctrb* genes in the intestine of *try*$^{-/-}$ medaka decreased significantly compared with that in the WT. Studies have shown that chymotrypsin plays a major role in growth restriction or inhibition in fish (Rungruangsak-Torrissen et al., 2006). Normally, chymotrypsin activation is regulated by trypsin (Ahsan et al., 2001; Darias et al., 2007).

The liver is an important location of protein metabolism, and the activity of transaminase in the liver can reflect the state of protein metabolism (Li et al., 2014). AST and ALT are the two most important transaminases involved in amino acid metabolism in the fish liver. They play an important role in the synthesis of nonessential amino acids and protein catabolism (Zhao et al., 2012). In this study, medaka *try*$^{-/-}$ resulted in a significant increase in AST and ALT activity in the serum. Under normal conditions, serum transaminase activity is low, but when fish are affected by adverse environmental stress or malnutrition, especially liver damage, it often leads to large amounts of transaminase from liver cells being released into the blood, resulting in a significant increase in AST and ALT activity in the serum (Sun et al., 2013; Cai et al., 2017). H&E staining of *try*$^{-/-}$ medaka liver tissue showed vacuolar degeneration of hepatocytes, significantly enlarged cells, blank cytoplasm, and nuclei squeezed to one side. The structure of healthy fish liver is uniform, and the liver cells are arranged regularly and tightly, unlike the damaged liver (Matsumoto et al., 2010; Topić Popović et al., 2023). Therefore, this study suggests that *try* deficiency may cause liver cell damage, thereby affecting the normal physiological function of the liver.

Protein synthesis in intestinal tissue is an important basis for fish growth and development (Jiang et al., 2009; Zhao et al., 2012). The ability of fish to digest and absorb food is largely determined by the development of their digestive organs. In addition, translation initiation is a rate-limiting step in protein synthesis, and the mechanistic target of rapamycin (mTOR) signaling pathway can regulate translation initiation and act as a regulatory center for intestinal protein synthesis (Feng et al., 2013). RT-qPCR showed that *try*$^{-/-}$ medaka

significantly reduced the expression of the *mtor* gene in medaka intestine, suggesting that *try*$^{-/-}$ medaka had adverse effects on the development of the intestine. Studies have shown that reduced protein deposition may affect the growth and development of intestinal tissues to some extent (Zhang et al., 2013). The integrity of vertebrate intestinal structure is an important basis for intestinal health and normal development (Zhang et al., 2013; Ullah et al., 2022). Observations of the intestinal tissue section showed that the intestinal wall was thinner, the intestinal villi were sparsely arranged and shorter, the microvilli were less numerous, and the striatum margin was damaged and incomplete.

We further investigated the effects of *try*$^{-/-}$ medaka on the gut microbiota of adult medaka using 16S rRNA high-throughput sequencing. The gut is a complex system, with obligate symbiosis and facultative parasitism between the microbes in the gut and the host, which play an indispensable role in growth and development, nutritional metabolism, immune function, and resistance to the invasion of pathogens (Wang et al., 2018). Microbial interactions in the gut are dynamic, not static. Studies have shown that factors such as environment, nutrient levels, and growth stage can affect the intestinal microbial composition of fish (Roeselers et al., 2011; Blaut, 2015). Here, alpha diversity analysis showed that the diversity of gut microbiota is significantly reduced in *try*$^{-/-}$ medaka. This suggests that damage to the intestinal structure will affect the colonization of intestinal flora and then lead to a reduction in diversity. At the phylum level, although Proteobacteria and Firmicutes are the dominant phyla of *try*$^{-/-}$ and WT medaka, they have different composition ratios. Compared with the WT, the proportion of Proteobacteria in *try*$^{-/-}$ medaka is lower, while that of Firmicutes is higher. Studies have shown that the intestinal flora of fish mainly consists of Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes, and Proteobacteria is the most important flora (Kormas et al., 2014). Firmicutes can effectively obtain energy from substrates, resulting in increased fat content. A large number of studies have shown that increased Firmicutes abundance is related to body weight and fat deposition (Zhang et al., 2013; Fujisawa et al., 2019). Therefore, changes in the diversity of *try*$^{-/-}$ medaka intestinal flora may affect the amino acid metabolism and lipid metabolism pathways and thus affect the growth and development of fish.

5 Conclusions

In this study, we established *try* and *amy* knockout medaka using CRISPR/Cas9 system. Our results demonstrate that *try* is indispensable for medaka larvae and continuously affects the growth, nitrogen metabolism, and intestinal development of medaka.

Data availability statement

All data and materials are available from the corresponding author upon request.

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Author contributions

Xiaodan JIA drafted the manuscript, designed the experiment, and performed the experiment. Shulin TANG drafted the manuscript, designed the experiment, and participated in some experiments. Hexiong FENG carried out the gene knockout. Dimei XU and Chenyuan ZHU participated in some experiments. Ke LU checked and revised the manuscript. Xufang LIANG designed the experiment and checked the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Xiaodan JIA, Shulin TANG, Hexiong FENG, Dimei XU, Chenyuan ZHU, Ke LU, and Xufang LIANG declare that they have no conflicts of interest.

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University (approval number: HZAUFI-2020-0042).

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Supplementary information

Tables S1 and S2