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RESEARCH ARTICLE

Effects of a *Lactobacillus* Mixture on Florfenicol Disposition, CYP3A Activity and Gut Microbiota in Nile Tilapia (*Oreochromis niloticus*)

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ABSTRACT

This study investigated the effects of *Lactobacillus delbrueckii* and *Lacticaseibacillus rhamnosus* (LD+LR) on the pharmacokinetics (PK) and withdrawal time (WDT) of florfenicol (FF) and the relationship of these effects to the gut microbiota in Nile tilapia. After 10 days of LD+LR or saline administration, fish received either a single dose of FF at 10 mg/kg body weight for PK analysis or five consecutive days of medication for WDT assessment. In the PK study, pretreatment with LD+LR reduced the elimination half-life ($t_{1/2\beta}$) by 5.1 hours, decreased the maximum serum concentration (C_{max}) by 40%, decreased the area under the concentration–time curve (AUC) by 49%, and increased clearance (CL/F) by 2.1-fold compared with controls. Under multiple-dose conditions, LD+LR pretreatment increased florfenicol amine (FFA) concentrations and FFA/FF ratios and reduced the WDT by two days. Next-generation sequencing of the gut microbiota revealed that LD+LR administration increased overall microbial richness and the relative abundance of Bacilli, coinciding with upregulation of the *Cyp3A40* gene and enhanced hepatic CYP3A activity. The results suggest that the *Lactobacilli* mixture may influence FF disposition, potentially in part involving microbiota-associated regulation of CYP3A activity, which may warrant consideration in dosage optimization.

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INTRODUCTION

Probiotic lactic acid bacteria (LAB) can offer various benefits to hosts by modifying intestinal microbiota, improving feed efficiency, and strengthening immune defenses against pathogens in aquaculture (El-Saadony *et al*., 2021). Among these, *Lactobacillus delbrueckii* and *Lacticaseibacillus rhamnosus* stand out as two of the five top probiotic species among 47 *Lactobacillus* strains in humans (Jacobsen *et al*., 1999). When used as feed additives, *L. delbrueckii* and *L. rhamnosus* can also provide significant benefits to fish. A study has shown that seabreams fed *L. delbrueckii* exhibit increased phagocytic and cytotoxic activity (Salinas *et al*., 2005). Further research suggests that supplementing fish diets with *L. rhamnosus* and *Lactococcus lactis* can enhance growth, modify gut microflora, and provide protection against *Streptococcus agalactiae* in Nile tilapia (Xia *et al*., 2018). Although probiotic LAB supplements clearly benefit fish health and immunity, antibiotic therapy is still necessary to control bacterial infections. Therefore, the use of antibiotics in probiotic-fed fish raises concerns about their interactions and potential effects on antibiotic effectiveness and safety (Hsieh *et al*., 2025; Wu *et al*., 2026). In mammals, research shows that probiotic-gut microbiota interactions can change the pharmacokinetics (PK) and pharmacodynamics (PD) of orally administered drugs, consequently influencing their therapeutic effectiveness

(Zhao *et al*., 2023). In fish, intestinal microflora is also important for various biological processes, including physiology, immunity and pathogen defense, making them critical for overall fish health (Madhulika *et al*., 2025). However, research on how probiotics affect the gut microbiota and drug metabolism in fish remains limited.

Florfenicol (FF) is one of the few bacteriostatic antibiotics that are approved for use in aquaculture (Anonymous, 2025). The European Medicines Agency (EMA) suggests a dosage of 10mg/kg of body weight per day for 10 days (EMA, 2000), and Taiwan's Ministry of Agriculture also recommends 10mg/kg daily for 5 days. In fish, florfenicol's main metabolite is florfenicol amine (FFA), which acts as the key marker residue for monitoring tissue depletion (Rairat *et al*., 2023). Different regulatory agencies have varying standards for what constitutes an acceptable marker residue for maximum residue limit (MRL) compliance, but most set a limit of 1μg/g in muscle-skin tissue based on a natural proportion. Depending on the authority, the marker residue might be considered as the FF parent drug in Taiwan or as the combined total of FF and its metabolites, expressed as FFA equivalents, in the EU (European Commission, 2025).

Cytochrome P450 (CYP) isozymes and/or the transporter P-glycoprotein (P-gp/MDR1) are believed to play important roles in the biotransformation and disposition of florfenicol (Liu *et al*., 2012). A previous study suggested that CYP3A29 could be a crucial enzyme involved in FF metabolism in pig liver (Xu *et al*., 2022). Similarly, CYP3A but not CYP1A is implicated in FF metabolism in chickens (Wang *et al*., 2018). A recent report also indicated that CYP isozymes and P-gp/MDR1 may be important in the metabolism and excretion of FF in Nile tilapia (Wu *et al*., 2026).

This study aimed to elucidate the effects of a mixture of *L. delbrueckii* and *L. rhamnosus* (LD+LR) on the pharmacokinetics, tissue deposition, and withdrawal time (WDT) of FF in Nile tilapia, a commercially important aquaculture species. The investigation also included assessments of changes in intestinal microbiota, transcriptional levels of *Cyp1A*, *Cyp3A40* and *P-gp/MDR1* genes, as well as hepatic CYP-mediated enzyme activities. These findings may inform antibiotic administration strategies for fish farms that supplement with probiotics.

MATERIALS AND METHODS

Experimental fish and probiotics: Seventy-eight Nile tilapia (300-350g) were reared at 25°C with dissolved oxygen ≥5.0 ppm and fed commercial pellets at 2% of their biomass daily. In the LD+LR-treated group, 39 fish received 0.1mL of the LD+LR mixture (containing 5×10^8 CFU of *L. delbrueckii* BCRC 14008 and 5×10^8 CFU of *L. rhamnosus* ATCC 53103) daily via oral gavage for 10 days, while another 39 fish received 0.1mL of saline daily as the control group. The Institutional Animal Care and Use Committee at National Chung Hsing University approved the studies (IACUC No.113-136).

Effect of LD+LR administration on FF pharmacokinetics (Trial 1): On the 11th day (one day after probiotic administration), six LD+LR-treated fish and six saline-treated fish were weighed, then gently given an FF solution by oral gavage at 10mg/kg of body weight. About 0.4mL blood samples were harvested from each fish at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 60 h after FF administration, following the protocol described by Wu *et al*. (2026).

Serum FF and FFA concentrations were quantified using the HPLC-FLD method described by Wu *et al*. (2026). Calibration curves were used to quantify FF and FFA, with validation data in Table S1. PK parameters were characterized using a two-compartment model with a 1/C weighting scheme, implemented in the PKSolver V2.0 program (Zhang *et al*., 2010).

Effect of LD+LR administration on the tissue depletion of FF and FFA (Trial 2): Twenty-eight LD+LR-treated fish and 28 saline-treated fish were weighed and then gavaged with FF solution at a dose of 10 mg/kg/day for five days. Fish's serum, muscle-skin tissue, liver, proximal intestine, and bile were harvested at days 1, 3, 5, and 8 after medication (7 fish/group/time point). FF and FFA concentrations in various tissues were quantified using the HPLC-FLD method by Wu *et al*. (2026). Calibration curves were used to quantify FF and FFA, with validation data in Table S1.

The sum of FF and FFA concentrations in muscle-skin served as the marker residue for determining the withdrawal time by using WT1.4 software, in accordance with EMA (2018) guidelines: WDT is the time when the 95% one-sided tolerance limit, with 95% confidence, falls below the MRL of 1μg/g.

Effect of LD+LR administration on gut microbiota (Trial 3): Five probiotic-treated fish and five saline-treated fish were euthanized and their intestines and livers were harvested. Total DNA was extracted from 200mg of intestinal contents from the midgut and hindgut. The hypervariable V3-V4 region of the prokaryotic 16S rRNA gene was amplified using the protocol described by Pan *et al*. (2023), and the Next-Generation Sequencing was performed by Tri-I Biotech Inc. (Taiwan).

Reverse transcription-Quantitative PCR analysis: Transcription levels of the *β-actin*, *P-gp/MDR1, Cyp1A* and *Cyp3A40* genes in tilapia livers and intestines were measured by RT-qPCR, using the method described by Wu *et al*. (2026). Specific primers for detecting the above genes are listed in Table S2.

Cytochrome P450 activities: Hepatic microsomal fractions from the fish in Trials 2 and 3 were collected. The CYP1A and CYP3A activities (pmol/min/mg) in hepatic microsomes were determined by using the ethoxy resorufin-O-demethylase (EROD) and BFC-O-debenzyloxylase (BFCOD) assays (Wu *et al*., 2026).

Statistical analysis: All results are expressed as mean ± standard deviation. Statistical differences in drug concentrations and PK parameters between the LD+LR- and saline-treated groups were assessed using an independent t-test ($P<0.05$). Significant differences in relative mRNA levels and CYP enzyme activities were determined by One-way ANOVA.

Table S1: Validation data of the HPLC-FLD method for FF and FFA analysis in different tissues of Nile tilapia (n=5).

Tissues	Target	Linear range (μg/g or mL)	r^2	LOQ (ng/g or mL)	LOD (ng/g or mL)	Recovery (%)	Precision (%)
Serum	FF	0.1-20	0.997	14	6	94-98%	3.9-7.5%
	FFA	0.1-20	0.998	40	13	76-86 %	4.2-8.8%
Muscle/skin	FF	0.1-20	0.998	62	20	79-95%	1.4-7.4%
	FFA	0.1-20	0.997	63	21	66-87%	1.6-8.9%
Intestine	FF	0.5-20	0.999	158	52	69-96%	2.2-9.8%
	FFA	0.5-20	0.998	93	30	43-58%	3.7-15.2%
Bile	FF	0.5-50	0.999	76	25	91-99%	5.3-10.3%
	FFA	0.5-50	0.997	133	44	67-82%	5.8-12.2%
Liver	FF	0.5-20	0.998	268	88	82-88%	3.9-4.7%
	FFA	0.5-20	0.998	239	78	74-85%	5.5-10.8%

Abbreviations: r^2, coefficient of determination; LOQ, limit of quantification; LOD, limit of detection.

Table S2: Primer sequences of genes of interest from *Oreochromis niloticus*.

=	Primer sequence (5'-3')	Amplicon length	GenBank number
Cyp1A	F: CTGACCTGTACAGCTTTCGC R: GCACATGAGTACTCTGGGGT	154	FJ389118.2
Cyp3A40	F: CACCTCTGGGAGATTGAAAGAG R: TGCTGGTTACCACATCCATAC	149	XM_019360130.2
P-gp/MDR1	F: CATCCTCTTTGCCACCACCATC R: CTCTGCTTTTGTCCTCCGCTC	183	AB699096.1
β-actin	F: TCAGGGTGTGATGGTGGGTATG R: CTCAGCTCGTTGTAGAAGGTGT	165	KJ126772.1

RESULTS

LD+LR affected the pharmacokinetics of FF: The serum FF concentrations in the LD+LR-treated group were significantly lower than those in the saline-treated group at 1, 2, 4, 8, 12, 24, 36, 48, and 60 hours after a single oral administration ($P<0.05$; Fig. 1). The maximum serum concentration (C_{max}) in the LD+LR-treated group (11.77 ± 4.07μg/mL) was notably lower than that of the saline-treated group (19.76 ± 2.67μg/mL; $P<0.01$), while the time to reach C_{max} (T_{max}) was kept similar. The LD+LR-treated group also exhibited a substantial decline in the area under the serum concentration-time curve (AUC; 132.2 ± 34.7h·μg/mL) compared with the saline-treated group (261.7 ± 48.5h·μg/mL; $P<0.001$; Table 1). Meanwhile, fish pretreated with LD+LR demonstrated a 2.1-fold increase in clearance (CL/F) and a 5.1-h reduction in elimination half-life ($t_{1/2\beta}$), indicating faster drug elimination ($P<0.01$). The mean residence time (MRT) was significantly reduced by 6.1 h (34%; $P<0.05$).

LD+LR affected tissue residues and withdrawal time: The saline-treated fish, the LD+LR-treated group showed significantly lower FF concentrations in the liver (−53%), muscle/skin (−42%) and serum (−26%), whereas there were no statistical differences in the bile or intestinal tissues on Day one after the multiple-dose FF treatments (Fig. 2A). In contrast, FFA levels were consistently higher in the LD+LR-treated group (1.6-2.3 folds, $P<0.05$), while maintaining the same tissue distribution rank order (bile > liver > proximal intestine > muscle-skin > serum (Fig. 2B). Consequently, FFA/FF ratios were 1.7-fold (serum) to 2.9-fold (muscle-skin) higher in the LD+LR-treated group across all tissues examined ($P<0.05$; Fig. 2C).

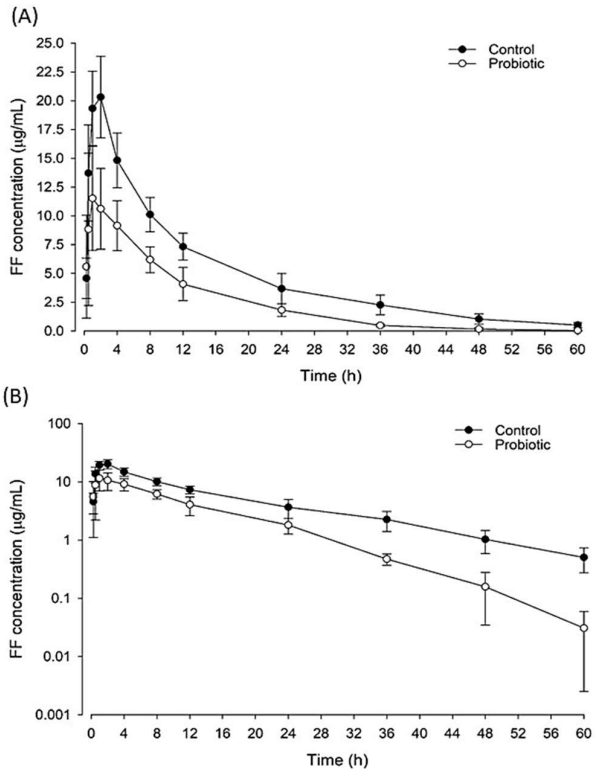

Fig. 1: (A) Linear and (B) semi-logarithmic plots of the serum concentration-time profile of florfenicol (FF) in Nile tilapia pretreated with LD+LR mixture (probiotic group) or saline (control group) following a single oral administration of FF (10mg/kg) at 25°C. The data are shown as Mean±SD (n=6).

Table 1: Pharmacokinetic parameters (Mean ± SD) of florfenicol in Nile tilapia pretreated with LD+LR mixture or saline following single oral administration at 10mg/kg (n=6).

PK parameters	Saline-treated	LD+LR-treated
Ka (l/h)	0.79±0.18	1.45±1.21
$t_{1/2ka}$ (h)	0.93±0.24	0.99±0.71
α (l/h)	0.52±0.21	0.43±0.10
$t_{1/2\alpha}$ (h)	1.63±0.82	1.71±0.41
β (l/h)	0.054±0.018	0.082±0.013*
$t_{1/2\beta}$ (h)	13.82±3.21	8.72±1.62**
K_{12} (l/h)	0.24±0.14	0.11±0.04
K_{21} (l/h)	0.19±0.05	0.26±0.11
K_{10} (l/h)	0.14±0.04	0.14±0.02
T_{max} (h)	2.05±0.34	2.19±1.06
C_{max} (μg/mL)	19.76±2.67	11.77±4.07**
AUC (h·μg/mL)	261.7±48.5	132.2±34.7***
Vz/F (L/kg)	0.69±0.11	1.01±0.34
Vc/F (L/kg)	0.27±0.05	0.58±0.22
Vss/F (L/kg)	0.58±0.1	0.84±0.27
CL/F (L/kg/h)	0.037±0.009	0.079±0.021**
MRT (h)	17.93±3.97	11.86±1.79*

Note: There were significant differences in the parameters between the control group and the probiotic group, as indicated by *P<0.05, **P<0.01, and ***P<0.001.

FF concentrations in muscle-skin were consistently and markedly lower in the LD+LR-treated group than in the saline-treated group across all sampling days after the multiple-dose FF administration (Table 2). In contrast, FFA concentrations tended to be higher in the LD+LR-treated group, leading to markedly increased FFA/FF ratios. Despite these opposing trends for FF and FFA, the total residue (FF+FFA) levels were generally comparable between treatment groups at most time points. Based on the EMA (2018) guideline and a MRL of 1μg/g, FF+FFA

residues fell below the MRL by Day 6 (5.62 days) in the LD+LR group but not until Day 8 (7.45 days) in the controls (Fig. 3). Table 3 indicated that serum FF, FFA and FFA/FF values followed the same overall pattern observed in muscle-skin; lower FF but higher FFA and FFA/FF ratio in the LD+LR-treated group, while the total serum residues (FF+FFA) remained similar between treatments. The minimum serum concentration of FF at steady state ($C_{min(ss)}$) in the LD+LR-treated group was 3.44 ± 1.11µg/mL, notably lower than in the saline-treated group (4.69 ± 0.99µg/mL; P<0.05). Note that when WDT was calculated using serum rather than muscle-skin depletion, it was 10 days in the control group and 9 days in the probiotic group (data not shown).

LD+LR altered expression of Cyp1A and Cyp3A40 gene: The transcription levels of *Cyp1A*, *Cyp3A40* and *P-gp/MDR1* genes in the livers and proximal intestines were determined (Fig. 4). The hepatic *Cyp1A* mRNA was markedly downregulated after LD+LR and/or FF treatment compared with the saline-treated group. Conversely, hepatic *Cyp3A40* mRNA was significantly upregulated in the LD+LR and LD+LR/FF group, but was reduced in the FF-treated group relative to the saline-treated group (Fig. 4A). Similar results were seen in the proximal intestinal tissues (Fig. 4B). In contrast, the treatments with LD+LR and/or FF did not change *P-gp/MDR1* gene expression (Fig. 4C). Taken together, the LD+LR supplement noticeably affected *Cyp1A* and *Cyp3A40* mRNA levels in the liver and intestine.

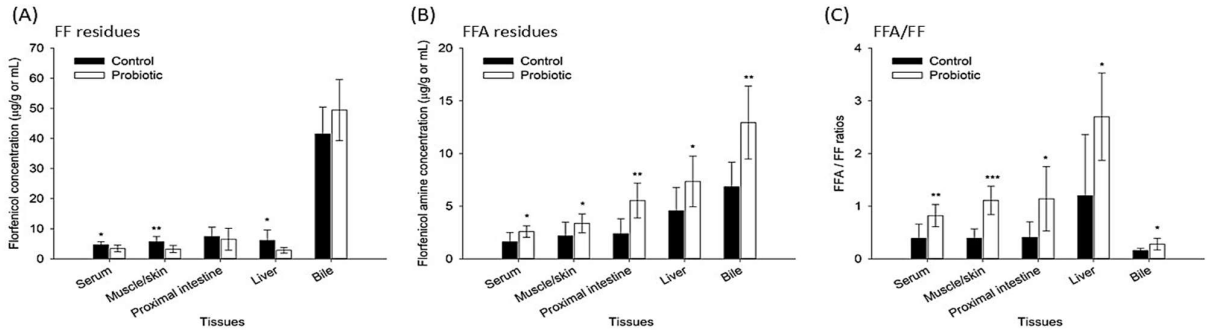

Fig. 2: (A) Florfenicol concentrations, (B) florfenicol amine levels, and (C) FFA/FF ratios in various tissues of Nile tilapia pretreated with the LD+LR mixture (probiotic) or saline (control) on Day 1 after 5-day oral administration of FF (10 mg/kg/day, n=7) at 25°C. Significant differences are indicated by *P<0.05, **P<0.01, and ***P<0.001.

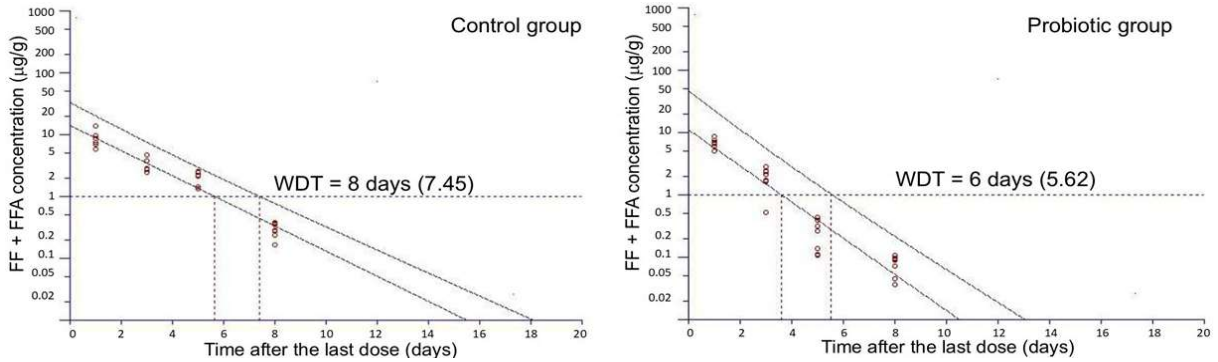

Fig. 3: Plots of WDT calculation showing total residues of florfenicol (FF) and florfenicol amine (FFA) in the muscle-skin of Nile tilapia pretreated with either normal saline (control) or LD+LR mixture (probiotic). These fish received oral doses of FF (10mg/kg daily) for five days (n=7 per group per time point). The lower line represents the linear regression, while the upper line marks the 95% upper tolerance limits with 95% confidence.

Table 2: Concentrations of florfenicol and florfenicol amine (FF and FFA, µg/mL) in the muscle-skin of Nile tilapia pretreated with LD+LR mixture or saline following oral administration of FF (10 mg/kg/day) at 25°C for 5 days (n=7/group/time point).

Time after the last dose	FF		FFA		FFA/FF		FF+FFA	
	Saline	LD+LR	Saline	LD+LR	Saline	LD+LR	Saline	LD+LR
Day 1	5.65±1.81**	3.27±1.18	2.17±1.31	3.37±0.9*	0.39±0.18	1.11±0.27**	7.81±2.69	6.64±1.93
Day 3	1.49±0.38**	0.39±0.23	1.32±0.71	1.53±1.03	0.99±0.61	4.41±3.42*	2.82±0.87	1.92±1.16
Day 5	0.99±0.54	< LOQ	0.93±0.17**	0.25±0.16	1.67±1.42	NA	1.92±0.51**	0.25±0.16
Day 8	0.19±0.06	< LOQ	0.09±0.04	0.07±0.02	0.59±0.26	NA	0.28±0.07**	0.07±0.02

Note: There were significant differences in the concentrations between both groups, as indicated by *P<0.05 and **P<0.01. < LOQ, below the limit of quantification; NA, not applicable.

Table 3: Concentrations of florfenicol and florfenicol amine (FF and FFA, µg/mL) in the serum of Nile tilapia pretreated with LD+LR mixture or saline following oral administration of FF (10 mg/kg/day) at 25°C for 5 days (n=7/group/time point).

Time after the last dose	FF		FFA		FFA/FF		FF+FFA	
	Saline	LD+LR	Saline	LD+LR	Saline	LD+LR	Saline	LD+LR
Day 1	4.69±0.99	3.44±1.11*	1.62±0.88	2.59±0.54*	0.39±0.27	0.82±0.21**	6.31±0.94	6.04±1.55
Day 3	0.96±0.56	0.45±0.26*	0.59±0.39	1.43±0.73**	0.87±0.75	4.17±2.49**	1.55±0.49	1.87±0.94
Day 5	0.11±0.02	0.09±0.05	0.34±0.18	0.58±0.15*	3.46±1.86	10.6±8.7*	0.45±0.19	0.67±0.18*
Day 8	0.11±0.04	0.09±0.05	0.43±0.04	0.43±0.07	4.46±1.57	6.67±3.3	0.54±0.05	0.52±0.09

Note: There were significant differences in the concentrations between both groups, as indicated by *P<0.05 and **P<0.01

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LD+LR altered hepatic CYP1A and CYP3A enzyme activities: Compared with the saline-treated group, the CYP1A-mediated EROD activity was substantially lower in the LD+LR-treated group (−42.7%), the FF-treated group (−53.2%), and the LD+LR/FF group (−35.4%; Fig. 5). However, CYP3A-mediated BFCOD activity was significantly increased by 1.4-fold in the LD+LR-treated group in contrast to the saline-treated group (23.46 vs. 17.18 pmol/mg/min), whereas it markedly decreased by 40% in the FF-treated group (10.26 pmol/mg/min). Additionally, the BFCOD activity in the LD+LR/FF group (20.86 pmol/mg/min) was similar to that in the saline-treated group but approximately twofold higher than in the FF-treated group.

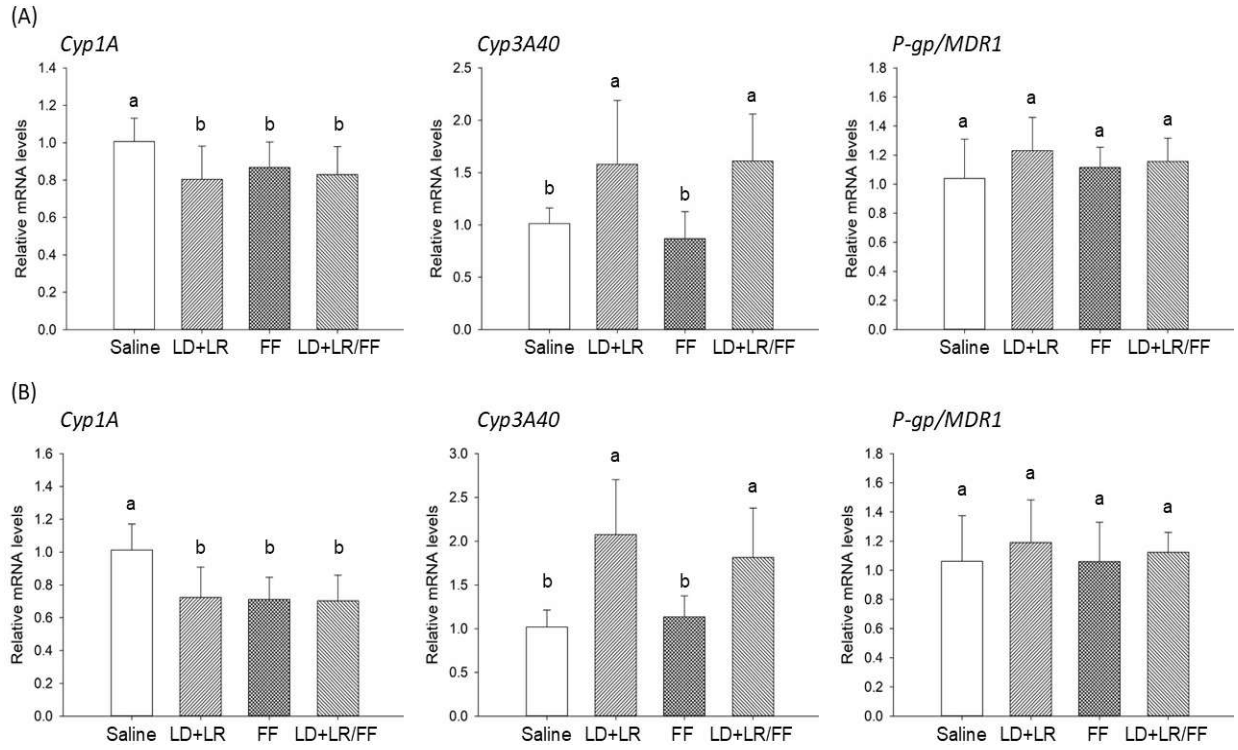

Fig. 4: Relative mRNA levels of *Cyp1A*, *Cyp3A40* and *P-gp/MDR1* genes in the liver (A) and proximal intestine (B) of Nile tilapia across different treatments. The saline and LD+LR groups included fish treated only with saline or the probiotic for 10 days (n=5). The FF and LD+LR/FF groups consisted of fish pretreated with saline or the LD+LR mixture for 10 days, then given multiple doses of FF for 5 days (n=7). Significant differences among various groups are indicated by different lowercase letters (P<0.05).

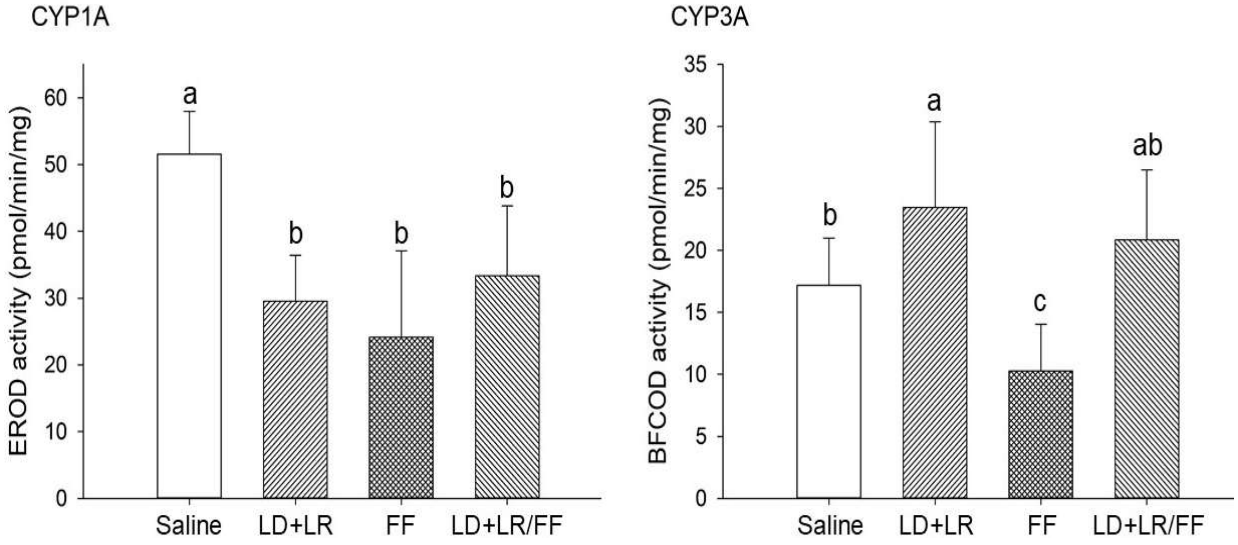

Fig. 5: Hepatic cytochrome P450 (CYP) 1A and 3A enzyme activities in Nile tilapia. The saline and LD+LR groups consisted of fish treated with only saline or LD+LR mixture for 10 days (n=5). The FF and LD+LR/FF groups consisted of fish that were pretreated with saline or LD+LR mixture for 10 days, then given multiple doses of FF for 5 days (n=7). Significant differences among various treatments are indicated by different lowercase letters (P<0.05).

LD+LR modified gut microbiota: Next-generation sequencing of validated reads from LD+LR-treated and saline-treated fish (n = 5/group) identified 367 amplicon sequence variants (ASVs) spanning 16 phyla and 144 genera. Venn analysis showed that 60.5% of ASVs were unique to the LD+LR-treated group, compared with only 23.7% in the saline-treated group, indicating greater species richness following LD+LR treatment (Fig. 6A). The alpha-diversity analysis (observed species and the Chao1 index) also indicated high richness in the LD+LR-treated group, but no significant difference was observed (Fig. 6B). The non-metric multidimensional scaling (NMDS) (stress = 0.081, highly reliable when <0.2; Dexter *et al*., 2018) demonstrated clear separation between groups (Fig. 6C). Bacterial community compositions of saline-treated fish clustered closely, while those of LD+LR-treated fish showed more variation between individuals. The phylum-level microbiota composition was dominated by *Fusobacteriota*, *Bacteroidota*, *Proteobacteria*, and *Firmicutes* in both groups. *Fusobacteriota* was the most abundant phylum in saline-treated (64.4%) and LD+LR-treated fish (66.1%, Fig. 6D). Compared with saline-treated fish, LD+LR supplementation substantially reduced *Bacteroidota* abundance (5.1% vs. 13.5%; P<0.05) and increased *Actinobacteriota* by 7.3-fold (2.2% vs. 0.3%; P<0.05). The genus-level taxonomic composition is shown in Fig. 7A; *Cetobacterium* predominated in both groups (~65%). In saline-treated fish, *Romboutsia* (9.4%), *Plesiomonas* (9%), *Macellibacteroides* (4.7%) and *Akkermansia* (1.7%) each exceeded 1% relative abundance, whereas LD+LR-treated fish were enriched in *Plesiomonas* (13%), *Romboutsia* (12.2%), *Macellibacteroides* (2.2%) and *Mycobacterium* (1.8%), indicating mild shifts in dominant genera.

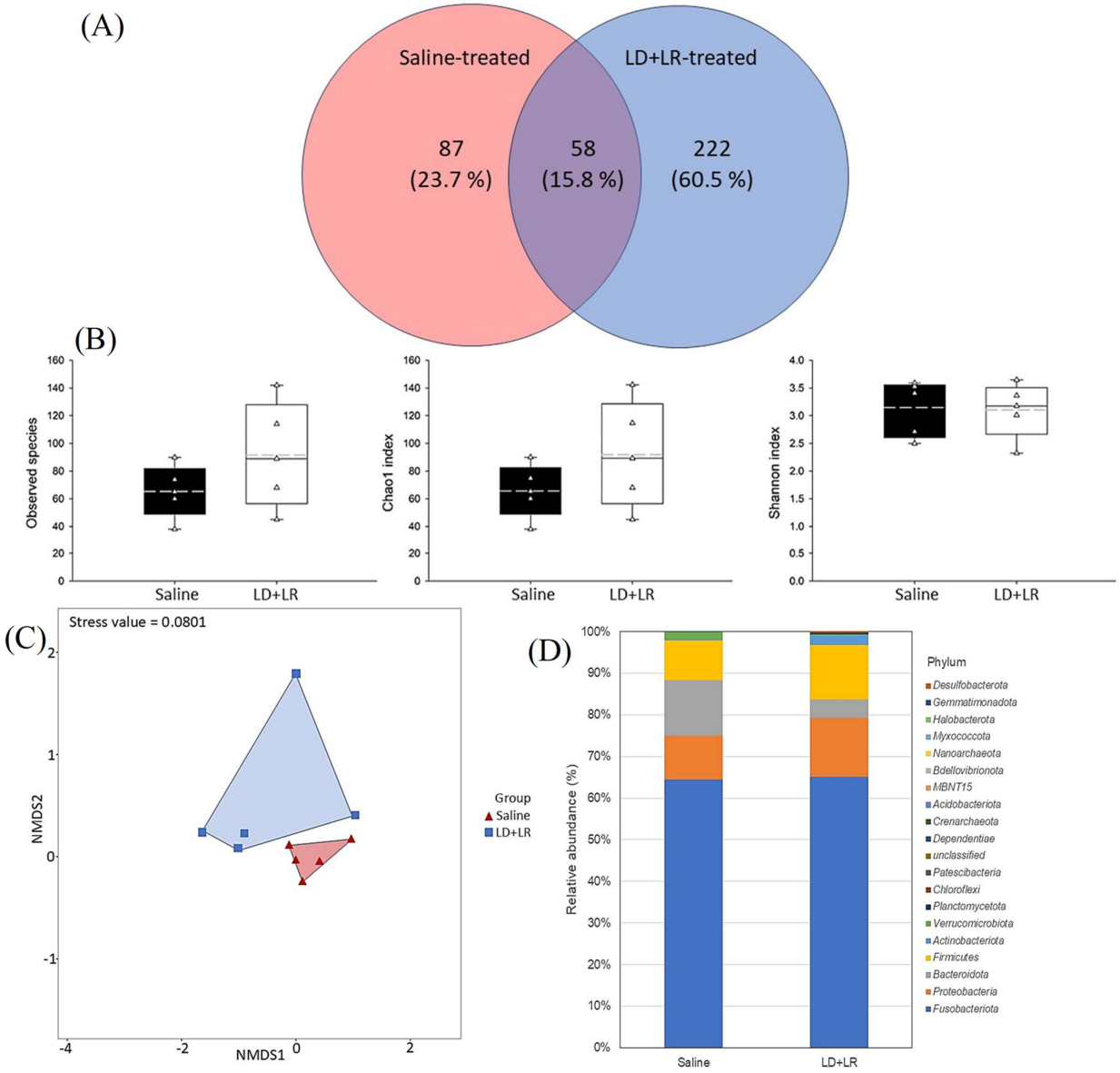

Fig. 6: Effects of treatments with LD+LR or saline on the gut microbiota of Nile tilapia. (A) Venn diagram analysis of microbiota with ASV; (B) Alpha diversity analysis includes observed species count, the Chao1 index, and the Shannon index; (C) Non-metric multidimensional scaling analysis showing the beta diversity of the two groups; (D) Mean relative abundance at the phylum level (n=5).

The linear discriminant analysis (LDA) identified one biomarker taxon enriched in the LD+LR-treated group and four enriched in the saline-treated group (LDA≥3.5, P<0.01; Fig. 7C). The LD+LR-associated biomarker was classified as *Bacilli* (LDA score = 3.66, P = 0.009), consistent with the presence of *Bacillus* (3 of 5 probiotic-treated fish) and *Lactobacillus* (2 of 5 probiotic-treated fish) observed exclusively in the heatmap (Fig. 7B). In comparison, *Bacteroides* (3 of 5 control fish), *Parabacteroides* (3 of 5 control fish), *Akkermansia* (4 of 5 control fish), and *Edwardsiella* (5 of 5 control fish) were enriched in saline-treated group (Fig. 7B). Overall, LD+LR supplementation altered tilapia microbial composition and increased the relative abundance of *Bacilli*. It should be noted that the microbiota analysis was primarily conducted to characterize compositional differences between treatment groups rather than to identify differential taxa across multiple conditions. Therefore, emphasis was placed on consistent patterns observed across diversity indices, NMDS ordination, and LDA-based biomarker analysis (LDA≥3.5), while individual taxonomic comparisons were interpreted cautiously.

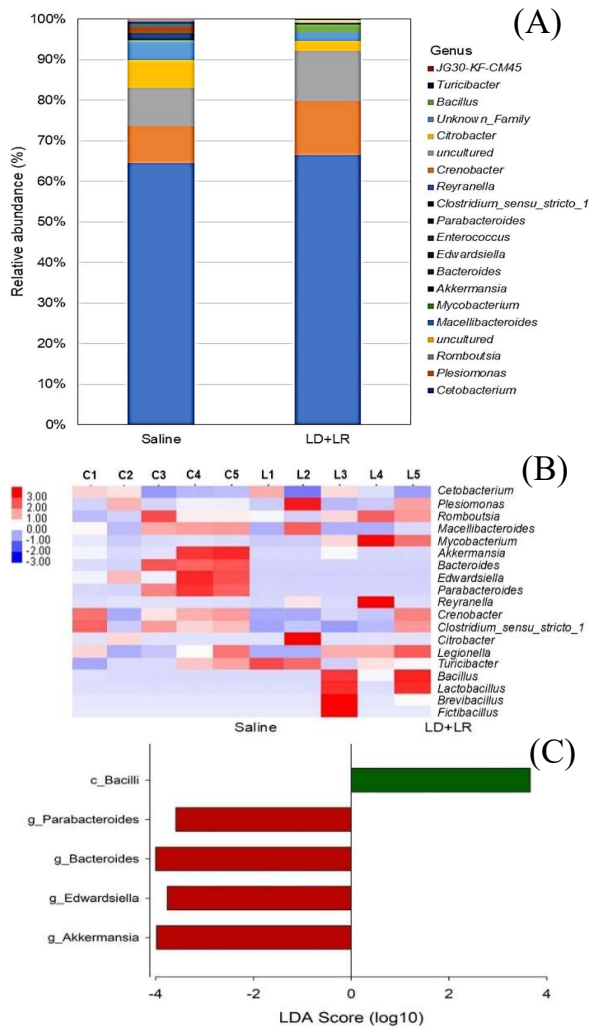

Fig. 7: Impact of treatment with LD+LR or saline on the gut microbiota of Nile tilapia. (A) Mean relative abundance at the genus level. (B) Genus-level heatmap of the core microbiota (C1~5: saline-treated fish; L1~5: LD+LR-treated fish); (C) Linear discriminant analysis for the two groups. Horizontal bars indicate the effect size for each taxon. Values are shown as the mean (n=5).

DISCUSSION

To the authors' knowledge, although probiotic supplementation has been widely investigated for its effects on intestinal microbiota in aquatic species, its influence on the pharmacokinetics of antibiotics remains largely unexplored. Given the increasing use of probiotics in aquaculture, understanding their potential interaction with antibiotic disposition is of practical importance. The present study therefore evaluated the effects of *L. delbrueckii* and *L. rhamnosus* (LD+LR) supplementation on the pharmacokinetics and withdrawal time (WDT) of florfenicol (FF) in Nile tilapia, with additional assessment of gene expression, enzyme activity, and gut microbiota to explore possible underlying mechanisms.

The concentration–time profiles of FF were better described by a two-compartment model, as indicated by visual inspection, lower Akaike's information criterion (AIC) values, and improved precision of parameter estimates (Gabrielsson and Weiner, 1999). Specifically, the AIC values for the two-compartment model (control: 19.96; probiotic: 16.45) were lower than those for the one-compartment model (control: 23.15; probiotic: 18.16). In addition, the coefficients of variation for major PK parameters were generally lower under the two-compartment model in both groups, indicating improved parameter stability. Therefore, the two-compartment model was considered more appropriate and was applied for subsequent analysis. Inter-individual variation was comparable between groups and, in the context of residue depletion, is inherently accounted for in WDT estimation based on the 95% one-sided tolerance limit with 95% confidence (EMA, 2018). Accordingly, the observed variability is unlikely to compromise the reliability of the depletion assessment.

Compared with saline-treated fish, LD+LR supplementation markedly changed the pharmacokinetics of FF, as evidenced by significantly lower serum and tissue concentrations, resulting in reduced C_{max} and AUC. The concomitant 2.1-fold increase in CL/F, together with a shortened elimination half-life, indicates accelerated elimination of FF in LD+LR-treated fish. These data demonstrate that pretreatment with LD+LR substantially changes FF disposition in Nile tilapia and are consistent with reports in terrestrial animals showing that probiotics can alter drug pharmacokinetics by modulating host metabolic processes (Liu *et al*., 2021).

A recent study indicates that LAB supplementation reduced systemic exposure and WDT by decreasing intestinal FF absorption in Asian seabass (Hsieh *et al*., 2025). To determine whether a similar mechanism contributed to the observed changes in PK in Nile tilapia, FF and its metabolite FFA were quantified in the intestinal tissue. Our results showed that FF concentrations in the intestine were higher than in the liver, muscle-skin, and serum in the LD+LR-treated group, but did not differ considerably from those in controls, indicating that LD+LR supplementation did not reduce intestinal FF uptake. This conclusion is further supported by RT-qPCR data showing that LD+LR treatment does not affect the expression of P-glycoprotein, a key efflux transporter that limits the absorption of xenobiotics across intestinal epithelia (Xie *et al*., 2016). These results show that reduced intestinal

absorption is unlikely to be the primary driver of the reduced systemic FF exposure observed in LD+LR-treated tilapia. Although intestinal FF levels were unchanged, intestinal FFA concentrations and FFA/FF ratios were markedly higher in the LD+LR-treated fish. This finding indicates that FF may undergo increased intestinal metabolism, possibly because of probiotic-induced activation of local CYP3A enzymes, rather than decreased absorption.

Consistent with enhanced metabolic conversion, significantly higher FFA levels and FFA/FF ratios were also exhibited in bile, liver, serum, and muscle-skin of LD+LR-treated fish. These results show that LD+LR supplementation may promote systemic biotransformation of FF. In mammals and poultry, FF metabolism is known to be intensely dependent on hepatic CYP3A enzymes (Wang *et al*., 2018; Wang *et al*., 2021; Xu *et al*., 2022). Accordingly, the effect of LD+LR supplementation on CYP expression and activity in tilapia was investigated. RT-qPCR analysis showed that LD+LR treatment substantially upregulated *Cyp3A40* expression in both liver and proximal intestine. These transcriptional changes were accompanied by a considerable increase in CYP3A enzyme activity, as measured by the BFCOD assay. Importantly, elevated CYP3A activity remained even after repeated FF administration, indicating that probiotic-induced enzyme activation was not overridden by drug exposure. Collectively, these findings suggest that LD+LR supplementation may reduce systemic FF exposure, potentially in association with increased *Cyp3A40* mRNA levels and enhanced CYP3A-mediated metabolism; however, the functional role of CYP3A40 isoform in tilapia has yet to be fully characterized and warrants further study. These observations closely parallel findings in land animals (Liu *et al*., 2021). The present study expands these probiotic-CYP3A interactions to fish and provides mechanistic evidence linking probiotic supplementation to altered antibiotic PK via metabolic induction. Interestingly, both *Cyp1A* expression and CYP1A-mediated EROD activity were obviously reduced in the LD+LR-treated group, suggesting selective downregulation of this pathway. However, the contribution of CYP1A to FF metabolism remains unclear, and its downregulation is unlikely to explain the increased formation of FFA observed in this study. Nevertheless, our findings are among the very few reports showing that probiotic supplementation exerts enzyme-specific regulatory effects rather than a generalized induction of xenobiotic metabolism in the teleost.

Interestingly, hepatic CYP1A and CYP3A activities were significantly lower in the FF-treated fish than in the controls, implying possible enzyme inhibition or downregulation by FF itself. Similar effects have been reported in broilers, where FF administration suppressed hepatic CYP1A1 expression (Han *et al*., 2021). Moreover, structurally related compounds, such as chloramphenicol, strongly inhibit hepatic CYP3A4 activity in humans (Park *et al*., 2003). Consistent with this, Wei *et al*. (2016) found that FF may enhance the antimicrobial or therapeutic effects of co-administered antibiotics, potentially by inhibiting drug-metabolizing enzymes. The results raise the possibility that FF directly suppresses CYP enzyme activity. Overall, these results suggest that LD+LR supplementation changes FF pharmacokinetics primarily by enhancing metabolic conversion rather than reducing intestinal absorption, with CYP3A activation playing a central role.

Three PK–PD indices are commonly used to evaluate antibiotic efficacy: T>MIC, C_{max}/MIC, and AUC/MIC. In our previous study, optimal dosing regimens for FF in Nile tilapia were determined using T>MIC as the PK–PD index, which is appropriate for time-dependent bacteriostatic agents such as FF (AliAbadi and Lees, 2000), based on PK parameters derived from single-dose studies (Rairat *et al*., 2019). In the present study, these PK parameters (Ka, β, and Vz/F) were applied to multiple-dose simulations to estimate the dosage required to maintain target exposure. Using a representative MIC value of 2μg/mL for common tilapia pathogens (Rairat *et al*., 2019), the predicted optimal dosage was approximately 1.85-fold higher in the LD+LR group (11.07 mg/kg/day) than in the control group (5.97 mg/kg/day; data not shown). In addition, AUC/MIC was evaluated, as it has been suggested as an appropriate PK–PD index for FF in certain fish pathogens (San Martín *et al*., 2019). At an MIC of 2μg/mL, the AUC/MIC values were 130.85 in the control group and 66.1 in the LD+LR group, indicating a reduced exposure in the probiotic-treated fish. Similarly, the minimum serum concentration at steady state ($C_{min(ss)}$) after multiple dosing was lower in the LD+LR group (3.44 ± 1.11μg/mL) than in controls (4.69 ± 0.99μg/mL; $P<0.05$). A comparable reduction in $C_{min(ss)}$ was also observed in our recent study using *Enterococcus faecium* (Wu *et al*., 2026), suggesting that the magnitude of probiotic–drug interaction may vary among strains. Taken together, these findings indicate that LD+LR supplementation reduces PK–PD indices associated with FF exposure. However, as $C_{min(ss)}$ remained above the reference MIC of 2μg/mL, the current dosing regimen may still achieve effective exposure under the conditions tested. The potential impact on therapeutic efficacy warrants further evaluation in dedicated PK–PD and infection-challenge studies.

From a food-safety standpoint, the faster metabolism and removal of FF in fish pretreated with LD+LR might provide some benefits. As per EMA (2018) guidelines, the sum of FF and FFA levels in muscle-skin declined below the MRL by Day 6 in the LD+LR-treated group, whereas it took until Day 8 in the saline-treated group. Similar trends in Nile tilapia showed administration of probiotic *E. faecium* shortened WDT from 7 to 6 days (Wu *et al*., 2026). Although the magnitude of WDT reduction observed here is mild, these outcomes warrant attention, as the effects may vary depending on antibiotic class, probiotic strain, and fish species.

Emerging evidence stresses the central role of the intestinal microbiota in changing the PK of orally administered drugs in mammals (Zhao *et al*., 2023). However, how LAB modulate intestinal microbial communities and, in turn, influence drug disposition in aquaculture species remains poorly understood. In this study, Next-Generation Sequencing of 16S rDNA was used to characterize LD+LR-induced alterations in the tilapia gut microbiota, with the aim of providing insights into potential microbiota-mediated mechanisms. First, Venn diagram analysis showed that the LD+LR supplement increased the number of gut microbiota species, although

there was no significant difference in the alpha-diversity index. The NMDS analysis also indicated differences in bacterial community composition between the two groups. Second, LD+LR administration notably increased the relative abundance of the phylum *Actinobacteriota,* which might improve growth in tilapia (Lubis *et al*., 2024). Compared with saline-treated controls, LD+LR administration mildly increases the relative abundances of *Cetobacterium* (65% vs. 64%) and *Romboutsia* (12% vs. 9%). *Cetobacterium* is a primary and beneficial intestinal bacterium in healthy farmed tilapia (Ofek *et al*., 2022; Zhang *et al*., 2022). *Romboutsia* can ferment carbohydrates into short-chain fatty acids (SCFAs), which can also improve growth performance (Wang *et al*., 2022; Huang *et al*., 2024). Third, LDA analysis identified the class *Bacilli* as a key microbial feature enriched by LD+LR supplementation (LDA = 3.66, P<0.01), with the genera *Bacillus* and *Lactobacillus* detected exclusively in some of the LD+LR-treated fish. Members of the class *Bacilli*, including *Bacillus*, *Enterococcus*, and *Lactobacillus*, are not only beneficial gut microbes but also known producers of bile salt hydrolase (BSH) (Kang *et al*., 2025). Luo *et al*. (2023) reported that oral administration of *Bacillus cereus* could modulate a gut microbiota–BSH–related pathway in Nile tilapia. BSH activity can convert primary bile acids into lithocholic acid, which activates the pregnane X receptor (PXR) and induces CYP3A expression (Klaassen and Cui, 2015). In the present study, LD+LR-treated fish exhibited elevated hepatic *Cyp3A40* mRNA levels, increased CYP3A enzymatic activity, and enrichment of *Bacilli* class in the gut microbiota. These concurrent changes may be consistent with involvement of a microbiota–bile acid–CYP3A–related pathway; however, further investigation, supported by more comprehensive fish-specific microbiota and metabolic reference datasets, will be required to clarify this potential linkage. In addition, LDA analysis indicated that the genera *Akkermansia*, *Bacteroides*, *Edwardsiella*, and *Parabacteroides* were notably enriched in saline-treated fish relative to the LD+LR-treated group. Although *Akkermansia, Bacteroides,* and *Parabacteroides* are core components of the human intestinal microbiota and contribute to host immune regulation and metabolic homeostasis (Cui *et al*., 2022; Rodrigues *et al*., 2022), their functional roles in the tilapia intestine remain unclear. Noticeably, LD+LR supplementation markedly reduced the abundance of *Edwardsiella*, a major fish pathogen, denoting a protective effect against pathogen colonization. Taken together, LD+LR supplementation modified the gut microbiota by altering bacterial community composition, selectively enriching *Bacilli*, and suppressing the potential colonization of pathogenic bacteria.

Conclusions: In summary, LD+LR pretreatment substantially altered the pharmacokinetics of florfenicol in Nile tilapia, resulting in reduced systemic exposure and a shortened withdrawal time. These changes were accompanied by increased CYP3A transcription and elevated CYP3A enzyme activity, together with consistently higher FFA levels and FFA/FF ratios across tissues, suggesting enhanced metabolic conversion of FF. At the same time, gut microbiota composition was modified, including enrichment of the class *Bacilli* and shifts in dominant taxa. While the relative contribution of intestinal absorption and metabolic processes cannot be fully distinguished, the combined evidence indicates that probiotic supplementation may influence both processes in a coordinated manner. The concurrent changes in microbiota composition and host metabolic activity further suggest a microbiota-associated modulation of drug disposition, although the specific pathways involved remain to be clarified. From a practical perspective, the reduced systemic and tissue drug levels may compromise therapeutic efficacy. Therefore, the potential interaction between probiotic supplementation and antibiotic pharmacokinetics should be considered when establishing dosing regimens in aquaculture.

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