Supplementation With Dietary Lacto-Sacc in Earthen Pond Water Reduced Nitrofuran Derivatives in Mud Crab (*Scylla Olivacea*)

Hasan M.J.[1,3*]; Munir M.B.[2*]; Asdari R.B.[1]; Supian A.S.A. B[2]; Akhter S.T.[3]; Rasel M.S.E.[3]; Haidar N.[3]; Reza M.F.[4]; Sarder L.[5]; Talukder S.[6]

[1]Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak,Malaysia
[2]Faculty of Agriculture, Universiti Islam Sultan Sharif Ali, Sinaut Campus, TB1741, Jalan Tutong, Tutong, Brunei Darussalam
[3]Quality Control Laboratory, Khulna, Department of Fisheries, Bangladesh
[4]District Fisheries Office, Meherpur, Department of Fisheries, Bangladesh
[5]Fish Inspection and Quality Control, Khulna, Department of Fisheries, Bangladesh
[6]Aleya Feeds Ltd. Gazipur, Bangladesh

***Corresponding Author:** Munir M.B*, Hasan M.J.
Email: hsjewel730@yahoo.com OR ruhinufo@yahoo.com

Abstract: Antibiotics, particularly nitrofuran derivatives, in aquaculture have raised major health concerns because to their carcinogenic and mutagenic properties. This study looks at how lacto-sacc, a probiotic supplement, decreases nitrofuran derivative residues in mud crabs (*Scylla olivacea*). Gravid female mud crabs were divided into three groups: control (0% lacto-sacc), 1% lacto-sacc, and 1.5% lacto-sacc. They were housed in breeding boxes inside earthen mangrove broodstock cages designed to simulate natural settings. The crabs were fed lacto sacc supplemented diets for 84 days before being tested for nitrofuran metabolites (SEM, AHD, AMOZ, and AOZ) by liquid chromatography-tandem mass spectrometry. The results revealed that lacto Sacc-treated groups had considerably lower levels of nitrofuran metabolites than the control group. After 84 days, SEM levels in the 1.5% lacto-sacc group (T3) dropped to 0.49±0.03 ppm, AHD to 0.027±0.001 ppm, AMOZ to 0.008±0.012 ppm, and AOZ was absent. The study exhibited that lacto-sacc supplementation significantly reduced harmful nitrofuran compounds in mud crabs, promoting safer aquaculture operations.

Keywords: mud crab (*Scylla olivacea*), supplementary food, probiotics lacto-sacc, nitrofuran, earthen pond, LC-MS/MS

Introduction

Aquaculture is crucial to global food security since it accounts for a major portion of the supply of aquatic products. Mud crabs, particularly *Scylla olivacea*, are among the most valuable species due to their high market demand and great nutritional value (Sakib et al.,2022). However, the growing popularity of mud crab aquaculture offers challenges, particularly in disease management (Liew *et al*., 2023). Bacterial infections, if untreated, can result in severe economic losses. Antibiotics, particularly nitrofuran derivatives, are commonly used to mitigate these risks (Pancu *et al*., 2021). Despite their effectiveness in treating bacterial infections, nitrofuran derivatives have significant health risks, including carcinogenicity and mutagenicity, leading their restriction in numerous countries (Pacholak *et al*., 2021; Vass *et al*., 2008). The metabolization of nitrofuran antibiotics, including furazolidone, furaltadone, nitrofurazone, and nitrofurantoin, produces various compounds, including 3-amino-2-oxazolidinone (AOZ), 1-aminohydantoin (AHD), semicarbazide (SEM), and 5-methylmorpholino-3-amino-2-oxazolidinone (AMOZ) (European Food Safety Authority (EFSA) *et al*., 2021; Jia *et al*., 2022). Because of their covalent binding to tissue proteins, these metabolites are difficult to detect and may be highly hazardous to human health (Alam & Haque, 2021; Cooper & Kennedy, 2007). Moreover, aquaculture uses chloramphenicol (CAP), an additional antibiotic that is banned in animals used for food production, which carries comparable dangers (Karikalan *et al*., 2023).

Water quality is another critical factor in aquaculture, directly influencing the health and growth of mud crabs (Pati *et al*., 2023). Parameters such as pH, dissolved oxygen, salinity, and ammonia levels can significantly impact both the effectiveness of probiotics and the absorption of harmful antibiotic residues (Sumon *et al*., 2022). For instance, poor water quality can exacerbate stress in mud crabs, making them more susceptible to bacterial infections and increasing the reliance on antibiotics. Ensuring optimal water conditions is essential not only for reducing the prevalence of diseases but also for promoting the healthy metabolism of both probiotics and antibiotics within aquatic species (Tabassum *et al*., 2021).

A safer and more sustainable alternative is being sought for due to the increasing concern over antibiotic residues in aquaculture products (Okeke *et al*., 2022; Watts *et al*., 2017). Given their many advantages over antibiotics and lack of side effects, probiotics have gained attention as a potentially effective treatment (Tegegne and Kebede, 2022). Research conducted by Farliana Wan Alias *et al*. (2023) has demonstrated that lacto-sacc, a probiotic supplement, may boost

immune response, improve gut health, and stimulate growth performance in a variety of aquatic species. By improving nutritional absorption, suppressing the growth of harmful bacteria, and altering the host's gut microbiota, probiotics help to lessen the need for antibiotics (Tegegne and Kebede, 2022; Wang *et al*., 2021).
Recent study suggests that dietary probiotics might help reduce antibiotic residues in aquaculture species (Hoseinifar *et al*., 2018). Probiotics have been demonstrated to degrade or restrict the absorption of hazardous chemicals, such as nitrofuran derivatives, potentially decreasing their deleterious effects (El-Saadony *et al*., 2021; Feng *et al*., 2018; Fijan, 2014; Hoseinifar *et al*., 2018). This study looked at the effectiveness of feeding lacto-sacc in lowering nitrofuran derivative residues in mud crab (*Scylla olivacea*), especially AOZ, AHD, SEM, and AMOZ. Understanding the influence of lacto-sacc on nitrofuran residual levels in mud crabs is critical for establishing safer and more sustainable aquaculture operations. This study's findings give important insights into the use of probiotics to reduce antibiotic residues, hence improving food safety and consumer health. Furthermore, the study emphasizes the viability of probiotics as an alternative to antibiotics in aquaculture, supporting ecologically responsible and health-conscious agricultural techniques.

Materials and Methods

Experimental Design and Diet Preparation

Gravid female mud crabs (*Scylla olivacea*) were collected from the Sundarbans mangrove forest's local river in Bangladesh. They were randomly divided into three groups: T_1 (0% lacto-sacc as control), T_2 (1% lacto-sacc), and T_3 (1.5% lacto-sacc). Each treatment group consisted of 20 gravid females, each housed individually in bamboo-made breeding boxes measuring 2 ft (L) × 1.5 ft (W) × 1.5 ft (H). The crabs were acclimatized in experimental ponds and were fed a formulated diet without lacto-sacc for the first seven days. Following this acclimation period, they were fed lacto-sacc-based diets for the duration of the experiment according to our another experiment. These breeding boxes were placed in earthen mangrove broodstock pens measuring 50 ft (L) × 24 ft (W) × 3 ft (D), which were designed to mimic the natural mangrove habitat of the Sundarbans region. The pen bottoms were muddy and planted with salt-tolerant grasses. The dykes were enhanced with the planting of two mangrove species, *Avicennia officinalis* (locally called "Baim") and *Bruguiera gymnorrhiza* (locally called "Kankra"), in a zigzag pattern to simulate a natural mangrove environment. All pens were equipped with inlet and outlet systems and supported by full aeration.

The immunostimulant diets for the mud crabs were prepared according to the method described by Munir *et al*. (2016 a, b) using various feed ingredients, including fish meal, rice polish, maize, palm oil, wheat flour, vitamin mix, mineral mix, mycotoxin binder, pellet binder, and lacto-sacc (Table 1). The lacto-sacc contained *Lactobacillus acidophilus* (1.2×10^8 cfu/g), *Enterococcus faecium* (7.3×10^7 cfu/g), and live yeast *Saccharomyces cerevisiae* (2.7×10^9 cells/g), which were thoroughly blended using a mixer homogenizer before adding distilled water.
The feeds were manually processed into 2 mm pellets, which were then dried overnight under aseptic conditions and stored at -20 °C until use. The pellet samples were analyzed in the laboratory for protein, fat, and ash content. Crude protein was determined using the Kjeldahl method after block digestion with a copper catalyst and steam distillation into boric acid (AOAC Official Method 990.20) (Thiex *et al*., 2002). Crude fat was measured using diethyl ether in a classic Soxhlet extraction method (AOAC Official Method 920.39) (Thiex *et al*., 2003) , and crude fiber was assessed using the Fibertec fibercap system (AOAC 962.09) (Fahey *et al.*, 2019) . The gravid female mud crabs had an average length of 6.61±0.21 cm, a width of 9.39±0.46 cm, and a weight of 122.33±1.53 g.

Table 1: Experimental diets preparation using dietary lacto-sacc

Ingredients	**T_1 (control) (0% lacto sacc)**	**T_2 (1 % lacto-sacc)**	**T3 (1.5 % lacto-sacc)**
Fish meal (g)	75.90	75.90	75.90
Rice polish (g)	4.00	4.00	4.00
Maize (g)	4.00	4.00	4.00
Palm oil (g)	4.00	4.00	4.00
Wheat flour (g)	3.00	2.00	1.50
Vitamin mix (g)	2.00	2.00	2.00
Mineral mix (g)	2.00	2.00	2.00
Pellet binder (g)	5.00	5.00	5.00
Mycotoxin binder (g)	0.10	0.10	0.10
Lacto-sacc (g)	0.00	1.00	1.50
Total (g)	100.00	100.00	100.00

Extraction and analysis of Nitrofuran Derivatives of Crab and water

Nitrofuran derivatives were determined according to (Cooper *et al*., 2005). For the extraction procedure used to analyze nitrofuran metabolites (SEM, AHD, AMOZ, and AOZ) via liquid chromatography-tandem mass spectrometry (LC-MS/MS), 1.00 ± 0.05 g portions of soft-shell crab (*Scylla olivacea*) meat and shell samples, along with known negative tissue, were weighed into 50 ml centrifuge tubes for matrix blank and spiked recovery samples. For SEM analysis of water 1.00 ± 0.05 ml portions were taken in the same way. Protein precipitation was achieved by adding 8 ml of cold methanol,

vortexing for 1 minute, and centrifuging at 4000 rpm for 4 minutes, with methanol subsequently discarded and repeated using 4 ml of methanol. Chemical treatment involved adding 5 ml of 0.2 M HCl and 50 µl of nitrobenzaldehyde, along with 200 µl of 10 ng/ml d5-AMOZ and 100 µl of the 10 ng/ml working spiking standard to the recovery tubes. Samples were incubated at 37 ± 2 °C for 16 ± 2 hours, avoiding light. Neutralization was achieved by adding 500 µl of 0.3 M KH2PO4 to each tube and adjusting the pH to 7.0 ± 0.5 with 1 M NaOH solution. The extraction process involved adding 4 ml of ethyl acetate, vortexing, and centrifuging at 4000 rpm for 8 minutes, with organic layers combined through repeated extractions. The extract was evaporated to near dryness under nitrogen at 45°C, then reconstituted with 1 ml of 50% methanol, vortexed, filtered through a 0.45 µm syringe filter, and collected in a vial. The resulting derivatives were analyzed using an Acquity UPLC (R) BEH C18 column (1.7 µm, 2.1x100 mm) on an A10UPH2878 LC system (Waters Singapore) coupled with a QBB 933 triple quadrupole mass spectrometer (Waters UK) in positive electrospray mode. Chromatographic separation and mass spectrometric analysis facilitated the identification and quantification of SEM, AHD, AMOZ, and AOZ. AMOZ-d5 was used throughout the procedure to account for any analyte loss and ion suppression during MS analysis. Results were calculated against standard curves, with concentrations expressed as nanograms per gram (ng/g) of wet weight soft-shell crab meat and shell, ensuring comprehensive assessment of the nitrofuran metabolites using advanced analytical techniques.

Water quality assessment

Dissolved oxygen (DO), temperature, pH, salinity, total alkalinity, and ammonia contents of the pond water was measured between 9.00 and 10:00 am after seven-day intervals. Salinity was measured using a transportable refractometer (ATAGO). A common centigrade thermometer was used to measure the surface water's temperature. A digital multimeter (HQ 40d digital multimeter, HACH) was used to record the water's pH and dissolved oxygen evels. Titrimetric analysis was utilized to calculate the total alkalinity (APHA, 2000). An ammonia test kit (HANNA) was used to determine the ammonia nitrogen level.

Statistical Analysis

The Statistical Package for the Social Sciences, Version 25 (SPSS, Chicago, IL, USA) was used to compute basic descriptive statistics, such as minimum, maximum, mean, and standard error for every location and treatments. The statistical significance between the experimental groups was assessed using one-way ANOVA, followed by an LSD post hoc test, with a 95% significance level. The analysis was conducted using SPSS (Version 25).

Results

Proximate composition of experimental diets

The proximate composition of the lacto-sacc diet was analyzed for three different feed types. The diet containing 1.5% lacto-sacc comprises 45% protein, 12% fat, 3% ash, and 40% carbohydrates. The diet with 1% lacto-sacc includes 40% protein, 16% fat, 3% ash, and 41% carbohydrates. Lastly, the diet without lacto-sacc (0%) consists of 35% protein, 16% fat, 3% ash, and 46% carbohydrates.

Water quality of culture ponds

Water quality of culture ponds is presented in Table 2. The dissolved oxygen (DO) level was ranged from 5.53 to 6.0 mg/L. The water salinity level was between 25.19 to 25.50 ppt. The pH measurements fell within the range of 7.80 to 8.12. Furthermore, the total dissolved solids (TDS) exhibited a range between 908 to 1000 mg/L. the total ammonia nitrogen was below 0.5 mg/L. The most found nitrofuran SEM was almost absence.

Table 2: The mean value (mean±SE) of different water quality parameters and nitrofuran metabolite in experimental ponds.

Parameters	T_1	T_2	T_3	Optimum level
Salinity (ppt)	25.41±0.11	24.19±0.70	25.50±0.25	10-25
pH	7.84±0.02	7.80±0.08	8.12±0.05	7.5-9.0
DO (mg/L)	5.53±0.44	5.47±0.53	6.00±0.41	>5
TDS (mg/L)	1000±48.80	942.86±64.02	908.33±23.86	-
Ammonia (mg/L)	0.29±0.02	0.30±0.02	0.37±0.04	<3
SEM (ng/mL)	0.0001±0.00	0.0002±0.00	0.0001±0.00	-

Source: (Shelley & Lovatelli, 2011)

Determination of Nitrofuran Derivatives

During the study period, the metabolites of nitrofuran (SEM, AHD, AMOZ, and AOZ) were evaluated. After 84 days of feeding, the study measured the levels of nitrofuran metabolites in the control and lacto-sacc diets of crab in brood stock. The SEM was 2.12–3.14 ppm, AHD was 0.061–0.063 ppm, AMOZ was 0.041–0.045 ppm, and AOZ was 0.0011–0.0013 ppm in broodstock while stocked (Table 3). In the control diet, the concentrations of nitrofuran metabolites declined over time; however, in the formulated feed containing dietary lacto-sacc, the concentration decreased more quickly (Table 3). The SEM concentration started to significantly differ among treatments after 28 days of feeding (Table 3). After 84 days of feeding, the SEM concentration in T_3 was reduced to 0.49±0.03 ppm. In the case of AHD, T_2 showed an almost similar

concentration to the control diet, and T_3 showed a significantly higher reduction after 28 days of feeding. After 84 days of feeding, all the diets significantly differ from each other. The T_2 showed a two-time reduction of (0.047±0.01 ppm), and the T_3 showed a four-time reduction (0.027±0.01 ppm) from the control diet. The AMOZ concentration started to differ among treatments after 56 days of feeding. After 84 days of feeding, the lowest concentration of 0.008±0.012 ppm in T_3 was found. The AOZ concentrations of T_1 and T_2 were similar until 84 days of feeding, while T_3 showed the lowest value of 0.0004 ±0.00 ppm after 28 days of feeding and was completely absent after 56 days of feeding. The AOZ concentration was only under the MRL value.

Table 3: The mean (mean±SE) value of semicarbazide (SEM), 1-aminohydantoin (AHD), 3-amino-5-morpholino-methyl-1,3-oxazolidinone (AMOZ), and 3-amino-2-oxazolidinone (AOZ) (ppm) in broodstock fed with different doses of lacto-sacc diet.

Nitrofuran	Rearing stages	T_1(Lacto-sacc 0%)	T_2(Lacto-sacc 1%)	T_3(Lacto-sacc 1.5%)
SEM	Brood crab	3.12±0.06 [a]	3.10±0.05 [a]	3.14±0.07 [a]
	S_1(28days feeding)	3.07±0.07 [a]	2.11±0.01 [b]	1.83±0.08 [c]
	S_2(56days feeding)	2.81±0.10 [a]	1.34±0.01 [b]	0.91±0.03 [c]
	S_3(84days feeding)	2.52±0.13 [a]	0.66±0.02 [b]	0.49±0.03 [c]
AHD	Brood crab	0.061±0.00 [a]	0.063±0.001 [a]	0.062±0.001 [a]
	S_1(28days feeding)	0.057±0.02 [a]	0.053±0.004 [ab]	0.043±0.005 [b]
	S_2(56days feeding)	0.053±0.04 [a]	0.052±0.009 [ab]	0.036±0.009 [b]
	S_3(84days feeding)	0.041±0.01 [a]	0.047±0.01[b]	0.027±0.001 [c]
AMOZ	Brood crab	0.045±0.00 [a]	0.043±0.005 [a]	0.041±0.005 [a]
	S_1(28days feeding)	0.042±0.01 [a]	0.029±0.004 [b]	0.025±0.006 [b]
	S_2(56days feeding)	0.032±0.01 [a]	0.021±0.004 [b]	0.019±0.006 [c]
	S_3(84days feeding)	0.022±0.07 [a]	0.013±0.007 [b]	0.008±0.012 [c]
AOZ	Brood crab	0.0015±0.00 [a]	0.0014±0.00 [a]	0.0013±0.00 [a]
	S_1(28days feeding)	0.0007±0.00 [a]	0.0007±0.00 [a]	0.0004 ±0.00 [b]
	S_2(56days feeding)	0.0007±0.00 [a]	0.0005±0.00 [a]	0.00 [b]
	S_3(84days feeding)	0.0003±0.00 [a]	0.0001±0.00 [ab]	0.00 [b]

Values in each row with different superscripts are significantly different ($P < 0.05$).

After 84 days of feeding brood crab, the crablets produced started to be fed with the treatment diet is delineated in Table 4. The SEM concentration of crablets significantly differs among treatments after one week of feeding. After seven weeks of consecutive feeding, T_3 showed the lowest concentration of 0.01±0.00 ppm compared to T_1 (1.15±0.09 ppm) and T_2 (0.04±0.00 ppm), respectively. After seven weeks of feeding, the lowest concentration of AHD was in T_3 (0.001±0.00 ppm) compared to T_1 (0.020±0.011 ppm) and T_2 (0.012±0.011 ppm). The AMOZ concentration in T_3 was reduced to 0.001±0.01 ppm within five weeks of feeding, while T_1 and T_2 did not reach the RPA level of 0.001 ppm in seven weeks. The AOZ concentration was 0 ppm for T_3 and 0.001 ppm for T_2 from the first week of feeding, while the control diet showed 0.001 ppm from the fourth week.

Table 4: The mean (mean±SE) value of semicarbazide (SEM), 1-aminohydantoin (AHD), 3-amino-5-morpholino-methyl-1,3-oxazolidinone (AMOZ), and 3-amino-2-oxazolidinone (AOZ), (ppm) in crablets fed with different doses of lacto-sacc diet for seven weeks.

	Rearing stages	**T_1(Lacto-sacc 0%)**	**T_2(Lacto-sacc 1%)**	**T_3(Lacto-sacc 1.5%)**
SEM	Crablet W-1	1.08±0.07 [a]	0.39±0.00 [b]	0.17±0.00 [c]
	Crablet W-2	1.09±0.08 [a]	0.33±0.00 [b]	0.13±0.00 [c]
	Crablet W-3	1.12±0.07 [a]	0.29±0.00 [b]	0.11±0.00 [c]
	Crablet W-4	1.12±0.07 [a]	0.21±0.00 [b]	0.09±0.00 [c]
	Crablet W-5	1.12±0.07 [a]	0.19±0.00 [b]	0.07±0.00 [c]
	Crablet W-6	1.14±0.09 [a]	0.09±0.00 [b]	0.04±0.00 [c]
	Crablet W-7	1.15±0.09 [a]	0.04±0.00 [b]	0.01±0.00 [c]
AHD	Crablet W-1	0.039±0.004 [a]	0.028±0.001 [b]	0.020±0.006 [c]
	Crablet W-2	0.035±0.006 [a]	0.023±0.002 [b]	0.014±0.007 [c]
	Crablet W-3	0.029±0.006 [a]	0.019±0.006 [b]	0.009±0.008 [c]
	Crablet W-4	0.027±0.007 [a]	0.017±0.007 [b]	0.005±0.008 [c]
	Crablet W-5	0.023±0.008 [a]	0.013±0.008 [b]	0.003±0.01 [c]
	Crablet W-6	0.022±0.009 [a]	0.012±0.009 [b]	0.002±0.01 [c]
	Crablet W-7	0.020±0.011 [a]	0.012±0.011 [b]	0.001±0.01 [c]
AMOZ	Crablet W-1	0.018±0.002 [a]	0.012±0.004 [b]	0.006±0.006 [c]
	Crablet W-2	0.017±0.002 [a]	0.011±0.006 [b]	0.005±0.007 [c]
	Crablet W-3	0.016±0.002 [a]	0.010±0.006 [b]	0.003±0.008 [c]
	Crablet W-4	0.013±0.003 [a]	0.010±0.007 [b]	0.002±0.008 [c]

	Rearing stages	T_1(Lacto-sacc 0%)	T_2(Lacto-sacc 1%)	T_3(Lacto-sacc 1.5%)
	Crablet W-5	0.011±0.004 [a]	0.008±0.008 [b]	0.001±0.01 [c]
	Crablet W-6	0.010±0.005 [a]	0.007±0.009 [b]	0.001±0.01 [c]
	Crablet W-7	0.008±0.007 [a]	0.006±0.011 [b]	0.000±0.01 [c]
	Crablet W-1	0.0007±0.00 [a]	0.0001±0.00 [a]	0
	Crablet W-2	0.0005±0.00	0	0
	Crablet W-3	0.0002±0.00	0	0
AOZ	Crablet W-4	0.0001±0.00	0	0
	Crablet W-5	0	0	0
	Crablet W-6	0	0	0
	Crablet W-7	0	0	0

W=week; Values in each row with different superscripts are significantly different (P < 0.05).

Discussion

Nitrofurans are antimicrobial drugs that are not approved for use in food-producing animals, although their natural occurrence has been documented (Mack *et al*.,1999). Nitrofurans are rapidly metabolised and found in animal tissues as protein-bound metabolites. The produced metabolites (AOZ, AMOZ, AHD, and SEM) attach to proteins in the body and last for several weeks following therapy (Cooper *et al*., 2005). The SEM concentration was found highest in mud crab shell compared to its muscle tissue (Hasan *et al*., 2013). Therefore, the goal of the study was to minimize nitrofuran metabolites utilizing probiotics in diet.

Optimum water quality parameters are essential for molting, growth and survivability in arthropods. Therefore, maintaining ideal water quality for brood and hatchling is very imperative for fish culture (Ojwala *et al*., 2018). The studied water quality parameters (Table 2) of broodstock crab and hatchlings were maintained within the optimum range for crab nursery, according to (Shelley and Lovatelli, 2011). Moreover, the presence of nitrofuran metabolite was extremely low, indicates nearly absence during the study period. The minimal presence may be attributed to the use of river water during the stocking, as stated in other studies (Yu *et al*., 2012).

The reference point for action (RPA) of nitrofuran metabolites for foods of animal origin is 0.001 mg/kg, as stated in law (Commission Decision 2002/657/EC and Commission Decision 2005/34/EC) to safeguard public health (EFSA Panel on Contaminants in the Food Chain, 2015). In this study, high levels of Lacto-sacc (1.5%) in the diet promoted the reduction of Nitrofuran metabolites from brood to larval stages for 84 days of feeding (Table 3). Brood crab fed with Lacto-sacc (1.5%) in the diet (T_3) had a much greater decrease of nitrofuran metabolites. The AOZ was at RPA level in brood crab (0.0013 mg/kg), while with the with the application of lacto-sacc diet, the AOZ was removed completely at 56 days for T_3 and 84 days for T_2 (Table 3).

The EFSA found that the SEM has a natural occurrence of development in shellfish; thus, they reassessed and recommended the RPA level of 1.00 mg/kg for SEM in 2015. (EFSA Panel on Contaminants in the Food Chain. In case of crablets rearing, after seven weeks of feeding, the SEM concentration dropped to 0.01 mg/kg in T_3 compared to T_1 and T_2, respectively, and ensures a safe limit (CONTAM, 2015). The results were consistent with natural occurrences of SEM concentration in shellfish (Van Poucke *et al*., 2011).

The AHD concentration was dropped to 0.001 mg/kg in T_3 compared to T_1 and T_2, respectively, whereas the EFSA recommended RPA level of 4.8 mg/kg (CONTAM, 2015). The AMOZ concentrations were completely removed at seven weeks of feeding. The AOZ concentration was at 0 ppm at 84 days of feeding of the larval stage, and continuation of treatment found no presence of the AOZ in crablets.

The reduction process of nitrofuran metabolites could be attributed to the binding of nitrofuran, given that Lactobacilli exhibit an excellent binding capacity (Monachese *et al.*, 2012). In the study, Lactobacilli was one of the components of experimental probiotics (lacto-sacc diet). Although the effect of bioremediation by probiotics is strain-dependent and specific *(Feng et al.*, 2018), Moreover, some studies find Lactobacilli can inhibit the intestinal absorption of heavy metals (Zhai *et al*., 2013) and pesticides (Cao *et al.*, 2012); therefore, it could be inhibiting the absorption of metabolites. Some studies recorded that Lactobacilli protect against pesticide-induced oxidative stress and downstream cellular damage and stimulate the host's own immunity and detoxification mechanisms (Chiocchetti *et al*., 2019; Russell *et al*., 2011). Therefore, this defence mechanism could also assist in detoxifying the nitrofuran metabolites.

Probiotics have also been shown to increase epithelium mucin production, which is a critical element of the epithelium barrier (Mack *et al*., 1999; Willemsen *et al*., 2003). Probiotics also assist in producing antagonistic activity like bacteriocins against pathogenic bacteria and inhibiting bacterial translocation by competing for receptors or adhesion to endothelial cells (Balcázar *et al*., 2007; Servin, 2004).

Phenoloxidase and prophenoloxidase activity assay, hemocyte count, hemolymph clotting time, and histology of the gut can clearly draw the overall picture of how lacto-sacc diet probiotics dramatically reduced the nitrofuran metabolites in larval stages of crab, which were not considered in this study due to the limitation of facilities. The current study recorded

the variation of the concentration of nitrofuran metabolites changes due to incorporation of probiotics (lacto-sacc diet). Regardless, this study suggested a new scope of detoxification of nitrofuran metabolites by feeding probiotics and also recommended further in-depth research to uncover the cause of bioremediation of nitrofuran metabolites in crab.

Conclusion

The results obtained from the present study have shown that supplementation with lacto-sacc diet (probiotics) is best for reducing naturally occurring nitrofuran metabolites in brood mud crabs (*Scylla olivacea*) fed for 84 days and crablets fed for seven weeks. There was a significant difference in the performance of the different doses of probiotics and control in brood stock and crablets. Within seven weeks of feeding with a 1.5% lacto-sacc-contained diet, we can ensure a reduction of all nitrofuran metabolites to the RPA level, which is safe for public health.

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References

1. Alam, M.M and Haque, M.M. (2021) Presence of antibacterial substances, nitrofuran metabolites and other chemicals in farmed pangasius and tilapia in Bangladesh: Probabilistic health risk assessment. Toxicology Reports 8:248–257. https://doi.org/10.1016/j.toxrep.2021.01.007
2. AOAC (2000) Official Methods of Analysis, 17th ed. Association of Official Analytical Chemists, 18th edn. Gaithersburg, Maryland
3. Balcázar, J.L., Vendrell, D. and de Blas I. et al. (2007) In vitro competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens. Veterinary microbiology 122:373–380
4. Cao, L., Yang X.J. and Li Z.J., et al. (2012) Reduced lesions in chickens with Clostridium perfringens-induced necrotic enteritis by Lactobacillus fermentum 1.2029. Poultry science 9}"007C1:3065–3071
5. Chiocchetti, G.M., Jadán-Piedra, C. and Monedero, V. et al. (2019) Use of lactic acid bacteria and yeasts to reduce exposure to chemical food contaminants and toxicity. Critical reviews in food science and nutrition 59:1534–1545
6. Cooper, K.M. and Kennedy, D.G. (2007) Stability studies of the metabolites of nitrofuran antibiotics during storage and cooking. Food Additives and Contaminants 24:935–942. https://doi.org/10.1080/02652030701317301
7. Cooper, K.M., Mulder, P.P.J. and Van Rhijn, J.A. et al. (2005) Depletion of four nitrofuran antibiotics and their tissue-bound metabolites in porcine tissues and determination using LC-MS/MS and HPLC-UV. Food Additives and Contaminants 22:406–414. https://doi.org/10.1080/02652030512331385218
8. El-Saadon, y M.T., Alagawany, M. and Patra, A.K. et al. (2021) The functionality of probiotics in aquaculture: An overview. Fish & Shellfish Immunology 117:36–52. https://doi.org/10.1016/j.fsi.2021.07.007
9. European Food Safety Authority (EFSA), O'Keeffe M., Christodoulidou, A. and Nebbia, C. (2021) Presence of nitrofurans and their metabolites in gelatine. EFS2 19:. https://doi.org/10.2903/j.efsa.2021.6881
10. Fahey, G.C., Novotny, L., Layton, B. and Mertens, D.R. (2019b) Critical Factors in Determining Fiber Content of Feeds and Foods and Their Ingredients. Journal of AOAC INTERNATIONAL 102:52–62. https://doi.org/10.5740/jaoacint.18-0067
11. Farliana Wan Alias, S.L., Munir, M.B. and Asdari, R., et al. (2023a) Dietary lacto-sacc improved growth performance, food acceptability, body indices, and basic hematological parameters in empurau (Tor tambroides) fries reared in the aquaponics system. Journal of Applied Aquaculture 35:1131–1153. https://doi.org/10.1080/10454438.2022.2095239
12. Feng, P., Ye, Z. and Kakade, A. et al. (2018a) A review on gut remediation of selected environmental contaminants: possible roles of probiotics and gut microbiota. Nutrients 11:22
13. Feng, P., Ye, Z. and Kakade, A. et al. (2018b) A Review on Gut Remediation of Selected Environmental Contaminants: Possible Roles of Probiotics and Gut Microbiota. Nutrients 11:22. https://doi.org/10.3390/nu11010022
14. Fijan, S. (2014) Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature. IJERPH 11:4745–4767. https://doi.org/10.3390/ijerph110504745
15. Hassan, M.N., Rahman, M., Hossain, M.B., Hossain, M.M., Mendes, R. and Nowsad, A. (2013). Monitoring the presence of chloramphenicol and nitrofuran metabolites in cultured prawn, shrimp and feed in the Southwest coastal region of Bangladesh. The Egyptian Journal of Aquatic Research, 39(1), 51–58.
16. Hoseinifar, S.H., Sun, Y-Z., Wang, A. and Zhou, Z. (2018) Probiotics as Means of Diseases Control in Aquaculture, a Review of Current Knowledge and Future Perspectives. Front Microbiol 9:2429. https://doi.org/10.3389/fmicb.2018.02429
17. Jia, J., Zhang, H., Qu, J., et al. (2022) Immunosensor of Nitrofuran Antibiotics and Their Metabolites in Animal-Derived Foods: A Review. Front Chem 10:813666. https://doi.org/10.3389/fchem.2022.813666

18. Karikalan, N., Yamuna, A. and Lee, T.Y. (2023) Ultrasensitive detection of ineradicable and harmful antibiotic chloramphenicol residue in soil, water, and food samples. Analytica Chimica Acta 1243:340841. https://doi.org/10.1016/j.aca.2023.340841
19. Liew, K-S,, Yong, F.K-B. and Lim, L-S. (2023) An Overview of the Major Constraints in Scylla Mud Crabs Grow-out Culture and Its Mitigation Methods. Aquaculture Studies 24:. https://doi.org/10.4194/AQUAST993
20. Mack, D.R., Michail, S. and Wei, S, et al. (1999) Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. American Journal of Physiology-Gastrointestinal and Liver Physiology 276: G941–G950
21. Monachese, M., Burton, J.P. and Reid, G. (2012) Bioremediation and tolerance of humans to heavy metals through microbial processes: a potential role for probiotics? Applied and environmental microbiology 78:6397–6404
22. Munir, M.B., Roshada, H., Yam, H. C., Terence, L. M., and Azizah, S. (2016a). Dietary prebiotics and probiotics influence growth performance, nutrient digestibility and the expression of immune regulatory genes in snakehead (Channa striata) fingerlings. Aquaculture. Volume 460, 1 July 2016, Pages 59–68.
23. Munir, M.B., Roshada, H., Abdul-Manaf, M.S., & Azizah, S. (2016b). Dietary prebiotics and probiotics influence the growth performance, feed utilisation, and body indices of snakehead (*Channa striata*) fingerlings. Trop Life Science Research 27(2):111–125.
24. Ojwala, R.A. Otachi, E.O. and Kitaka, N.K. (2018) Effect of water quality on the parasite assemblages infecting Nile tilapia in selected fish farms in Nakuru County, Kenya. Parasitology Research 117:3459–3471,(2018). https://link.springer.com/article/10.1007/s00436-018-6042-0
25. Okeke, E.S., Chukwudozie, K.I. and Nyaruaba, R. et al. (2022) Antibiotic resistance in aquaculture and aquatic organisms: a review of current nanotechnology applications for sustainable management. Environ Sci Pollut Res 29:69241–69274. https://doi.org/10.1007/s11356-022-22319-y
26. Pacholak, A., Burlaga, N., Guzik, U. and Kaczorek, E. (2021) Investigation of the bacterial cell envelope nanomechanical properties after long-term exposure to nitrofurans. Journal of Hazardous Materials 407:124352. https://doi.org/10.1016/j.jhazmat.2020.124352
27. Pancu, D.F., Scurtu, A. and Macasoi, I.G. et al. (2021) Antibiotics: Conventional Therapy and Natural Compounds with Antibacterial Activity-A Pharmaco-Toxicological Screening. Antibiotics 10:401. https://doi.org/10.3390/antibiotics10040401
28. Pati, S.G., Paital, B. and Panda, F., et al. (2023) Impacts of Habitat Quality on the Physiology, Ecology, and Economical Value of Mud Crab Scylla sp.: A Comprehensive Review. Water 15:2029. https://doi.org/10.3390/w15112029
29. Russell, R.J., Scott, C. and Jackson, C.J. et al. (2011) The evolution of new enzyme function: lessons from xenobiotic metabolizing bacteria versus insecticide-resistant insects. Evolutionary Applications 4:225–248
30. Sakib, M.H., Ahmmed, S. and Washim, M.R. et al (2022) Population dynamics of mud crab, Scylla olivacea (Herbst, 1796) from the Sundarbans of Bangladesh. Aquaculture Fish & Fisheries 2:224–232. https://doi.org/10.1002/aff2.42
31. Servin, A.L. (2004) Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS microbiology reviews 28:405–440
32. Shelley, C., and Lovatelli, A. (2011). Mud crab aquaculture: A practical manual. FAO Fisheries and Aquaculture Technical Paper, 567, I
33. Sumon, T.A., Hussain, M.A. and Sumon M.A.A. et al. (2022) Functionality and prophylactic role of probiotics in shellfish aquaculture. Aquaculture Reports 25:101220. https://doi.org/10.1016/j.aqrep.2022.101220
34. Tabassum, T. Sofi Uddin Mahamud A.G.M. and Acharjee, T.K. et al. (2021) Probiotic supplementations improve growth, water quality, hematology, gut microbiota and intestinal morphology of Nile tilapia. Aquaculture Reports 21:100972. https://doi.org/10.1016/j.aqrep.2021.100972
35. Tegegne, B.A. and Kebede, B (2022) Probiotics, their prophylactic and therapeutic applications in human health development: A review of the literature. Heliyon 8:e09725. https://doi.org/10.1016/j.heliyon.2022.e09725
36. Thiex, N.J., Anderson, S. and Gildemeister, B. et al. (2003) Crude Fat, Diethyl Ether Extraction, in Feed, Cereal Grain, and Forage (Randall/Soxtec/Submersion Method): Collaborative Study. Journal of AOAC INTERNATIONAL 86:888–898. https://doi.org/10.1093/jaoac/86.5.888
37. Thiex, N.J., Manson, H. and Anderson, S. et al. (2002a) Determination of Crude Protein in Animal Feed, Forage, Grain, and Oilseeds by Using Block Digestion with a Copper Catalyst and Steam Distillation into Boric Acid: Collaborative Study. Journal of AOAC INTERNATIONAL 85:309–317. https://doi.org/10.1093/jaoac/85.2.309
38. Thiex, N.J., Manson, H. and Anderson, S. et al. (2002b) Determination of Crude Protein in Animal Feed, Forage, Grain, and Oilseeds by Using Block Digestion with a Copper Catalyst and Steam Distillation into Boric Acid: Collaborative Study. Journal of AOAC INTERNATIONAL 85:309–317. https://doi.org/10.1093/jaoac/85.2.309
39. Van Poucke, C., Detavernier, C., Wille, M., Kwakman, J., Sorgeloos, P., and Van Peteghem, C. (2011). Investigation into the possible natural occurence of semicarbazide in Macrobrachium rosenbergii prawns. Journal of Agricultural and Food Chemistry, 59(5), 2107–2112.
40. Vass, M., Hruska, K. and Franek, M. (2008) Nitrofuran antibiotics: a review on the application, prohibition and residual analysis. Vet Med 53:469–500. https://doi.org/10.17221/1979-VETMED
41. Wang, X., Zhang, P. and Zhang, X. (2021) Probiotics Regulate Gut Microbiota: An Effective Method to Improve Immunity. Molecules 26:6076. https://doi.org/10.3390/molecules26196076

42. Watts, J. Schreier, H., Lanska, L. and Hale, M. (2017) The Rising Tide of Antimicrobial Resistance in Aquaculture: Sources, Sinks and Solutions. Marine Drugs 15:158. https://doi.org/10.3390/md15060158
43. Willemsen, L.E., Koetsier, M.A., van Deventer, S.J. and van Tol, E.A. (2003) Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E1 and E2 production by intestinal myofibroblasts. Gut 52:1442–1447
44. Yu, H.-J., Li, B., Cai, Y.-Q., Ye, F.-T., Tai, J.-M., Hui, Y.-H., Xu, J., and Feng, B. (2012). Determination of Semicarbazide content in crustaceans by Liquid chromatography tandem mass spectrometry. Chinese J. Anal. Chem, 40, 1530–1535.
45. Zhai, Q., Wang, G., and Zhao, J., et al. (2013) Protective effects of Lactobacillus plantarum CCFM8610 against acute cadmium toxicity in mice. Applied and environmental microbiology 79:1508–1515