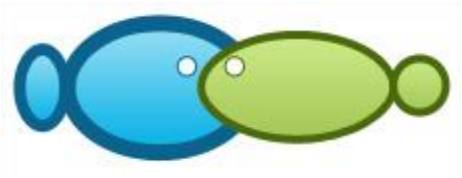

Mitigation of nitrofuran residues in *Scylla olivacea* (Herbst,1796) through dietary Lacto-sacc supplementation

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Abstract. Nitrofuran metabolites, particularly semicarbazide (SEM), 1-aminohydantoin (AHD), 5-methylmorpholino-3-amino-2-oxazolidinone (AMOZ), and 3-amino-2-oxazolidinone (AOZ), pose significant risks in aquaculture due to their carcinogenic properties and strict zero-tolerance regulations. This study investigated the effectiveness of a probiotic mixture of *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* (Lacto-sacc) in reducing nitrofuran residues in the range mud crab, *Scylla olivace*a (Herbst, 1796). Three diets were formulated: a control (0%) and treatments supplemented with Lacto-sacc at 1.0% and 1.5%. 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. Initial SEM concentrations in control crabs were 1.25±0.06 µg kg $^{-1}$, which increased to 1.42±0.04 µg kg $^{-1}$ after the experimental period. In contrast, SEM levels declined significantly with probiotic supplementation: from 1.24±0.07 to 0.61±0.02 µg kg $^{-1}$ in the 1.0% group, and 1.27±0.04 to 0.41±0.01 µg kg $^{-1}$ in the 1.5% group ($p < 0.05$). Reductions in other nitrofuran derivatives, including AOZ and AMOZ, followed similar trends. Notably, SEM concentrations in the 1.5% treatment fell below the recommended permissible allowance (RPA) (0.5 µg kg $^{-1}$) set by the European Food Safety Authority. These findings demonstrate that dietary supplementation with Lacto-sacc, particularly at 1.5%, can effectively mitigate nitrofuran metabolite accumulation in orange mud crabs. The use of probiotics offers a practical, eco-friendly approach to ensuring food safety, enhancing consumer confidence, and promoting sustainable aquaculture practices.

Key Words: Orange 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. Orange mud crab, *Scylla olivace*a (Herbst, 1796) is among the most valuable species due to their high market demand and great nutritional value (Sakib et al 2022). However, the growing popularity of orange 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 to their restriction in numerous countries (Vass et al 2008; Pacholak et al 2021).

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) (EFSA et al 2021; Jia et al 2022). Due to their covalent binding to tissue proteins, these metabolites are challenging to detect and may pose significant risks to human health (Cooper & Kennedy 2007; Alam & Haque 2021). Moreover, aquaculture uses chloramphenicol, an antibiotic banned in animals used for food production, which poses comparable risks (Karikalan et al 2023).

Water quality is another critical factor in aquaculture, directly influencing the health and growth of orange mud crabs (Pati et al 2023). Parameters such as pH, dissolved oxygen (DO), 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 orange 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 due to the increasing concern over antibiotic residues in aquaculture products (Watts et al 2017; Okeke et al 2022). Given their many advantages over antibiotics and lack of side effects, probiotics have gained attention as a potentially effective treatment (Tegegne & 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 (Wang et al 2017; Tegegne and Kebede 2022).

A recent study suggests that dietary probiotics might help reduce antibiotic residues in aquaculture species (Hoseinifar et al 2024). Probiotics have been demonstrated to degrade or restrict the absorption of hazardous chemicals, such as nitrofuran derivatives, potentially decreasing their deleterious effects (Fijan 2014; Feng et al 2018; Hoseinifar et al 2018; El-Saadony et al 2021).

This study looked at the effectiveness of feeding Lacto-sacc in lowering nitrofuran derivative residues in orange orange mud crab, especially AOZ, AHD, SEM, and AMOZ, to promote safer and more sustainable aquaculture practices.

Material and Method

Ethical approval. The present research was conducted with prior animal ethical approval of Sher-e-Bangla Agricultural University Animal Ethics Committee (SAU-AEC/FAMS/2022-78).

Experimental design and diet preparation. Gravid female orange mud crabs were collected from the Sundarbans mangrove forest's local river in Bangladesh. They were randomly divided into three groups: T1 (0% Lacto-sacc as control), T2 (1% Lacto-sacc), and T3 (1.5% Lacto-sacc). Each treatment group consisted of 20 gravid females; each was 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 another experiment. These breeding boxes were placed in earthen mangrove broodstock pens measuring 50 ft (L) × 24 ft (W) × 3 ft (H), 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 orange mud crabs were prepared according to the method described by Munir et al (2016) 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^{-1}), *Enterococcus faecium* (7.3×10^7 CFU g^{-1}), and live yeast *Saccharomyces cerevisiae* (2.7×10^9 CFU g^{-1}), 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 orange 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, which is also described in a published article (Hannan et al 2024) of the same project.

Table 1

Experimental diets preparation using dietary Lacto-sacc

Ingredients	*T1 (control) (0% Lacto sacc)*	*T2 (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 meat and shell samples, along with known negative tissue, were weighed into 50 mL centrifuge tubes for matrix blank and spiked recovery samples. For particularly semicarbazide (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^{-1} d5-AMOZ and 100 µl of the 10 ng mL^{-1} 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 KH_2PO_4 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, 2.1 x 100 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. The LC-MS/MS method was validated according to international guidelines, with limits of detection ranging from 0.05-0.10 ng g^{-1} and limits of quantification between 0.15-0.30 ng g^{-1} for SEM, AHD, AMOZ, and AOZ. Average recoveries for all metabolites in spiked crab tissue samples were within the range of 82-95%, demonstrating good accuracy and reliability of the analytical procedure. Results were calculated against standard curves, with concentrations expressed as nanograms per gram (ng g^{-1}) of wet weight soft-shell crab meat and shell, ensuring a comprehensive assessment of the nitrofuran metabolites using advanced analytical techniques. The unit mg/kg is used here to get the number after the decimal point.

Water quality assessment. DO, temperature, pH, salinity, total alkalinity, and ammonia contents of the pond water were 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 levels. Titrimetric analysis was utilized to calculate the total alkalinity (APHA 2005). 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 treatment. 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. The water quality of culture ponds is presented in Table 2. The DO level ranged from 5.53 to 6.0 mg L^{-1}. 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^{-1}. The total ammonia nitrogen was below 0.5 mg L^{-1}. The most found nitrofuran SEM was almost absent.

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^{-1})	5.53±0.44	5.47±0.53	6.00±0.41	> 5
TDS (mg L^{-1})	1000±48.80	942.86±64.02	908.33±23.86	-
Ammonia (mg L^{-1})	0.29±0.02	0.30±0.02	0.37±0.04	< 3
SEM (ng mL^{-1})	0.0001±0.00	0.0002±0.00	0.0001±0.00	-

Source of optimum level: (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 mg kg^{-1}, AHD was 0.061-0.063 mg kg^{-1}, AMOZ was 0.041-0.045 mg kg^{-1}, and AOZ was 0.0011-0.0013 mg kg^{-1} in broodstock while stocked (Figure 1). 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 (Figure 1).

The SEM concentration started to significantly differ among treatments after 28 days of feeding (Figure 1). After 84 days of feeding, the SEM concentration in T3 was reduced to 0.49±0.03 mg kg^{-1}. In the case of AHD, T2 showed an almost similar concentration to the control diet, and T3 showed a significantly higher reduction after 28 days of feeding. After 84 days of feeding, all the diets significantly differ from each other. The T2 showed a two-time reduction of (0.047±0.01 mg kg^{-1}), and the T3 showed a four-time reduction (0.027±0.01 mg kg^{-1}) 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 mg kg^{-1} in T3 was found. The AOZ concentrations of T1 and T2 were similar until 84 days of feeding, while T3 showed the lowest value of 0.0004±0.00 mg kg^{-1} after 28 days of feeding and was completely absent after 56 days of feeding. The AOZ concentration was only under the MRL value.

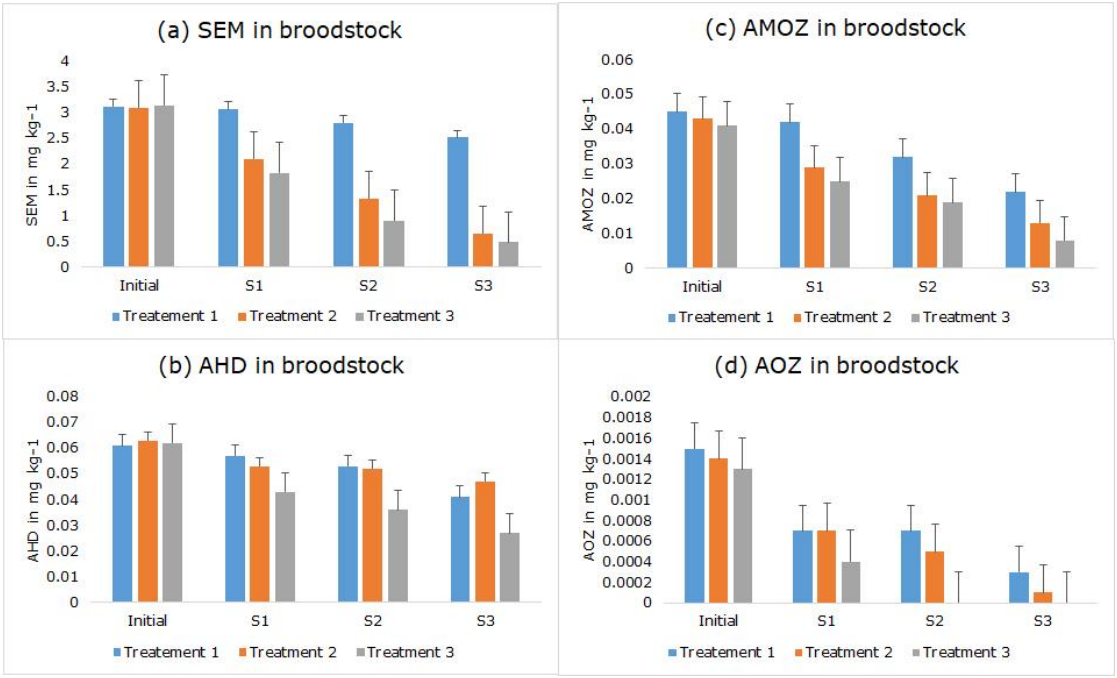

Figure 1. The concentration of nitrofuran metabolites (mg kg^{-1}) (SEM, AHD, AMOZ, AOZ) in broodstock fed with different doses of lacto-sacc diet (T1, T2, and T3) for a consecutive seven weeks.

After 84 days of feeding brood crab, the crablets produced started to be fed with the treatment diet, as delineated in Figure 2. The SEM concentration of crablets significantly differs among treatments after one week of feeding. After seven weeks of consecutive feeding, T3 showed the lowest concentration of 0.01±0.00 mg kg^{-1} compared to T1 (1.15±0.09 mg kg^{-1}) and T2 (0.04±0.00 mg kg^{-1}), respectively. After seven weeks of feeding, the lowest concentration of AHD was in T3 (0.001±0.00 mg kg^{-1}) compared to T1 (0.020±0.011 mg kg^{-1}) and T2 (0.012±0.011 mg kg^{-1}). The AMOZ concentration in T3 was reduced to 0.001±0.01 mg kg^{-1} within five weeks of feeding, while T1 and T2 did not reach the recommended permissible allowance (RPA) level of 0.001 mg kg^{-1} in seven weeks. The AOZ concentration was 0 mg kg^{-1} for T3 and 0.001 mg kg^{-1} for T2 from the first week of feeding, while the control diet showed 0.001 mg kg^{-1} from the fourth week.

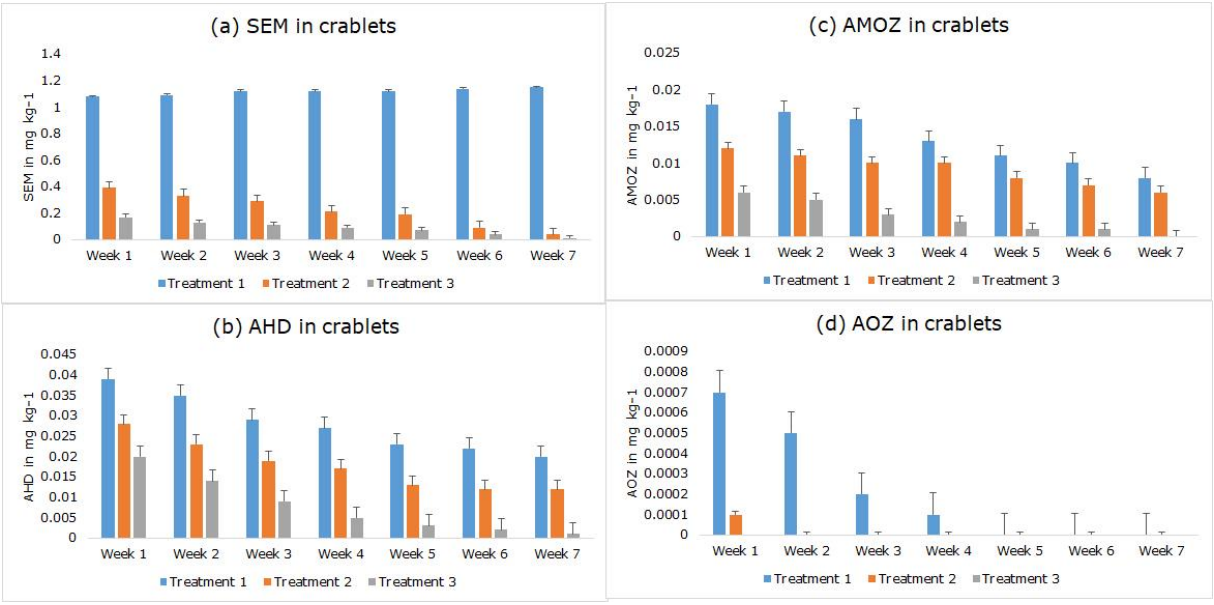

Figure 2. The concentration of nitrofuran metabolites (mg kg^{-1}) (SEM, AHD, AMOZ, AOZ) in crablets fed with different doses of lacto-sacc diet (T1, T2, and T3) for consecutive seven weeks.

Discussions. 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 to be highest in the orange mud crab shell compared to its muscle tissue (Hasan et al 2022). Therefore, the goal of the study was to minimize nitrofuran metabolites by utilizing probiotics in the diet.

Optimum water quality parameters are essential for molting, growth, and survivability in arthropods. Therefore, maintaining ideal water quality for brood and hatchlings is 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 & Lovatelli 2011). Moreover, the presence of nitrofuran metabolite was extremely low, indicating a near 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 2013).

The reference point for action RPA of nitrofuran metabolites for foods of animal origin is 0.001 mg kg^{-1}, as stated in law (Commission Decision 2002/657/EC and Commission Decision 2005/34/EC) to safeguard public health (EFSA CONTAM 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 (Figure 1). Brood crab fed with Lacto-sacc (1.5%) in the diet (T3) had a much greater decrease in nitrofuran metabolites. The AOZ was at the RPA level in brood crab (0.0013 mg kg^{-1}), while with the application of the Lacto-sacc diet, the AOZ was removed completely at 56 days for T3 and 84 days for T2 (Figure 1).

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^{-1} for SEM in 2015. In the case of crablets rearing, after seven weeks of feeding, the SEM concentration dropped to 0.01 mg kg^{-1} in T3 compared to T1 and T2, respectively, and ensures a safe limit (EFSA 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^{-1} in T3 compared to T1 and T2, respectively, whereas the EFSA recommended RPA level of 4.8 mg kg^{-1} (EFSA CONTAM 2015). The AMOZ concentrations were completely removed at seven weeks of feeding. The

AOZ concentration was at 0 mg kg^{-1} 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* were 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 that *Lactobacilli* can inhibit the intestinal absorption of heavy metals and pesticides (Zhai et al 2013; Cao et al 2007); therefore, it could inhibit 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 (Das et al 2022; Javanshir et al 2021). 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 (Monteagudo-Mera et al 2019; Plaza-Diaz et al 2019).

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 due to the 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 orange mud crabs 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-containing diet, dietary Lacto-sacc effectively reduced nitrofuran residues to safe levels. 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.

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Conflict of interest. The authors declare that there is no conflict of interest.

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