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DNA-BASED SPECIES IDENTIFICATION OF FOUR IMPORTANT CYPRINIDAE FISH IN THE NORTH OF VIETNAM

Luu Thi Ha Giang[1, *], Pham Hong Nhat[1], Vu Thi Trang[1], Vu Thi Huyen[1], Vu Van Sang[1]

ABSTRACT

The four Cyprinidae fish including *Cyprinus carpio, Semilabeo notabilis, Spinibarbus denticulatus* and *Bangana* sp. are valuable fish for aquaculture and fish conservation in the North of Vietnam. DNA-based molecular method is a potential technique for fish species identification and genetic analysis. In this study, 21 mitochondrial DNA cytochrome oxidase I (COI) sequences of the four Cyprinidae fish were collected from previous projects of Research Institute for Aquaculture No.1 and minned on GenBank for further analysis of molecular identification. The nucleotide discrimination showed higher average AT content (55.17%) compared to average GC content (44.84%) in the Cyprinidae fish. The obtained sequences were compared to the GenBank database (NCBI) using the Basic Local Alignment Search Tool (BLAST), a total of 37 sequences with high percentage identity (92.88 to 99.84%) were found. Phylogenetic tree analysis for 58 COI sequences revealed four main branches corresponding to the four genera in the Cyprinidae. The mean genetic Kimura 2 - parameter distances illustrated the close relationship diversity between species (0.004 to 0.058) and genera (0.105 to 0.151). Single nucleotide polymorphism (SNP) analysis of all species in the study revealed distinct features regarding unique sites (31 sites) for four species. DNA sequence based QR codes were generated based on the short sequence of COI containing SNP data for accurate identification of fish species. In addition, the results showed the high Potential of using the DNA-based method to identify Cyprinidae fish in Vietnam.

Keywords: *Bangana* sp., *COI, Cyprinus carpio, Semilabeo notabilis, Spinibarbus denticulatus, DNA-based species identification.*

Received: 10 June 2023; revised: 8 August 2023; accepted: 19 August 2023.

1. INTRODUCTION

Cyprinus carpio, Semilabeo notabilis, Spinibarbus denticulatus, Bangana sp. are freshwater fish belonging to the carp family (Cyprinidae), which are important cyprinid fish in the North of Vietnam. Common carp (*Cyprinus carpio*) is one of the main fish species cultured and of great economic importance in Northern Vietnam [1]. Meanwhile, *Semilabeo notabilis, Spinibarbus denticulatus* and *Bangana* sp. are unique and rare fish species and have high economic value in the Northern midlands and mountainous region. The distribution range of these fish is in large rivers of the Red river system (Thao River, Chay River, Lo River, Gam River) and the Thai Binh river system [1]. These are all species of high gastronomy and are considered the king of freshwater fish in the North of Vietnam. Nevertheless, along with the overfishing and the degradation of the environment, these fish are increasingly scarce as well as in danger of extinction, and have been named in the Vietnam Red data book (1992) [2].

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There was a report of several confusions among these species from different local areas in Vietnam due to the similar morphology of Ciprinid fish [3]. *Semilabeo notabilis, Spinibarbus denticulatus, Bangana* sp. have almost the same shape of body; they all live in the same habitat. These fish have the habit of eating moss on rocks with their mouths, it fromed the structure of the fish's mouth is quite special for the purpose of finding food. It is challenging for non-taxonomists to accurately recognize and identify these species based on the conventional methods. Therefore molecular identification can complement morphological identification data.

Mitochondrial DNA cytochrome oxidase I (COI) sequence information plays a vital role in identifying species [4]. This gene sequence is conserved within a species, and the rate of mutation is fast enough to distinguish between closely related species [5]. Therefore, the COI sequencing method, i.e., DNA barcoding, has been used as a standard tool for species identification [6]. DNA barcoding can help to eliminate existing misidentification and the availability of cryptic species that mimic and equally compromise the accuracy of fishes in research, fishery management and conservation [7]. A number of countries have had DNA barcoding done on freshwater and marine fishspecies. Until now, more than 502.000 specimens, belonging to 26.187 fish species, have been successfully identified in BOLD [8].

In Vietnam, seven hundred and sixty-five COI sequences of coastal ray-finned fishes were generated, belonging to 458 species, 273 genera, 113 families and 43 orders, was reported for DNA barcoding study by Pham The Thu *et al.* (2019) [9]. The mtDNA COI sequence analysis also has been used to understand genetics structure and species identification of several fish species including common carp [10]; the Mastacembelidae [11].

The present study was aimed to estimate genetic differences among the four cyprinid species collected from the North of Vietnam using the different computational methods e.g., data mining, nucleotide discrimination, distance, phylogenetics, and Single Nucleotide Polymorphism (SNP) of fish species in the carp family (Cyprinidae). The DNA sequence data generated from this study was used to develop a "Quick Response Code" (QRC). This study provides important information for future conservation and fisheries resource management of four important cyprinid fish species in Vietnam.

2. MATERIALS AND METHODS

2.1. Fish database of COI gene

Table 1. List of fish species (Order: Cypriniformes; Family: Cyprinidae) and sample sequences used in the analysis

Vietnamese name	Scientific name	Genus	Location	Genbank (Acession Number)	Source
Anh Vũ	*Semilabeo notabilis*	Semilabeo	Thai Nguyen, Ha Giang, Phu Tho, Cao Bang	OP269716 → OP269720	RIA1 (2023)
Cá Bỗng	*Spinibarbus denticulatus*	Spinibarbus	Ha Giang, Tuyen Quang, Hoa Binh	MW446147 → MW446157	RIA1 (2021)
Cá chép	*Cyprinus carpio*	Cyprinidae	Cao Bang	MW450995→ MW450999	RIA1 (2021)
Rầm xanh	*Bangana* sp.	Bangana	Ma river, Thanh Hoa	KY498533	[12]

COI gene sequences was collected from several previous reports which focused on the *Cyprinidae* in the mountainous of Northerm Vietnam, involving *Semilabeo notabilis; Spinibarbus denticulatus; Cyprinus carpio, Bangana* sp. The sampling and sequencing activities were conducted by our team in Research Institute for Aquaculture No. 1 from 2021 to 2023. In total, 20 sequence data (COI gene) of fish species (*Semilabeo notabilis; Spinibarbus denticulatus; Cyprinus carpio)* were collected and recently published in GenBank. The *Bangana* sp. isolated from Vietnam were minned on NCBI (https://www.ncbi.nlm.nih.gov/). Total 21 COI sequencens subjected to this study were shown in the table 1.

2.2. Nucleotide discrimination and BLAST annotation

Compositional features for COI sequences were calculated for the base number of the four nucleotides and GC content for each sequence as well as an overall average by using MEGAX software [13].

The BioEdit software [14] was also used to check and determine the degree of similarity of sequences and to create the consensus sequence of each species.

The Basic Local Alignment Search Tool (BLAST) is highly efficient for determining sequence similarities with reference sequences from GenBank. The input consensus sequences were compared with the maximum similarity data sets of fish species based on high BLAST identity percentage with the lowest E-value for the generated alignment. The 37 validated reference sequences for all fish species were downloaded from GenBank for utilization in the construction of a phylogenetic evolutionary tree (neighbor-joining tree).

2.3. Multiple sequence alignment, estimates of evolutionary divergence and phylogenetic analysis

A sequence file including the 21 Vietnamese sequences and 37 reference sequences was aligned through MEGAX using the CLUSTAL alignment tool. Additionally, all sequences were edited manually, i.e., similar, highly mismatched sites and gaps were removed; each base of the spliced sequence was checked before submission to other analysis. Analysis of genetic distance between populations was conducted using MEGA; the evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

The phylogenetic trees were constructed by using algorithm programs in MEGAX software. The bootstrap support of the Neighbor-Joining tree was assessed using 1000 replicates.

2.4. Single Nucleotide Polymorphism (SNP) Screening and DNA barcode generation

Based on the above alignment data and SNPs were detected manually for estimation of unique sites same as described by Yang *et al.* (2019) [15].

QR code is easily accessible two-dimensional barcode, readable by smartphones. It allows to encode over 4000 characters in a two-dimensional barcode. The COI fragments containing SNPs were used for the development of DNA barcodes for each species using an online QR code generator (https://www.online-qrcode-generator.com/).

3. RESULTS AND DISCUSSION

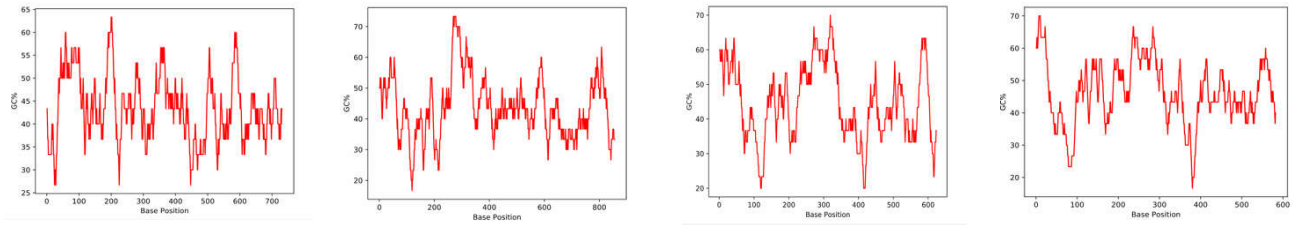
3.1. Nucleotide composition

A total of 21 COI sequences were generated from 4 Vietnamese cyprinid fish minned from previous reports and GenBank (Table 1). The sequence length ranged from 604 bp to 884 bp (mean 725 bp) and there was not any codon, insert or deletion in the sequences. The average nucleotide compositions (Table 2) were 26.32% adenine (A), 28.87% cytosine (C), 17.93% guanine (G) and 26.88% thymine (T). This result corresponds to previous studies in genus *Capoeta* (Family Cyprinidae*)* that showed the average nucleotide frequencies were C (29.24%), T (26.82%), A (26.53%), G (17.41%) [16].

Table 2. Comparison of the nucleotide composition among the 4 Vietnamese Cyprinidae

Fish species	Total (bp)	A (%)	T (%)	G (%)	C (%)	GC (%)	AT (%)
Semilabeo obscurus	652	26.17	28.65	17.82	27.36	45.16	54.84
Spinibarbus denticulatus	884	26.73	29.36	18.12	25.78	44.00	56
Cyprinus carpio	760	27.05	29.47	17.55	25.92	43.48	56.52
Bangana sp.	604	25.33	27.98	18.21	28.48	46.7	53.3
Average	725	26.32	28.87	17.93	26.88	44.84	55.17

The Cyprinidae fish species nucleotide discrimination revealed varied AT (the percentage of adenine + thiamine) and GC (the percentage of guanine+ cytosine) contents. Among the four species, the observed nucleotide base composition of all analyzed sequences was average at 55.17% AT (range from 53.3 to 56.52%) and 44.84% GC (from 43.48 to 46.7%) (Table 2, figure 1). Overall, the results demonstrated that for the Cyprinidae fish species, the average AT content (55.17%) was higher than average the GC content (44.84%). This result is consistent with previous studies in fish species that reported higher AT content than GC content [17]. Reviewed data from the 655 bp *COI* region was on average GC content in the 44 freshwater species about 45.2% [18]; from 89 fish species (GC content = 45.48%) [19], and from 79 species of fish with GC content=45.78% [20] .



Cyprinus carpio | *Spinibarbus denticulatus* | *Semilabeo obscurus* | *Bangana* sp.

Figure 1. Comparison of the average GC among the 4 Cyprinidae

Genomic DNA base composition and especially its GC content plays an important role in the functioning and regulation of genes [21]. The GC content is known to vary widely between genomes of different species and also serve as an important criterion in taxa delimitation [22]. This genomic trait has been widely studied, and its evolution has been proposed to be associated with numerous mutational and selective forces driven by genetic, metabolic, and ecological factors [23]. Our analysis revealed that the highest nucleotide base composition was 46.7% GC in *Bangana* sp. and 45.6% in *Semilabeo obscurus*. In contrast, *Spinibarbus denticulatus* has lower GC content with COI gene at 44% and lowest GC content was observed in *Cyprinus carpio* at 43.38%. It could be explained by the different habitats of the fish species because higher GC contents were found to be mostly associated with species inhabiting more stable environments. Hence, *Bangana* sp. and *Semilabeo obscurus* are sharing the same ecological conditions (high water quality, low water temperatures), less suffer from the stress of the environment and similar morphology. In the other hand, *Spinibarbus denticulatus* and *Cyprinus carpio* have a broader spectrum of habitats.

3.2. Similarity-based search of the GenBank databases

The results of comparing the nucleotide concensus sequences of studied fish samples with GenBank data through NCBI were shown in table 3. The BLAST results showed all of the taxa available in a database that have sequence similarity with the query sequence. A total of

fifteen COI sequences of fish were selected with percentage identity ranging from 92.88 to 99.84% across the fish sequences (Table 3). The expected value, e-value and maximum coverage obtained from the sequences ranged from 0.0 and 93 - 100% (online data), respectively. A total of 4 genera in the Cyprinidae were also detected among the fish samples. Total 37 sequences with maximum identity percent (ID) score and query cover were selected for estimating of evolutionary divergence and building phylogenetics tree. These were *Cyprinus carpio* (number, n=18), *Semilabeo* sp. (n=11), *Spinibarbus denticulatus* (n=5), and *Bangana* sp. (n=3). For the total number of species, 6 (*Cyprinus carpio, Semilabeo obscurus, Semilabeo notabilis, Spinibarbus denticulatus, Bangana decora, Bangana lemassoni)* of them were found among the fish sequences. According to the sampling countries, 22 sequences isolated from the fish belonging to China; 6 sequences from USA; 4 sequences from Vietnam, 2 sequences from Taiwan, one from Japan and one sequence was recorded in Japan.

Table 3. Similarity results and reference sequence (Accession no.) for fish species identified by BLAST/GenBank

Scientific name	BLAST hits in NCBI database	Percent identities (%)	GenBank Accession No.	Year of publication	Source of sequence
Cyprinus carpio	*Cyprinus carpio*	98.95	MK291479.1	2018	China
	Cyprinus carpio	98.95	MH202953.1	2019	China
	Cyprinus carpio	98.95	MG570435.1	2021	USA
	Cyprinus carpio	98.95	KP993139.1	2015	China
	Cyprinus carpio	98.95	KP993137.1	2015	China
	Cyprinus carpio	98.95	OM736811.1	2022	USA
	Cyprinus carpio	98.95	OL693871.1	2022	USA
	Cyprinus carpio	98.95	OL457418.1	2022	USA
	Cyprinus carpio	98.95	MZ713633.1	2022	Taiwan
	Cyprinus carpio	98.95	MW125611.1	2022	China
	Cyprinus carpio	98.95	OM234682.1	2022	China
	Cyprinus carpio	98.95	KJ511883.1	2014	Hungary
	Cyprinus carpio	98.95	KF856965.1	2014	China
	Cyprinus carpio	98.95	MW680808.1	2021	China
	Cyprinus carpio	98.95	NC_018039.1	2023	USA
	Cyprinus carpio	98.95	NC_018037.1	2023	USA
	Cyprinus carpio	98.95	JN673560.1	2016	Taiwan

	Cyprinus carpio	98.68	NC_018366.1	2013	China
Semilabeo obscurus	*Semilabeo notabilis*	99.84	KY615364.1	2017	Vietnam
	Semilabeo notabilis	99.69	NC_045916.1	2023	China
	Semilabeo obscurus	99.69	MN229749.1	2021	China
	Semilabeo obscurus	99.69	MN229748.1	2021	China
	Semilabeo obscurus	99.68	GU086581.1	2016	China
	Semilabeo notabilis	99.67	KY615365.1	2017	Vietnam
	Semilabeo obscurus	99.54	NC_037408.1	2023	China
	Semilabeo obscurus	99.54	MN229747.1	2021	China
	Semilabeo notabilis	96.39	KY615363.1	2017	Vietnam
	Semilabeo notabilis	96.26	KT633633.1	2017	Vietnam
	Semilabeo notabilis	95.98	JX074195.1	2012	China
Spinibarbus denticulatus	*Spinibarbus denticulatus*	97.99	JX042168	2013	China
	Spinibarbus denticulatus	97.97	GU086582	2016	China
	Spinibarbus denticulatus	97.96	AP013335	2016	Japan
	Spinibarbus denticulatus	97.79	KJ994631	2017	China
Bangana sp.	*Bangana decora*	95.7	NC 026221	2023	China
	Bangana decora	95.96	MG732840	2019	China
	Bangana lemassoni	92.88	GU86575	2016	China

The three species of *Semilabeo obscurus, Spinibarbus denticulatus* and *Bangana* sp. are native fish, distributing mainly in the highlands of Vietnam, Lao and China and rarely in other Asian countries. Therefore, it is predictable that fewer number of identified Cyprinid species in GenBank for these three species compared to *Cyprinus carpio.* In this study, there were two species found by BLAST for the *Semilabeo obscurus* (RIA1's specimen), which are *Semilabeo notabilis* and *Semilabeo obscurus*. Interestingly, the *Semilabeo notabilis* matched with higher percent identities (99.84% and 99.69%, respectively) and also lower percent identities (from 95.98 to 96.39%) compared to the *Semilabeo obscurus* from China (from 99.54 to 99,69% identities). It might be missidentification of this species of previous study, since literally only specimen with a sequence similarity of 97% or above are considered to be a single species. In addition, BLAST results (Table 3) indicated that

the *Bangana* sp. from Vietnam might be not one of *Bangana decora* or *Bangana lemassoni* species (from 92.88 to 95.7% identities), more studies and larger databases are needed to identify exactly the species name of *Bangana* specimens from Vietnam.

3.3. Genetic diversity among samples and evolutionary relationship Phylogenetic analysis

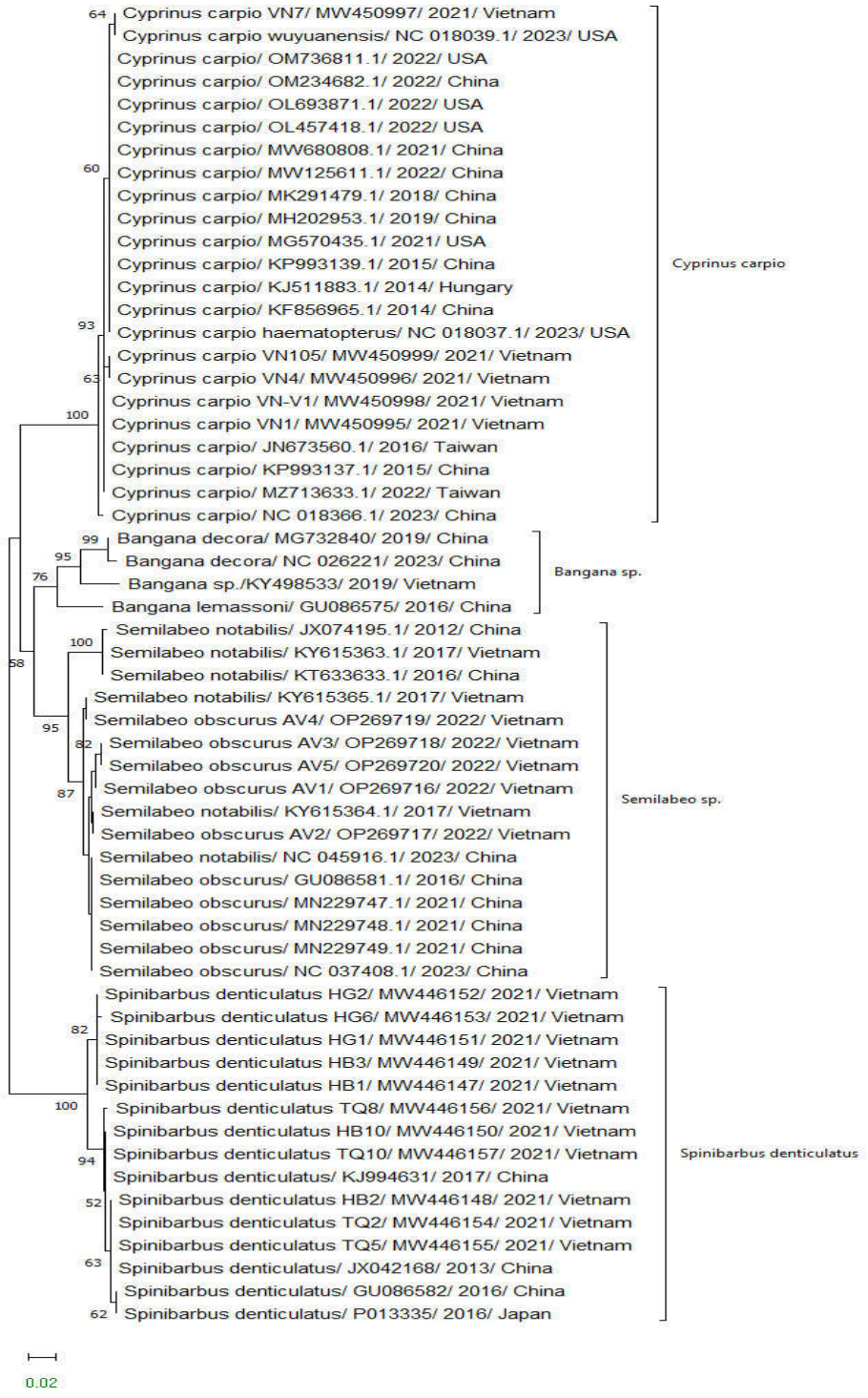


Figure 2. Neighbor-Joining tree of the Cyprinidae based on 58 COI sequences.

Note: Numbers at tree branches indicate the percentage of replicate trees in which the same clusters are found in the bootstrap tests of 1000 replicates.

The phylogenetic tree of the studied fish in figure 2 was divided into 4 main groups corresponding to the 4 subfamilies in the Cyprinidae. Therein, The first group is *Spinibarbus denticulatus* included 2 clusters indicated with the highest bootstrap value (100%). Interestingly, among 11 fish samples isolated from Vietnam, there are 5 haplotypes from Ha Giang and Hoa Binh provinces which were grouped into separate clusters, while other 6 haplotypes have closer relationship with *Spinibarbus denticulatus* from China and Japan. Group 2 is *Cyprius carpio*, which show close relationship between fish from variety regions China, Taiwan, The USA and Vietnam (100% value of bootstrap). Group 3 included two branches of *Semilabeo* (95% value of bootstrap); but it seems to be no distinction between *Semilabeo obscurus* and *Semilabeo notabilis* in both countries. Group 4 indicated with lowest bootstrap value (76%) included 4 divergence *Bangana* sp. The isolated from Vietnam could be more closed to *Bangana decora,* scine it cluster with two *Bangana decora* isolated from China with well supported by bootstrap value with 95%, the BLAST search (Table 3) showed similar result that Vietnamese *Bangana* sp. is 95.96% identities with Chinese *Bangana decora.*

The phylogenetic relationship of Cyprinidae has also been published by many authors. In which, Thai *et al.* (2007), examined the phylogenetic relationships in the subfamilies within Cyprinidae using mitochondrial 16S rRNA, D-loop and cytochrome b gene sequences from 25 species of cyprinids collected from Vietnam combined with sequences of cyprinids available in GenBank, the result showed that there are two principal lineages within the Cyprinidae: Cyprinines and Leuciscines, but many of the subfamily boundaries remained unclear [24]. Other study using 16S rRNA by Sharma *et al.* (2014), indicated that the family Cyprinidae of Agra region was resolved as a paraphyletic group which shows the divergence may occur, but they share the same common ancestor [25]. In recent study, Alam *et al.* (2021) provided more information about the phylogenetic relationship between cyprinid fishes species in Bangladesh, in trees, Leuciscinae subfamily showed monophyletic lineage - where Garrinae, Schizothoracinae, Rasborinae, Cultrinae, Cyprininae subfamilies were polyphyletic [26]. These studies all revealed a complex evolutionary relationship of the Cyprinidae and sugested further studies on phylogenetic analysis of Cyprinidae fishes below the level of family.

In Vietnam, several studies conducted in RIA1 have been reported the phylogenetics relationship within species of *Semilabeo obscurus* [27], *Spinibarbus denticulatus, Cyprinus carpio* [10]. In this study, the combination in the Neighbor - Joining tree indicated the relationship between these species, *Semilabeo* sp. might be closely related to *Bangana* sp., since two genera were placed in one branch supported by bootstrap value with 58% (Figure 2).

3.4. Genetic Divergence (K2P) Among Taxa

The estimates of evolutionary divergence between sequences of the COI were determined using Kimura's two-parameter model of nucleotide substitution (Table 4). Intergeneric genetic distances ranged from 0.105 to 0.151. The highest estimates of evolutionary divergence among groups were found between *Spinibarbus denticulatus* and *Bangana* sp. (0.151 ± 0.027), and the lowest between *Spinibarbus denticulatus* and *Cyprinus carpio* (0.105 ± 0.019). The divergence between *Cyprinus carpio* and *Semilabeo* sp. and the divergence between *Cyprinus carpio* and *Bagana* sp. were similar value at 0.135 ± 0.023 compared to 0.136 ± 0.023, respectively.

The mean K2P distance of species within genus was from 0.004 to 0.058. The highest K2P genetic distances was found in *Bangana* sp. (0.058 ± 0.011), then *Semilabeo* sp. (0.018 ± 0.005), *Spinibarbusi denticulatus* (0.013 ± 0.005), and lowest genetic distances was detected in *Cyprinus carpio* (0.004 ± 0.002).

Table 4. Summary of Kimura 2-parameter genetics divergence within (the diagonal) and between species (below the diagonal) of cyprinid fishes based on 58 COI sequences

	Cyprinus carpio	*Semilabeo* sp.	*Spinibarbus denticulatus*	*Bangana* sp.
Cyprinus carpio	0.004 ± 0.002			
Semilabeo sp.	0.135 ± 0.023	0.018 ± 0.005		
Spinibarbus denticulatus	0.105 ± 0.019	0.120 ± 0.023	0.013 ± 0.005	
Bangana sp.	0.136 ± 0.023	0.145 ± 0.026	0.151 ± 0.027	0.058 ± 0.011

In case of the *Semilabeo* sp., the BLAST result illustrated two species with high percentage of identities (Table 3). Additionally, the output of the phylogenetic tree (Figure 2) and the genetics divergence (Table 4) showed conservative sequences of COI gene within the *Semilabeo* genus. These findings showed close evolution and lower genetic distances of *Semilabeo* species between different regions (0.018 ± 0.005). In contrast, Ha T.T.T *et al.* (2018) indicate higher genetic distance (0.04) between two *Semilabeo* groups from different provinces in Vietnam [28].

In the case of other species *Cyprinus carpio, Spinibarbus denticulatus, Bangana* sp., the Kimura 2 - parameter, genetics divergence result in this study is consistent with previous studies, the intraspecific genetic distances based on K2P are usually low (below 1) and are rarely greater than 2 across a broad range of taxa [29]. All the genetics distances in this study showed less than 1 sequence diversity within species and showed less than 2 sequence diversity between species, which indicated no increase in genetic variability relative to species (Table 4).

3.5. Single Nucleotide Polymorphism screening and QR Codes generation

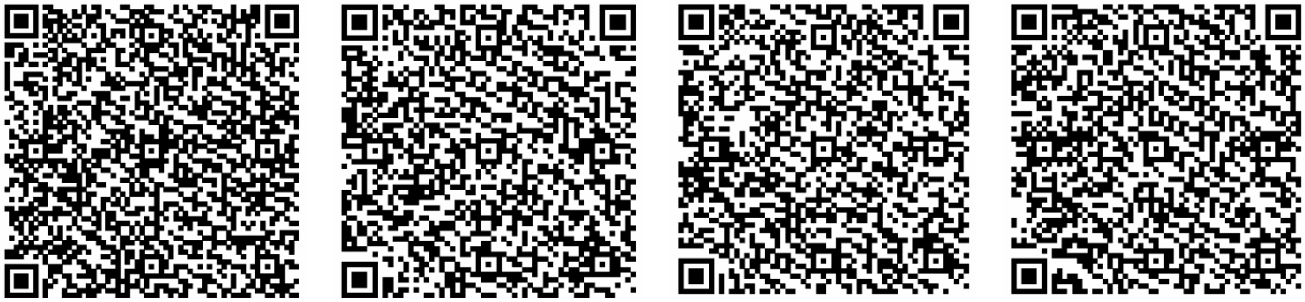
From the sequence alignments, there were genetic variations at a nucleotide level as determined at different positions of the representative sequences. Base on the result of phylogenetics tree, the four species including 51 sequences was selected for identification of variable sites (Table 5). Consequently, the original data of COI - studied sequences were selected into a specific short segments (235 bp) of COI barcode (from site 465 to site 699). There were 31 character attribute sites were observed over the DNA barcode. The sample data generated in the present study were showing lowest number of variable sites (4 positions) for the *Bangana* genus. Whereas, there was nine diverse sites detected for each species involving *Semilabeo obscurus, Spinibarbus denticulatus, Cyprinus carpio*. These differences may be due to the replacement mutation occurred in the groups that can cause the phenotypic differences in fish species, which could be used as potential markers for identification of the Cyprinidae.

At the nucleotide level, the sequence alignment revealed much polymorphism at different positions and this similar degree of variations had been previously observed in several fish species. Previous reports yielded 56 variable sites in 43 sequences of *Steindachneridi on scriptum* [30]; and 76 variable sites in 74 fish of *Engraulis encrasicolus* [31]. The existing variations show a high degree of heterogeneity within the studied fishes. In this study, the polymorphic sites were observed for different species, however it could not represent for different countries.

Table 5. Single Nucleotide Polymorphism observed in the different species of the Cyprinidae

	Nucleotide positions	465	474	475	483	486	498	504	519	525	531	547	549	555	561	564	573	576	582	585	588	595	597	600	624	636	645	651	672	684	693	699
1	Bangana decora/ MG732840/ 2019/ China	C	T	T	T	C	T	C	C	C	C	C	A	C	C	A	T	C	A	T	T	C	A	T	G	T	A	C	A	A	C	C
2	Bangana decora/ NC 026221/ 2023/ China	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	Bangana sp./KY498533/ 2019/ Vietnam	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	Cyprinus carpio haematopterus/ NC 018037.1/ 2023/ USA	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
5	Cyprinus carpio VN-V1/ MW450998/ 2021/ Vietnam	T	.	C	A	.	.	.	.	.	.	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
6	Cyprinus carpio VN1/ MW450995/ 2021/ Vietnam	T	.	C	A	.	.	.	.	.	.	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
7	Cyprinus carpio VN105/ MW450999/ 2021/ Vietnam	T	.	C	A	.	.	.	.	.	.	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
8	Cyprinus carpio VN4/ MW450996/ 2021/ Vietnam	T	.	C	A	.	.	.	.	.	.	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
9	Cyprinus carpio VN7/ MW450997/ 2021/ Vietnam	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
10	Cyprinus carpio wuyuanensis/ NC 018039.1/ 2023/ USA	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
11	Cyprinus carpio/ JN673560.1/ 2016/ Taiwan	T	.	C	A	.	.	.	.	.	.	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
12	Cyprinus carpio/ KF856965.1/ 2014/ China	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
13	Cyprinus carpio/ KJ511883.1/ 2014/ Hungary	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
14	Cyprinus carpio/ KP993137.1/ 2015/ China	T	.	C	A	.	.	.	.	.	.	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
15	Cyprinus carpio/ KP993139.1/ 2015/ China	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
16	Cyprinus carpio/ MG570435.1/ 2021/ USA	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
17	Cyprinus carpio/ MH202953.1/ 2019/ China	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
18	Cyprinus carpio/ MK291479.1/ 2018/ China	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
19	Cyprinus carpio/ MW125611.1/ 2022/ China	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
20	Cyprinus carpio/ MW680808.1/ 2021/ China	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
21	Cyprinus carpio/ MZ713633.1/ 2022/ Taiwan	T	.	C	A	.	.	.	.	.	.	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
22	Cyprinus carpio/ NC 018366.1/ 2023/ China	T	.	C	A	.	.	.	.	.	.	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
23	Cyprinus carpio/ OL457418.1/ 2022/ USA	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
24	Cyprinus carpio/ OL693871.1/ 2022/ USA	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
25	Cyprinus carpio/ OM234682.1/ 2022/ China	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
26	Cyprinus carpio/ OM736811.1/ 2022/ USA	T	.	C	A	.	.	.	.	.	T	.	G	.	.	G	C	.	G	C	.	T	.	.	.	C	T	.	.	.	T	.
27	Semilabeo obscurus AV1/ OP269716/ 2022/ Vietnam	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	G	.	T	T
28	Semilabeo obscurus AV2/ OP269717/ 2022/ Vietnam	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	G	.	T	T
29	Semilabeo obscurus AV3/ OP269718/ 2022/ Vietnam	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	G	.	T	T
30	Semilabeo obscurus AV4/ OP269719/ 2022/ Vietnam	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	.	.	T	T
31	Semilabeo obscurus AV5/ OP269720/ 2022/ Vietnam	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	G	.	T	T
32	Semilabeo obscurus/ GU086581.1/ 2016/ China	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	G	.	T	T
33	Semilabeo obscurus/ MN229747.1/ 2021/ China	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	G	.	T	T
34	Semilabeo obscurus/ MN229748.1/ 2021/ China	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	G	.	T	T
35	Semilabeo obscurus/ MN229749.1/ 2021/ China	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	G	.	T	T
36	Semilabeo obscurus/ NC 037408.1/ 2023/ China	A	.	C	C	T	.	.	.	.	.	T	.	T	A	.	C	.	.	.	.	.	G	C	.	.	T	.	G	.	T	T
37	Spinibarbus denticulatus HB1/ MW446147/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	.	T	.
38	Spinibarbus denticulatus HB10/ MW446150/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	T	T	.
39	Spinibarbus denticulatus HB2/ MW446148/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	T	T	.
40	Spinibarbus denticulatus HB3/ MW446149/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	.	T	.
41	Spinibarbus denticulatus HG1/ MW446151/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	.	T	.
42	Spinibarbus denticulatus HG2/ MW446152/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	.	T	.
43	Spinibarbus denticulatus HG6/ MW446153/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	G	T	.
44	Spinibarbus denticulatus TQ10/ MW446157/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	T	T	.
45	Spinibarbus denticulatus TQ2/ MW446154/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	T	T	.
46	Spinibarbus denticulatus TQ5/ MW446155/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	T	T	.
47	Spinibarbus denticulatus TQ8/ MW446156/ 2021/ Vietnam	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	G	.	A	.	C	T	.	T	T	.
48	Spinibarbus denticulatus/ GU086582/ 2016/ China	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	T	T	.
49	Spinibarbus denticulatus/ JX042168/ 2013/ China	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	T	T	.
50	Spinibarbus denticulatus/ KJ994631/ 2017/ China	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	T	T	.
51	Spinibarbus denticulatus/ P013335/ 2016/ Japan	.	C	C	C	.	C	T	T	T	.	.	.	.	T	.	C	T	.	.	C	.	.	.	A	.	C	T	.	T	T	.

Note: "."Represented the similarity of sequences, and Character attributes were highlighted in red. Nucleotide positions noted from the trimmed COI sequence after alignment, and based on the complete COI gene reference of Cyprinus carpio 1551 bp from the GenBank database (accession number OM736811.1)



Cyprinus carpio | *Semilabeo obscurus* | *Spinibarbus denticulatus* | *Bangana* sp.

Figure 3. QR codes generated using the 235 bp COI gene fragments

Moreover, all the Cyprinidae COI barcoding fragment (235 bp) were used to generate scannable QR codes. DNA sequence based QR codes for the Vietnamese *Bangana* sp. *Semilabeo obscurus, Spinibarbus denticulatus, Cyprinus carpio* are given in figure 3. We have developed DNA sequence based QR codes that can be scanned using mobile phone applications in the same way that barcodes are scanned in supermarkets. This study generated QR codes for the identi fication of 4 fish species belonging to the family of Cyprinidae based on molecular approaches. The DNA QR code can be used as the tag or label in the tracking system, this techinique can be applied to develop the native fish producs on blockchains for different Cyprinid, however it is not suitable for different contries. According to the previous reports, Yang *et al.* (2019) developed one-dimensional DNA barcode as an example for the precise identification of Teleost fish species [15]. Ghouri *et al.* (2020) targeting 17 commercially available freshwater and marine fish species of Pakistan, based on using QR barcodes of DNA unique SNP [32]. The use of species authentication supported by DNA barcoding could provide an effective approach for monitoring, management, and conservation of the fisheries sector.

4. CONCLUSION

DNA-based molecular method showed the efficiency in identification of the four Cyprinidae fishes at the species level in Vietnam. In this study, 21 Vietnamese specimens and 37 sequences minned from Genbank were analyzed of nucleotide composition, phylogenetic relationship, and SNP using DNA-based method. *Semilabeo notabilis* and *Bangana* sp. showed genetically closer relationship compared to *Spinibarbus denticulatus* and *Cyprinus carpio*. The 31 polymorphic sites were observed for different species, however it could not represent for different countries. DNA sequence based QR codes were generated based on the short sequence of COI containing SNP data for accurate identification of fish species The protocol of DNA-based analysis in this present study may serve as a reference for accurate identification of fishes that may facilitate ichthyological research, biodiversity management and authentication of fish products in Vietnam.

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INVESTIGATING WATER SURFACE TEMPERATURE AT SHRIMP FARMS IN QUYNH LUU DISTRICT, NGHE AN PROVINCE BY USING REMOTELY SENSED DATA IN GOOGLE EARTH ENGINE

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ABSTRACT

Temperature is a fundamental environmental element of all aquatic ecosystems, including aquaculture ponds. However, the availability of in situ water temperature data typically only represent a small portion of shrimp farms' thermal profile, and this often leads to limitation in investigating impacts of global warming and climate change on shrimp farming. For this aim, satellite-derived water surface temperature (WST) at shrimp farms in Quynh Luu district, Nghe An province, Vietnam were investigated using Landsat data in Google Earth Engine. Results showed that there was a seasonal variation of WST at shrimp farms, with monthly mean WST ranged from 21.8°C (December) to 30.7°C (July). Annual WST at shrimp farms in Quynh Luu district showed an increasing trend from 2000 to 2022. A remarkable warming trend of WST was detected during shrimp farming season (often from April to October each year) and this may lead to high risk for shrimp farms there. Findings of this study indicate that greater awareness of shifts in WST regime at shrimp farms is required if this activity is to be sustainable under climate change.

Keywords: *Water surface temperature, shrimp farming, Landsat, Google Earth Engine.*

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1. INTRODUCTION

Temperature is a fundamental environmental element of all aquatic ecosystems in general and aquaculture ponds in particular. Water temperature affects the survival, growth, maturation and reproduction of all aquatic organisms - the majority of which are poikilothermic (cold-blooded) organisms that can only thrive when water temperatures are within their preferred thermal range [1, 2]. Water temperature also regulates many physical, chemical, and biological processes in water bodies, such as the maximum dissolved oxygen concentration, the percentage of toxic unionized ammonia form - NH_3, the rate of chemical reactions, metabolic rates and nutrient cycling [2, 3]. In addition, global warming and weather extremes due to climate change (e.g., heat waves and droughts) lead to increases in water temperature as well as changes of its seasonal patterns on the earth [4], especially for shallow water bodies [5 - 7]. Therefore, evaluating water temperature is essential to understanding its direct and indirect effects on aquatic ecosystem health as well as cultured species in the climate change context.

A conventional way to assess water temperature is by using in situ thermometers or sensors to measure temperatures at specific monitoring stations. However, this approach requires the appropriate infrastructure and fieldwork, which is time consuming and expensive, especially applying for large aquatic ecosystems (e.g., lakes, large aquaculture farms). In Vietnam, environmental monitoring programs for aquaculture have been using in situ

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measurement of water temperature and such programs are mainly implemented at some concentrated aquaculture areas with few times per year. Although the monitoring sites and frequency in environmental monitoring programs in aquaculture have improved and extended recently [8], in situ measurements are unable to cover for a vast number of aquaculture farms in Vietnam in general and shrimp farms in particular. Therefore, the availability of in situ water temperature data typically only represent a small portion of shrimp farms' thermal profile.

Besides conventional methods for water temperature measurements, data from satellite sensors may provide additional information on spatiotemporal variability of water surface temperature (WST), or "skin temperature" [9]. Several satellite sensors are capable of collecting thermal emission data which can be used for retrieving WST. Coarse spatial and high frequency satellite sensors (e.g., the Advanced Very High Resolution Radiometer (AVHRR), Visible Infrared Imaging Radiometer Suite (VIIRS) and the MODerate resolution Imaging Spectroradiometer (MODIS)) have been commonly used to retrieve and map sea surface temperature [10 - 14] and large lakes [15 - 17]. Recently, higher spatial resolution sensors have been used for retrieving and mapping WST in oceans, large lakes as well as small water bodies (e.g., smaller lakes and reservoirs, streams, rivers, ponds). For example, Tonooka and Hirayama (2010) used Advanced Spaceborne Thermal Emission and Reflection Radiometer (ASTER) thermal infrared data to retrieve WST in small Lake Senba in Japan [18]. Teggi (2012) developed an algorithm for improving the spatial resolution of ASTER from 90 to 30 m to map WST in coastal waters and of watercourses in Italy [19]. Moreover, the Landsat Data Continuity Mission program holds an impressive continuous record of imagery data, Landsat (i.e., Landsat 4 and 5 TM, Landsat 7 ETM+, Landsat 8 TIRS and Landsat 9 TIRS-2) is a prime platform for studies on thermal properties of waters [20 - 25].

In addition, the Google Earth Engine (GEE), a cloud-based platform for earth science data and analysis developed by Google has enabled to process large scale satellite images that available free for public, such as Landsat [26, 27]. GEE has been used to conduct various global and regional scale studies, on variety issues [26, 27], including WST [28 - 32]. Study of Pedreros-Guarda *et al.*, (2021) [28] also indicated that using the method developed by Ermida *et al.*, (2020) [33] to retrieve WST in small inland water bodies from Landsat 7 and 8 data in GEE produced the best results without calibration [28].

As with many coastal areas in Vietnam, shrimp farming has rapidly developed in Quynh Luu district, Nghe An province over the last two decades [34]. However, the availability of water temperature data at shrimp farms is sparse due to limitations of in situ environmental monitoring programs in aquaculture as mentioned previously. Therefore, this study aimed to investigate multitemporal WST at shrimp farms in Quynh Luu district, Nghe An province using Landsat data in GEE. The study will provide the first detailed record of WST information at concentrated shrimp farming areas, contributing to sustainable development of shrimp farming in Vietnam in the climate change context.

2. METHODOLOGY

2.1. Study area

Quynh Luu district is located in the Northeast of Nghe An province, in the North Central of Vietnam (Figure 1). The district is bordered by Hoang Mai town in the North, the East Vietnam Sea in the East, Dien Chau, Tan Ky and Yen Thanh districts in the South and Southwest, and Thai Hoa town and Nghia Dan district in the West. Quynh Luu district has a diverse topography, including plains, coastlines and low hills [35]. The district is characterized by the tropical monsoon climate, which is hot, humid and rainy during summer (May - September), and cool and dry in winter (October - April). Annual mean air temperature is about 30°C and the highest air temperature may be up to 40°C. Annual rainfall is about 1,459 mm (920 - 2,047 mm) [35].

Shrimp farming has rapidly developed in terms of land use and shrimp production in Quynh Luu district over the last two decades. Total area of shrimp farms in Quynh Luu district is about 786.15 ha in 2020 [34]. Shrimp farms are mainly located along tidal creeks of Mai Giang, Hau and Thai rivers in coastal communes of Quynh Luu district (Figure 1).

Shrimp farming is promoted by the local government for producing valuable aquatic products, raising local living standards and thereby contributing to sustainable development in coastal areas. According to the Master Plan for Fisheries Development of Nghe An province to 2020 for vision to 2030, Quynh Luu district is one of main districts for the development of coastal shrimp farming [36]. Based on data from Department of Fisheries of Nghe An province in 2020, total area of shrimp farming of the province was 2,234 ha, produced 7,896 tonnes of commercial shrimp production; of which, Quynh Luu district shared a vital part for both shrimp culture area as well as commercial shrimp production [37].

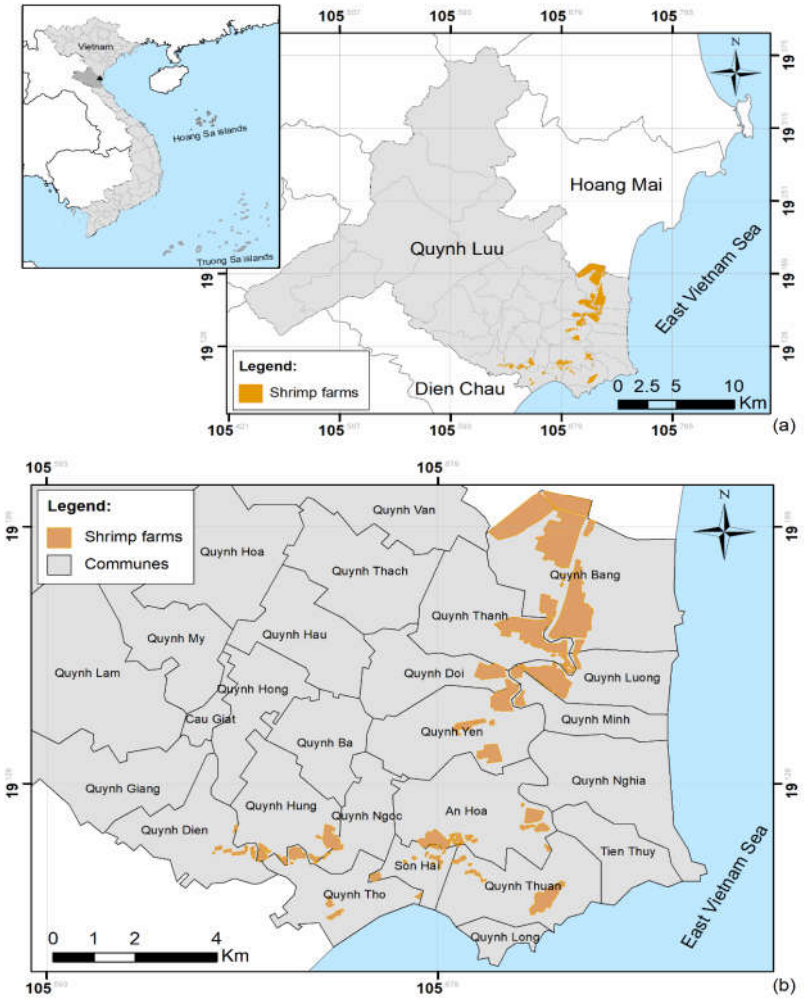

Figure 1. Shrimp farms in Quynh Luu district, Nghe An province, Vietnam in 2020 (a) and the enlargement showing communes with shrimp farms (b) (redraw from figure 1 and 4 in [38])

2.2. Datasets and methods

Landsat 5 TM, Landsat 7 ETM+, and Landsat 8 OLI/TIRS datasets in the GEE platform were used in this study (https:// developers. google. com/ earth-engine/datasets/catalog/landsat) [39]. Since shrimp farming in Quynh Luu district, Nghe An province has developed from 2000 [34], imagery data of the study area (paths 126 and 127, row 47), acquired from 01st January 2000 to 30th June 2023 were collected to retrieve land and water surface temperature (L/WST) (Table 1). In addition, Google Earth, digital topographic maps of the

study area were used to provide ancillary data. A global positioning system (GPS) Garmin 72 was also used for ground truthing in the study area.

Table 1. Satellite, Google Earth Engine dataset, path/row of the study area, equator crossing time (ECT), and available period for each Landsat satellite

Satellite	*Dataset*	*Path/ Row*	*ECT*	*Available period*
Landsat 5 (TM)	T1-SR, T1-TOA	126/47, 127/47	10:00 - 10:30 AM (16-day)	01/01/1984 – 05/05/2012
Landsat 7 (ETM+)	T1-SR, T1-TOA	126/47, 127/47	10:00 - 10:30 AM (16-day)	01/01/1999 – present
Landsat 8 (OLI; TIRS)	T1-SR, T1-TOA	126/47, 127/47	10:00 - 10:30 AM (16-day)	11/04/2013 – present

Satellite data were processed by the method developed by Ermida *et al.*, (2020) [33]. JavaScript codes in GEE platform were used for extracting and analyzing L/WST in Quynh Luu district. The intersection of the geometries of L/WST and shrimp farming areas was applied to detect WST at shrimp farms in the study area (Figure 2).

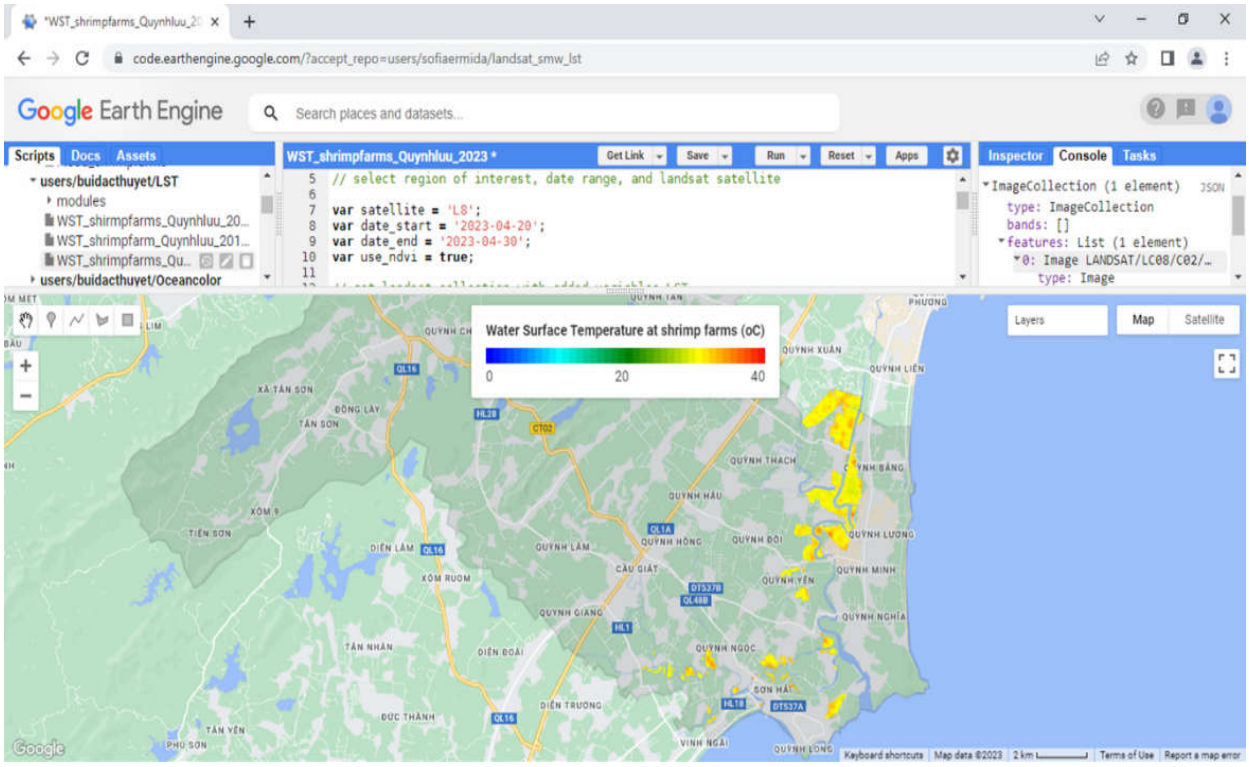

Figure 2. GEE platform interface of water surface temperature at shrimp farms in Quynh Luu district, Nghe An province, Vietnam

3. RESULTS AND DISCUSSION

3.1. Temporal variation of water surface temperature at shrimp farms

Satellite-derived WST at shrimp farms in Quynh Luu district ranged from 12.3°C to 39.4°C, mainly concentrated at 20 - 33°C (around 80% retrieved WST from Landsat data acquired 2000 - 2023) (Figure 3). WST was also found to show seasonal variation, reaching a maximum in July (defined as summer seasons) with monthly mean WST of 30.7°C, and its minimum in December (defined as winter season) with monthly mean WST of 21.8°C (Figure 4). Monthly mean WST at shrimp farms in Quynh Luu district aligns with the pattern of monthly mean air temperature in the North Central of Vietnam - Vinh Hydrometeorological Station in Nghe An province [40].

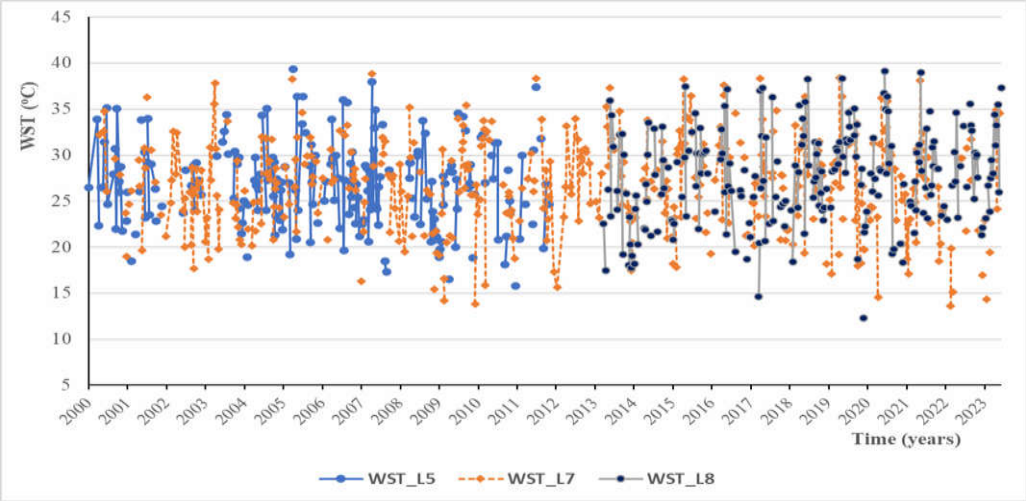

Figure 3. Water surface temperature (WST) at shrimp farms in Quynh Luu district, Nghe An province, Vietnam from 2000 to 2023. Data retrieved from Landsat 5 (L5), Landsat 7 (L7) and Landsat 8 (L8)

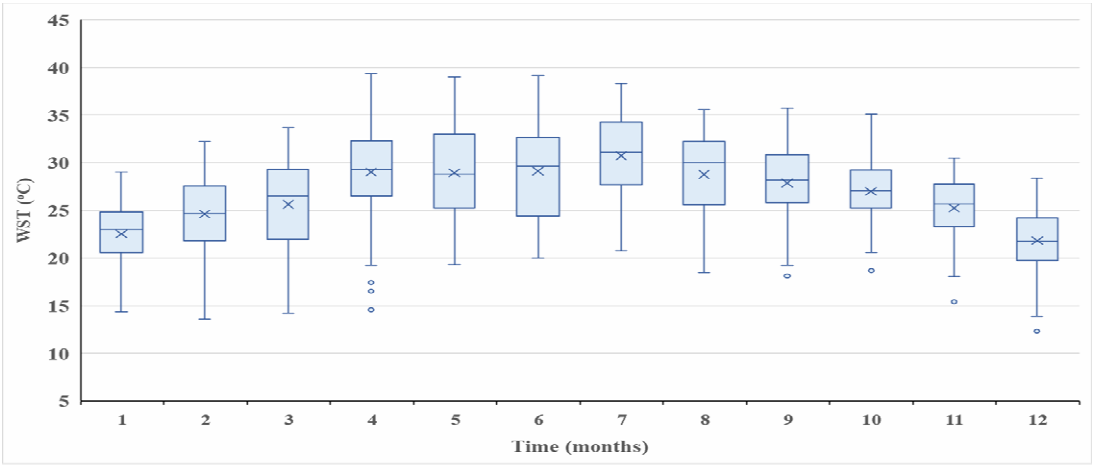

Figure 4. Boxplot of the monthly water surface temperature (WST) at shrimp farms in Quynh Luu district, Nghe An province, Vietnam. Data retrieved from Landsat 5, 7 and 8 from 2000 to 2023

Satellite-derived WST detected in this study was more fluctuated and higher at some certain days during summer months (e.g., reaching 38 - 39°C in April 2005 and May 2021) in the comparison with in situ measurements of water temperature at some shrimp farms in Quynh Luu district (e.g., 28 - 34°C in April - July 2015) [41]. This was due to WST retrieved from remotely sensed data was measured in the top layer of water bodies (i.e., approximately the upper 100 µm, called 'skin temperature') [9, 42] and it was highly affected by solar radiation and atmospheric temperature [43]. Thus, skin temperature may differ from the in situ temperature measured at depth, also known as (bulk) surface temperature [42]. Further study for assimilation of satellite-derived temperature observations with in situ monitoring data are therefore highly recommended in order to understanding water thermal patterns, contributing for water quality monitoring at shrimp farms in Quynh Luu district, Nghe An province.

3.2. Water surface temperature at shrimp farms under climate change

Annual WST at shrimp farms in Quynh Luu district was found to show a warming trend from 2000 to 2022 (Figure 5a). Using a linear regression model, the rate of change is defined by the slope of regression line which in this case was about 0.0103°C during the period of 2000 - 2022 (Figure 5a). The finding aligns with research on global warming and climate change of IPCC (2022) in which water temperature has increased both inland water bodies (e.g., up to 1°C per decade in

rivers and 0.45˚C per decade in lakes) as well as sea water surface (0.68 - 1.01˚C from 1850 - 1900 to 2011 - 2020) [4].

In addition, there was a seasonal variation of long-term thermal trend at shrimp farms in Quynh Luu district from 2000 to 2022, in which WST has decreased in winter and spring seasons (Figure 5b) and increased in summer and autumn seasons (Figure 5c). This means that WST at shrimp farms in Quynh Luu district is warmer during shrimp farming season there (normally form April to October each year) in the context of climate change. Shifts in WST regime not only directly affects cultured shrimps but also causes changes of water quality in shrimp ponds due to its alteration in many physical, chemical, and biological processes (e.g., dissolved oxygen concentration, the percentage of toxic unionized ammonia form - NH_3, the rate of chemical reactions, metabolic rates and nutrient cycling) [2, 3]. In other words, shrimp farming in Quynh Luu district were found to be affected by climate change in relation to warming trend of WST during shrimp farming season.

Research revealed that global warming, reaching 1.5˚C in the near-term, and more frequent and intense extreme weather events (e.g., heat waves) has caused widespread adverse impacts to nature and people, across sectors and regions [4]. Based on climate change scenarios (RCP4.5 and RCP8.5) for Vietnam developed by Ministry of Natural Resources and Environment, mean annual temperature in Nghe An province is predicted to increase 0.8 - 2.2°C (RCP4.5) and 1.3 - 2.8°C (RCP8.5) in 2046 - 2065, and 1.0 - 2.9°C (RCP4.5) and 2.0 - 4.8°C (RCP8.5) in 2080 - 2099 [44]. Thus, aspect of global warming and climate change should be considered in planning for shrimp farming in Quynh Luu district, Nghe An province.

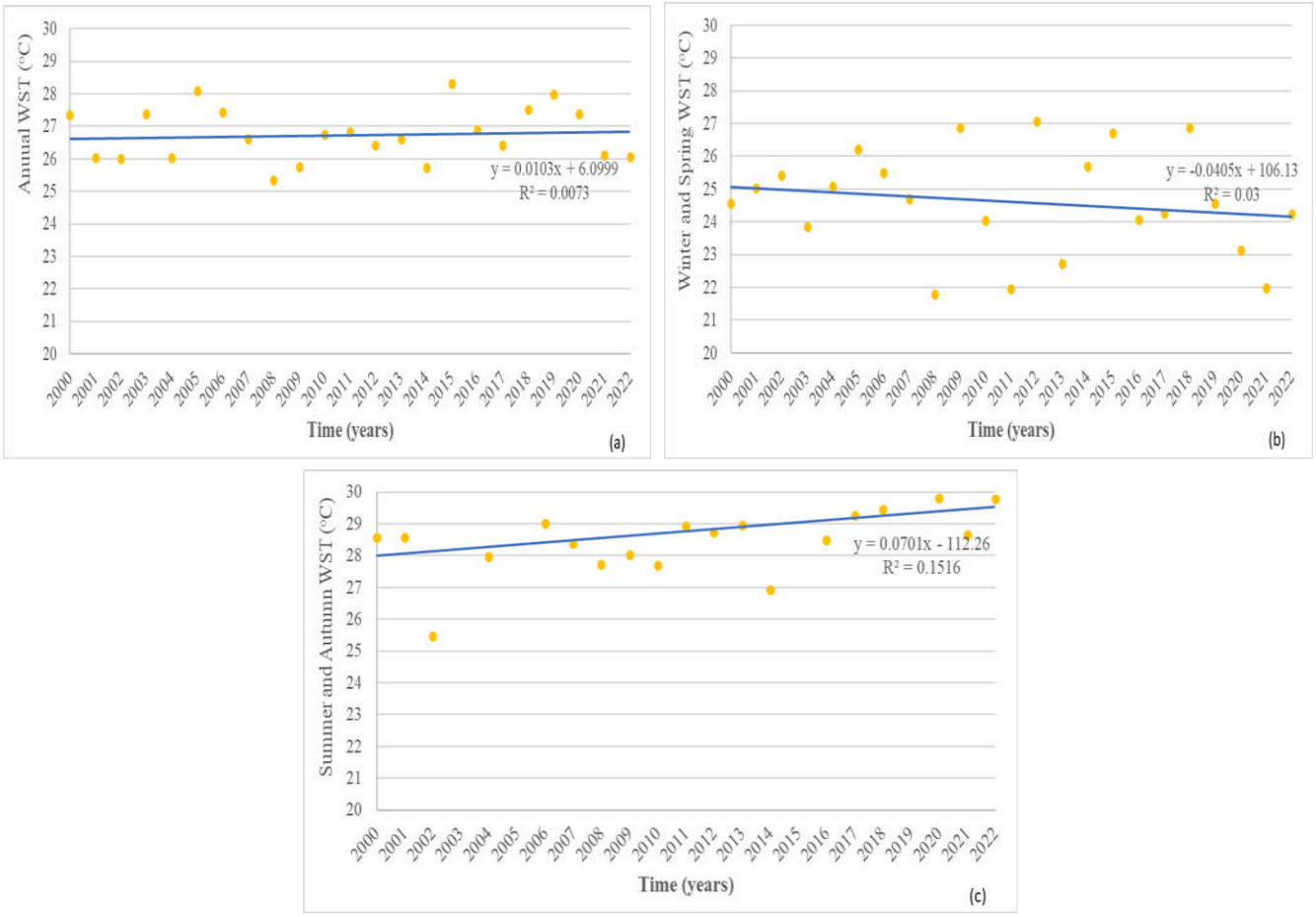

Figure 5. Water surface temperature (WST) trend at shrimp farms in Quynh Luu district, Nghe An province, Vietnam for the period 2000-2023: a) Annual; b) Winter and Spring; c) Summer and Autumn

4. CONCLUSION

Finding of this study showed that satellite-derived WST at shrimp farms in Quynh Luu district ranged from 12.3°C to 39.4°C. There was a seasonal variation of WST at shrimp farms in the study area with the highest monthly mean WST in July (30.7°C) and the lowest monthly mean WST in December (21.8°C). Annual WST at shrimp farms in Quynh Luu district showed an increasing trend from 2000 to 2022. A remarkable warming trend of WST was detected during shrimp farming season (often in April to October each year) and this may lead to high risk for shrimp farms there. Besides limited availability of in situ water temperature data, satellite-derived WST at shrimp farms in Quynh Luu district, Nghe An province has provided essential information to inform the sustainable development of shrimp farming in the context of climate change.

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ISOLATION AND CHARACTERIZATION OF BACTERIAL PATHOGENS CAUSING HEMORRHAGIC DISEASE IN ASIAN SWAMP EEL (*Monopterus albus* Zuiew, 1793) IN THE MEKONG DELTA

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ABSTRACT

Several types of diseases occur during Asian swamp eel (*Monopterus albus*) culture, the most prevalent being bacterial hemorrhagic disease. In the Mekong Delta, farming Asian swamp eel (*M. albus*) is severely affected by a highly fatal hemorrhagic disease that causes massive economic losses. This study aimed to determine the causative agent of hemorrhagic disease in Asian swamp eels. Several clinical signs were observed, including red swollen vents, head edema, abdomen and/or internal organ hemorrhage. A total of 52 bacterial isolates were recovered on Tryptone Soya Agar from different diseased eel farms in the Mekong Delta, including Hau Giang, Can Tho, An Giang, Vinh Long, and Bac Lieu provinces. A bacterial morphology examination showed that the isolates were Gram-negative, short rod-shaped, 0.1 - 0.3 mm colony size with creamy yellow color after 24 hours incubation at 28°C. Furthermore, an assessment of the biochemical identification system indicated the presence of motile *Aeromonas* spp. In addition, the 16S rRNA gene sequencing results confirmed that the majority of the isolates were *Aeromonas veronii*. A challenge test was carried out to determine the pathogenicity of *A. veronii* isolates collected from the eels. The calculated LD_{50} value of the CT07 isolate was $1.51x10^4$ CFU/eel after a 14-day injection in eels with clinical signs similar to those observed in diseased eels on the farms. Moribund eels were also sampled for histopathological examination. Moreover, antibiotic susceptibility testing of 19 isolates was performed using eight antibiotics in this study.

Keywords: *Aeromonas veronii, Asian swamp eel, challenge test, hemorrhagic disease, antibiotic susceptibility.*

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1. INTRODUCTION

The Asian swamp eel (*Monopterus albus*) is a commercially crucial cultured freshwater fish in China and Southeast Asian countries, including Vietnam [1]. In Vietnam, seafood is considered a key economic sector, particularly in the Mekong Delta. Scientists are especially interested in the eel since it has the ability to change females [2]. Along with that development, farmers also face a big problem of disease in Asian swamp eels. In particular, a group of bacteria, *Aeromonas* spp., was commonly recorded as the main pathogen in freshwater fish in the Mekong Delta.

Aeromonas veronii has caused significant losses in many cultured freshwater and marine fish species worldwide. It has infected almost all freshwater and some brackish water fish species and caused mass mortalities in severe production and economic losses [3]. *Aeromonas* spp. isolated from EUS-infected fish was found virulent in fish on artificial infection studies [4]. Among different motile *Aeromonas* species, *A. veronii* has recently emerged as one of the important fish pathogens causing infections in catfish, *Ictalurus punctatus* [5], tilapia, *Oreochromis niloticus* [6], European seabass, *Dicentrarchus labrax* [7], goldfish,

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Carassius auratus [8], common carp, *Cyprinus carpio* [9]. However, there have been no specific studies on bacterial hemorrhagic diseases in Asian swamp eels in the Mekong Delta. Due to the diversity of pathogens, the prevention and treatment of diseases in aquatic animals is only effective when the causative agent of the disease is accurately identified. To find out more information on the causes of hemorrhagic disease in swamp eels, the study entitled "Isolation and characterization of bacterial pathogens causing hemorrhagic disease in Asian swamp eel (*Monopterus albus* Zuiew, 1793) in the Mekong Delta)" was carried out.

2. MATERIALS AND METHODS

2.1. Sample collection

Asian swamp eels with clinical signs such as hemorrhage at internal organs and/or head edema were collected from eel farms in the Mekong Delta, including Hau Giang, Can Tho, Vinh Long, and Bac Lieu provinces from April 2021 to May 2023. The eel samples were collected directly at the farm and delivered to the laboratory at the Faculty of Aquatic Pathology (CTU) for further analysis.

2.2. Bacterial isolates

The liver and kidney of eel samples were streaked onto tryptone soy agar (TSA, Merck), glutamate starch phenol red (GSP, Merck), and incubated at 28°C for bacterial isolation. After 24 hours of incubation, the predominant colonies were subcultured until pure colonies were obtained. Then, the isolates were preserved at -80°C in tryptone soya broth (TSB, Merck) containing 25% glycerol for further study.

2.3. Morphological and biochemical identification

The obtained colonies were examined for morphological features, including color, Gram staining, motile, oxidase, catalase, and oxidative-fermentative (O/F) tests [10]. Biochemical characterization of the isolates was performed using the API® 20 E (Biomerieux, France) according to the manufacturer's instructions.

2.4. 16S rRNA sequencing and phylogenetic analysis

The isolated colonies were species-identified using 16S rRNA sequences. The bacterial 16S rRNA amplification was conducted using the universal primers 27F (5′-AGAGTTTGATCCTGG CTCAG-3′) and 1492R (5′-GGTTACCTTGTTACG ACTT-3′). The multiple alignments of the 16S rRNA sequences achieved in this study and their closely related species retrieved from GenBank were conducted using the Clustal W method, and the phylogenetic tree was performed using the Neighbor-Joining method with the bootstrap set to 10,000 replicas in MEGA 7.0 software [11].

2.5. Experimental determination of the bacterial virulence

All isolates were evaluated for their capacity to cause disease in eels using 10^6 and 10^5 CFU/mL injection doses. The CT07 strain was selected for further investigation due to its potential to cause eel death (data not shown). Healthy eels (6 - 9 g) were handled in accordance with the Animal Ethics Committee of Can Tho University. The eels were randomly arranged at a density of 10 eels/tank (35 L) with a water column around 10 cm high, aeration, and nylon rope as substrates.

The pathogenic bacteria strain CT07 was recovered from glycerol stocks and streaked directly onto TSA. After 24 hours, a pure colony of each isolate was cultured in 10 mL of TSB overnight at 28°C, 200 rpm. To determine the 50% lethal dose of pathogenic bacteria based on a previous study [12], five groups of healthy eels (n = 150) were injected intraperitoneally with the bacterial strain at concentrations of $7.73x10^2$, $7.73x10^3$, $7.73x10^4$, $7.73x10^5$ CFU/eel. In contrast, the control group was injected with sterile physiological saline (0.9% NaCl). Thereafter, cumulative mortality was monitored for the next 14 days. The liver and kidney of moribund eels were streaked onto TSA medium for bacterial isolation, and eel tissues (liver, kidney, and intestine) were collected for histopathology examination. These re-isolated strains were

biochemically compared to the CT07 strain used in the challenge.

2.6. Histopathological analysis

Internal organs (liver, kidney, and intestine) were collected from moribund eels and fixed in a 10% NBF (Neutral buffered formalin) solution. After 24 hours, the samples were rinsed with running water, transferred to 75 - 100% ethanol for dehydration, cleared with xylene, and finally infiltrated with paraffin wax. The standard paraffin embedding procedure was applied to the section with a thickness of 5 µm, and the slices were stained with hematoxylin and eosin (H&E) methods. Light microscopy (ECLIPSE E200, Nikon) was utilized to detect pathological changes in stained histological sections.

2.7. Investigation of antibiotic susceptibility

Antibiotic susceptibility of 19 bacterial isolates was carried out using the disc diffusion method under the Clinical and Laboratory Standards Institute guidelines [13]. A total of 8 antibiotics, including amoxicillin/clavulanic (AMX/30 µg), cefotaxime (CTX/30 µg), cephalexin (CL/30 µg), colistin sulfate (CT/50 µg), doxycycline (DO/30 µg), florfenicol (FFC/30 µg), rifampicin (RIF/30 µg), trimethoprim/sulfamethoxazole (SXT/1.25/ 23.75 µg), were employed to evaluate bacterial susceptibility. The inhibition zones of diameter were classified as (S) susceptible, (I) intermediate, and (R) resistant [13].

3. RESULTS AND DISCUSSION

3.1. Clinical symptoms and sample collection

The clinical signs of infected Asian swamp eels are shown in figure 1, mainly as a hemorrhage at internal organs and/or head edema. In the nursing stage, abdominal bleeding was the primary clinical symptom (Figure 1.A). In the adult stage, the body of infected eels appeared with sores and/or head edema (Figure 1.B-C).

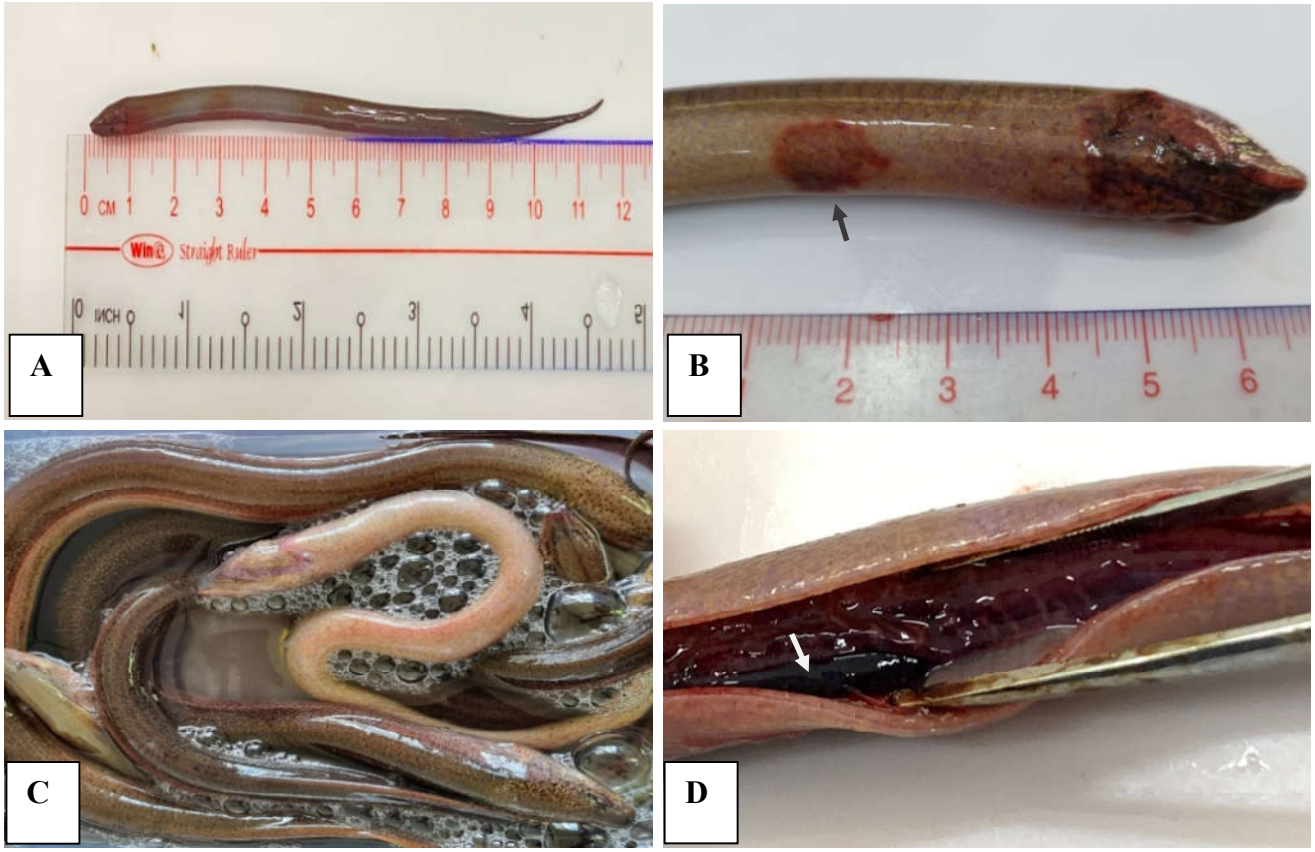

Figure 1. Clinical symptoms of infected Asian swamp eel

(A) Abdominal bleeding in the nursing stage; (B, C) eel with ulceration and edema of the head in the adult stage; (D) clinical sign of internal organ hemorrhage in diseased eels

The results of the bacterial isolation from diseased eels are shown in Table 1. A total of 158 diseased eels were used, and 52 isolates of *Aeromonas* spp. were obtained from the eel liver and kidney.

Table 1. Summarised results of *Aeromonas* spp. isolation from Asian swamp eels infected with hemorrhagic disease

Time	Location	Number of eels sampled	Number of *Aeromonas* spp. isolates
April to June 2021	An Giang province	7	2
June to October 2022	Vinh Long province	21	7
March 2022 to May 2023	Hau Giang province	92	32
April to December 2022	Can Tho city	28	9
May 2023	Bac Lieu province	10	2
Total		158	52

3.2. Bacterial identification

All isolates grew well on TSA after 24 hours incubation at 28°C. The colonies were round, slightly raised, creamy yellow on TSA (Figure 2.A) and yellow on GSP agar (Figure 2.B). The bacterial isolates were capable of causing β-hemolysis on BA medium supplemented with 5% sheep blood (Figure 2.C). The morphological characteristics of the isolated bacteria showed that they were motile, giving positive reactions to catalase, oxidase, oxidizing, and fermenting (O/F) glucose. Gram-staining showed that the bacterial cells stained red/pink and appeared short rod-shaped, indicating that the strain was Gram-negative (Figure 2.D).

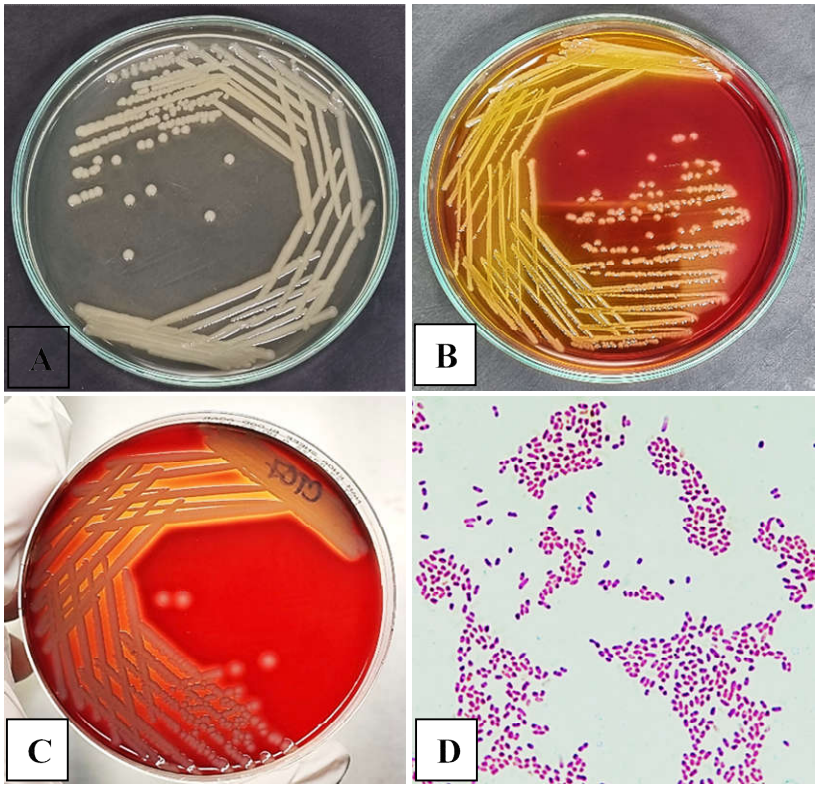

Figure 2. Characterization of the isolated bacteria

(A) Colonies on TSA; (B) Colonies on GSP; (C) Colonies on BA medium supplemented with 5% sheep blood; (D) Gram-stained bacterial cells (100X).

The physiological and biochemical characteristics of 52 isolates are presented in Table 2. According to the biochemical results using the API 20E kit, the bacterial isolates collected from hemorrhagic eels exhibited biochemical features compatible with *Aeromonas* spp., as described by previous studies [14], [15]. The isolated bacteria were phenotypically identical except for the ADH, ODC, TDA, VP, AMY, and ARA reactions (Table 2).

Table 2. Results of physiological and biochemical characteristics of isolated bacteria

Identification test (s)	*Aeromonas veronii* ATCC 35604^T [14]	*Aeromonas veronii* [15]	*Aeromonas* spp. (This study)
Gram	-	-	-
Shape	Rod-shaped	Rod-shaped	Rod-shaped
Motility	Motile	Motile	Motile
Oxidase	+	+	+
Catalase	+	+	+
O/F test	+/+	+/+	+/+
ONPG	+	+	+
ADH	-	+	+
LDC	+	+	+
ODC	+	-	-
CIT	+	+	+
H_2S	-	-	-
URE	-	-	-
TDA	+	-	+
IND	+	+	+
VP	+	-	+
GEL	+	+	+
GLU	+	+	+
MAN	+	+	+
INO	-	-	-
SOR	-	-	-
RHA	-	-	-
SAC	+	+	+
MEL	-	-	-
AMY	+	-	+
ARA	-	-	+

Notes: (+) positive; (-) negative.

In addition to the API 20E examination, 16S rRNA sequencing and phylogenetic analysis are used for species identification. The BLASTn results showed that the CT07 strain was 99.7%

identical to *Aeromonas veronii* strain HN1903 (Accession: MT990643.1) in the NCBI database. After multiple alignments of highly similar sequences, the phylogenetic results indicate that the 16S rRNA gene sequence of *A. veronii* forms a distinct cluster (Figure 3). As a result, the strain was designated as *A. veronii* based on its morphological, physiological, biochemical, and molecular properties.

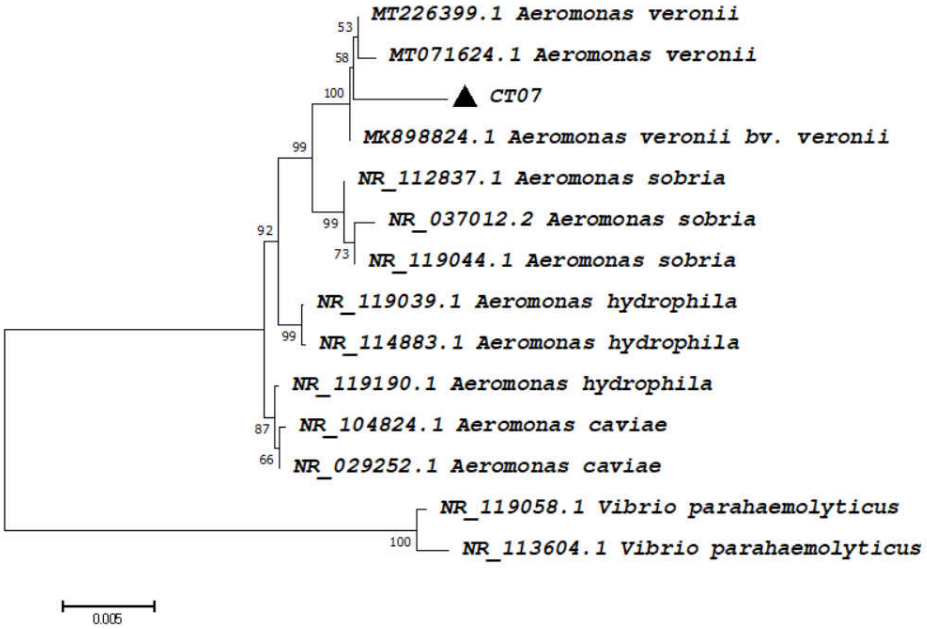

Figure 3. Phylogenetic tree between isolate (CT07) and other reference strains based on 16S rRNA gene sequences. At branching nodes, the bootstrap support values are provided

Motile *Aeromonas* species are diverse and widely distributed in freshwater environments [16]. Previous studies reported that *A. veronii* was an opportunistic bacterium that caused high mortality in aquatic animals [17], [18]. To date, several freshwater species were infected by *A. veronii,* such as channel catfish, *Ictalurus punctatus* [5], tilapia, *Oreochromis niloticus* [6], European seabass, *Dicentrarchus labrax* [7], goldfish, *Carassius auratus* [8], common carp, *Cyprinus carpio* [9]. In this study, the clinical symptoms (Figure 1) were remarkably comparable to those observed in a previous study on Asian swamp eels infected with *A. veronii* in China, including red swollen vents, head edema, abnormal swimming, abdomen hemorrhage and hemorrhage at internal organs [19].

3.3. Pathogenicity of *Aeromonas veronii* in Asian swamp eels

Figure 4. Clinical signs of eels post-challenge with strain CT07. (A) surface hemorrhage, (B) abdomen hemorrhage, (C) head edema, (D) red swollen vents

To calculate the lethal dose of the CT07 strain in Asian swamp eels, the challenge experiment was conducted at doses of $7.73x10^2$, $7.73x10^3$, $7.73x10^4$, $7.73x10^5$ CFU/eel (with abbreviated codes as CT07-2, CT07-3, CT07-4, and CT07-5, respectively) and treatment was injected with physiological saline (0.9% NaCl) as a control treatment.

The challenged eels exhibited typical symptoms of diseased eels similar to those observed at the farms, including lethargic swimming, anorexia, loss of mucus, abdominal hemorrhage, head edema, red swollen vents, liver, and renal hemorrhage (Figure 4), which were similar to previous study report [19]. Previous studies showed that *Aeromonas* spp. infection was the most dangerous causative agent in several aquatic animals [15], [20].

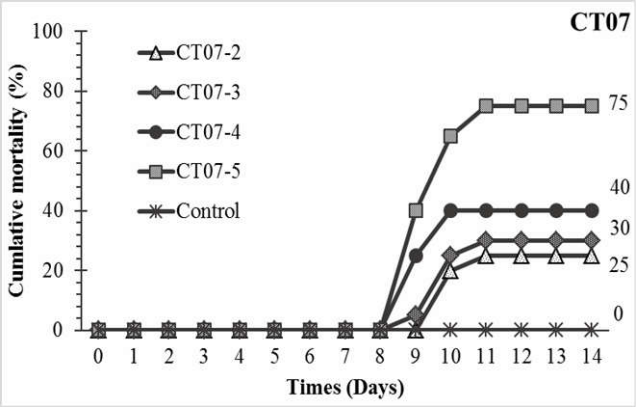

Figure 5. Cumulative mortality (%) of healthy Asian swamp eel injected with CT07

All challenge treatments exhibited mortality during the 14-day experimental monitoring, whereas no mortality was noted in the control group. In particular, the eels began to show signs of lethargy swimming and slow response to noise on the first day. At all bacterial challenge treatments, the mortality started on day 8, where the CT07-6 treatment had the highest mortality. Afterward, the cumulative mortality percentage increased dramatically in all treatments, and the eels ceased dying on day 12 post the injection (Figure 5). The challenge results showed that the calculated LD_{50} value was 1.51×10^5 CFU/mL and lower than the previous study of 1.52×10^7 CFU/mL [19]. Therefore, farmers should be aware of the presence of *Aeromonas* spp. in the Asian swamp eel hatchery and have suitable treatments to prepare a pond before stocking. Similarly, acute mortality was also found in the experimental challenge of red tilapia (*Oreochromis* sp.) with *A. veronii* [21]. Furthermore, *A. veronii* severely threatens freshwater species and could harm fish health [22]. The discrepancies in the clinical signs and pathological changes in the infected fish might be associated with the kind and degree of stress exerted on the fish, the level of bacterial virulence, the genetic disease resistance of the fish species, and different strains of the bacteria [19].

3.4. Histopathology of infected eels

Histological observations at the liver, kidney, and intestine showed that hemorrhage appeared on all three organs when the eels were infected with *A. veronii* (Figure 6). In the liver, congestion was recorded in the blood vessels and capillaries, followed by the degeneration of hepatocytes and the concentration of inflammatory cells (Figure 6.A-B). In addition, the hemorrhage was presented in the renal tissue, and proximal tubules were observed with necrosis leading to loss of tubular structure. Concentrations of inflammatory cells, melano-macrophages, and necrotic tissues around the hemorrhagic zone were also recorded (Figure 6.C-D). The mucous membranes in the intestine were shed and the intestinal villi were collapsed, leading to a disruption in the absorption of nutrients. Red blood cells hemorrhagic from the inside of the intestinal villi and connective tissue layer slough into the intestinal lumen, accompanied by sloughing of mucous-secreting cells and intestinal microvilli (Figure 6.E-F). In this study, the histological structure of eels infected with *A. veronii* was similar to previous studies on other species [15], [23]. Moreover, all internal organs (especially intestinal tissue) of cultured eels infected with *A. veronii* in China were also observed [19]. Multi-organ hemorrhage of infected eels is suggested to be related to the hemolytic virulence of *Aeromonas* species [15], and the bacteria hemolysis was confirmed when grown on blood agar plates. The signs of coagulation necrosis and renal structural disorder

in the previous study were similar to this study [19]. In addition, fragmentation, shrinkage, and fading of kidney cell nuclei were also observed. (Figure 6.D). Pathological signs were seen in renal tissue-specific to *A. veronii* infection.

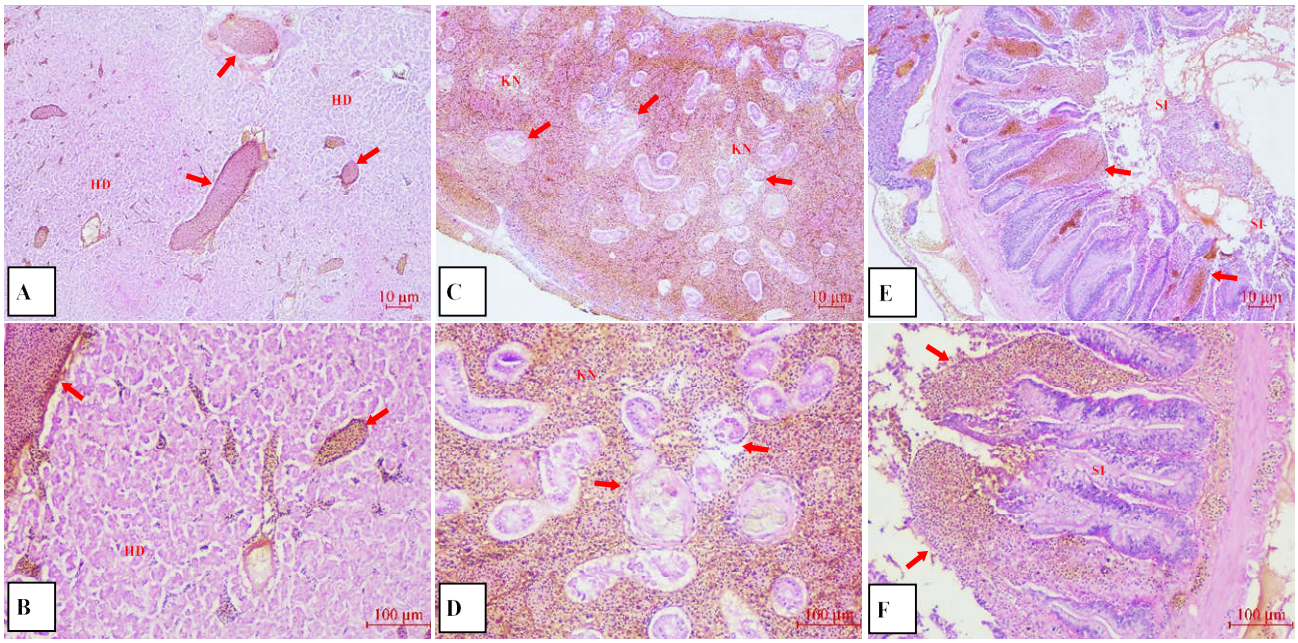

Figure 6. Histopathological changes of the liver, kidney, and intestine from the infected eels ***(A, B): liver of diseased eel with hepatocyte degeneration (HD) and congestion in the blood vessels (red arrow); (C, D): kidney of diseased eel observed inflammatory kidney tissue (KN) and tubular coagulation necrosis (red arrow); (E, F): intestine of diseased eel with the shedding of the mucous membrane (SI) and hemorrhage from the connective tissue (red arrow).***

3.5. Antibiotic susceptibility

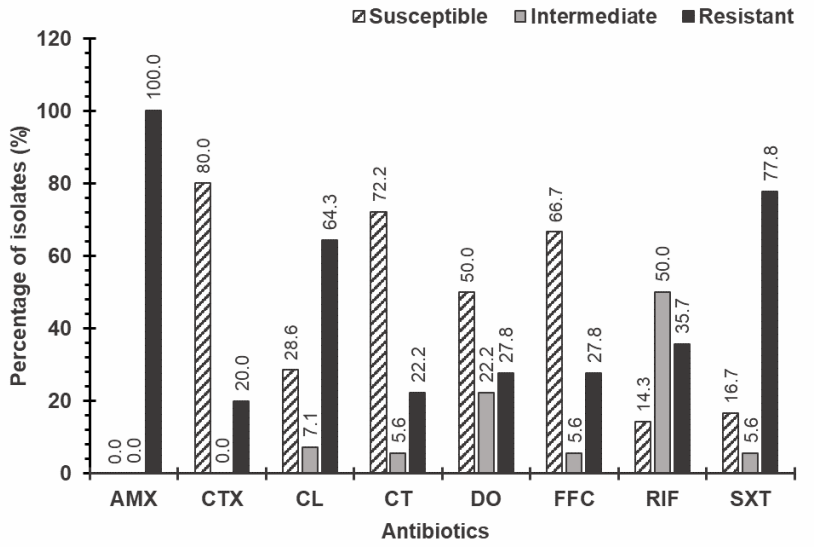

Figure 7. Antibiotic susceptibility evaluations on isolates

Amoxicillin/clavulanic (AMX/30 µg), cefotaxime (CTX/30 µg), cephalexin (CL/30 µg), colistin sulfate (CT/50 µg), doxycycline (DO/30 µg), florfenicol (FFC/30 µg), rifampicin (RIF/30 µg), trimethoprim/sulfamethoxazole (SXT/1.25/23.75 µg).

The antibiotic susceptibility data of 19 *A. veronii* isolates against eight antibiotics are shown in figure 7. The results revealed that the majority of the isolates were susceptible to cefotaxime

(72.22%) and colistin sulfate (80%). In contrast, *A. veronii* isolates exhibited a more significant percentage of resistance to cephalexin (64.28%), trimethoprim/sulfamethoxazole (77.77%), and amoxicillin/clavulanic (100%). The previous study suggested that the first option of treatment for *Aeromonas* infections is cephalosporins, aminoglycosides, quinolones, tetracyclines (excluding tetracyclines), phenicols, nitrofurans, and sulfonamides [19]. In addition, *A. veronii* was also found highly susceptible to cefotaxime, nevertheless resistant to two other medicines, ampicillin and nalidixic acid [24].

Overall, the principle of utilizing drugs should not only consider drug sensitivity but also strictly comply with regulations for aquatic products. Therefore, we recommend florfenicol and cefotaxime to control hemorrhagic disease in Asian swamp eels.

4. CONCLUSION

The main pathogen causing hemorrhagic disease in diseased Asian swamp eels in the Mekong Delta was confirmed to be *Aeromonas veronii* by morphological, biochemical identification, and 16S rRNA sequencing. The experimental injection challenge study was performed and fulfilled Koch's postulates with the LD_{50} of $1.51x10^5$ CFU/eel. The pathological changes were noted in the liver, kidney, and intestines of Asian swamp eels infected with *A. veronii*. Antibiotic sensitivity testing showed that the isolated bacteria were susceptible to several antibiotics, and we recommend florfenicol and cefotaxime to control hemorrhagic disease in Asian swamp eels.

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VIBRIO - BACTERIA - CONTROLLING POTENTIAL OF A SEDIMENT BIOELECTROCHEMICAL SYSTEM INTEGRATED IN A TEN-LITER-SCALED BRACKISH AQUACULTURE MODEL - AN INITIAL UPSCALE STUDY

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ABSTRACT

Vibrio bacteria are among the most serious pathogens of aquatic creatures. Furthermore, they can cause diseases at any stage in the lifecycle of aquaculture animals with mortality up to 100%, which results in great economic losses. To control these agents, the currently widely-applied solution is to use antibiotics, which cause environmental pollution and drug resistance in microorganisms. Another solution is biological products, which have not yet been proven highly effective. In this context, there have been studies showing that a promising new solution is to use a sediment bioelectrochemical system (SBES) though only at small scales (<1 L). In this study, we evaluated the *Vibrio*-bacteria-controlling capability of a sediment bioelectrochemical system (SBES) integrated into a 10 L scale model that mimics a water column in a real-life brackish aquaculture pond. The viability of three strains *Vibrio harveyi* NBRC15634 (*Vh*), *Vibrio parahaemolyticus* NBRC12711 (*Vp*), and *Vibrio parahaemolyticus* CED (wild-type, isolated from actual ponds) when exposed to the filtrates obtained from the cathode solution and the anode solution of the system, was determined over time by plate counting on the specific TCBS medium. The results showed that the density of *Vibrio* bacteria did not change when exposed to the filtrate from the cathode but decreased significantly, up to 99% when treated with the filtrate from the anode of SBES, while no similar phenomenon occurred with the control. Even when the two *Vibrio* strains were present simultaneously, the SBES reduced the densities of *Vp* (in CFU/mL) by approximately 2 logs, and completely eliminated *Vh* after 3 hours. In summary, using the SBES will be a potential method to efficiently control pathogenic *Vibrio* bacteria in brackish aquaculture while offering simple implementation.

Keywords: *Sediment bioelectrochemical system, Vibrio harveyi, Vibrio parahaemolyticus.*

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1. INTRODUCTION

Aquaculture brings many benefits to Vietnam, such as creating millions of jobs, providing food, and earning great export value, thereby promoting our economy [1]. Nevertheless, this vital economic sector often faces several challenging issues, such as environmental pollution, inconsistent product quality, and aquatic diseases. One of the most common diseases is vibriosis caused by *Vibrio* bacteria [2]. For example, shrimp vibriosis is a highly destructive disease

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that affects many species of shrimp and is caused by many *Vibrio* species [3].

Vibrio bacteria are Gram-negative, have rod-shaped or comma-shaped cells, and usually inhabit environments with high salinity [4]. *Vibrio* species often interact in shared habitats, leading to *Vibrio* infections with high mortality rates both in the wild and aquaculture systems [5]. Negative changes in environmental factors can trigger the rapid multiplication of pathogenic bacteria already present at low levels in shrimp, leading to disease outbreaks and causing up to 100% production loss [6]. They cause various problems in shrimp during their different life stages. These problems may range from growth retardation to sporadic mortality and, eventually, mass mortality [7].

Among the *Vibrio* species, *Vibrio parahaemolyticus* and *Vibrio harveyi* are serious pathogens responsible for disease outbreaks, which infect from the first days of shrimp stocking [8]. Infection with *V. harveyi* can result in various diseases in shrimp, including red tail syndrome, luminous disease, …[9], while infection with *V. parahaemolyticus* causes acute hepatopancreatic necrosis disease (AHPND) in shrimp. The latter has affected the shrimp farming industry for nearly a decade, resulting in billions of dollars of losses [10].

Several measures have been taken to reduce the presence of *Vibrio* bacteria in aquaculture ponds, such as disinfectants, antibiotics, and microbial products [2]; however, each method has its disadvantages, the most serious of which are low efficiency and environmental pollution. Disinfectants can reduce the density of *Vibrio* bacteria but often cause secondary pollution and can affect the health of fish and shrimp, so they can be usually only applied before or after rearing [11]. Antibiotics are commonly used today, but the abusive use of antibiotics destroys beneficial bacteria in the animals' digestive tracts and the environment. In particular, the overuse of antibiotics leads to the widespread development of antibiotic-resistant bacteria, which becomes a significant threat to humans [12]. *V. harveyi* strains that are multi-resistant to streptomycin, erythromycin, and cotrimoxazole have been found to cause mass mortality in shrimp larvae [7]. In addition, recent studies have shown that most *V. parahaemolyticus* isolated from both wild and aquaculture environments are resistant to many drugs [13]. That negative consequence has led to the proposition for the use of probiotics. However, the reality shows that the improper use of probiotics and the loss of their quality control make this measure ineffective [14]. Therefore, viable, practical, economical, and environmentally safe alternatives are urgently required to control *Vibrio* bacteria in aquaculture.

A promising new solution recently discovered is the use of bioelectrochemical systems (BESs), which are known to have antibacterial capabilities. In bioelectrochemical systems, energy-rich organic substances are often used as electron donors at the anode, where they are degraded by electrochemically active microorganisms; and the released electrons are transferred to the anode and then move through the external circuit to the cathode to be accepted by an electron acceptor, usually oxygen (thereby forming water), thereby completing a redox reaction [15]. Recent studies have shown that a sediment BES works well in brackish pond culture and enhances the ability of *in situ* treatment of organic pollution in the water and the sediment of aquaculture ponds [16]. Another promising application potential of BESs is their ability to treat pathogenic bacteria. Pioneering studies found that the bactericidal effect of BESs might be related to the production of H_2O_2, pH increases, or oxidant residues due to reactions at the cathode [17 – 19]. Subsequently, Ieropoulos *et al.* (2019) [20] discovered an apparent bactericidal effect of the anode medium. This is further demonstrated by the study of Vasieva *et al.* (2019) [21] on the microbial community in the anode of BESs, which showed a substantial reduction in the amount of DNA of common pathogenic bacteria such as *Vibrio*, *Shigella*, *Yersinia*, *Haemophilus*, and *Pseudomonas* bacteria.

The above-mentioned findings are the basis for the idea of integrating BESs in aquaculture

ponds to reduce pathogenic *Vibrio* bacteria. Such integration is practically possible using a sediment BES (SBES), which has a sediment anode and a floating cathode, as demonstrated in previous studies [16], [22]. Such an SBES integrated into a lab-scale brackish aquaculture model displayed an impressive ability of controlling *Vibrio* bacteria at a small scale (< 1 L), eliminating the *Vibrio* bacteria entirely after only 5 minutes; while the control did not have such an ability. We also discovered that such an effect was solely due to the SBES aqueous solution, rather than physical activities or biological contacts. In order to be able to apply such a promising application potential in practice, in this study, we have built and operated an integrated model of a 10-liter-scaled bio-electrochemical system with a sediment electrode and a height of 1.5 m, mimicking a water column in a brackish water aquaculture pond. We then evaluated the inhibitory ability of SBES against several reference strains of *V. harveyi* and *V. parahaemolyticus*, as well as a wild-type strain of *V. parahaemolyticus* isolated from nature. The results of this study could be the basis for an improved technique to control *Vibrio* bacteria in aquaculture.

2. MATERIALS AND METHODS

2.1. Microbial strains and growth media

In this study, the strains *Vibrio harveyi* NBRC15634 (abbreviated as Vh), *V. parahaemolyticus* NBRC12711 (abbreviated as Vp) (purchased from NBRC, Japan) and *V. parahaemolyticus* CED (abbreviated as Vp CED) (wild type, isolated from shrimp ponds, provided by Research Institute for Aquaculture No.1) were used as representatives of the respective species in the experiments.

The selective thiosulfate-citrate-bile salts-sucrose (TCBS) medium was used for counting the *Vibrio* bacteria. For culturing and maintaining *Vibrio*, Lubria-Bertani (LB) medium was used, with an exceptional NaCl concentration of 3% (w/v) (a concentration previously verified by the team to be the most favorable for the growth of the *Vibrio* strains used).

2.2. Tank models and operation

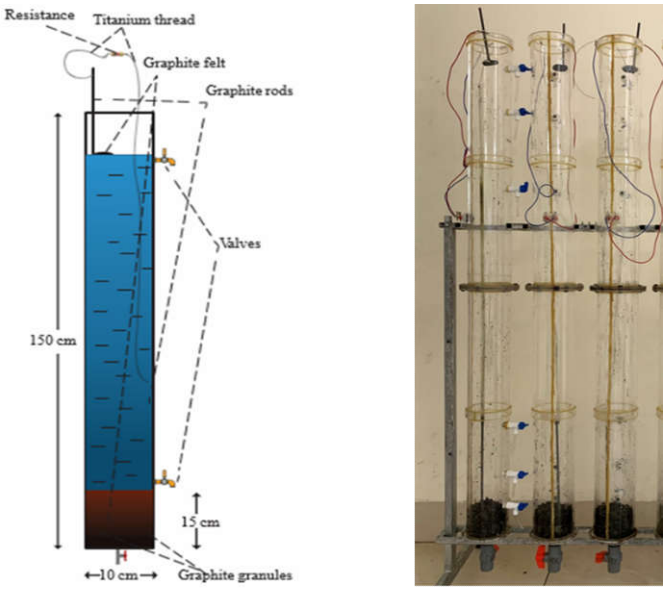

Figure 1. Drawing (A) and actual setups (B) of the column tank model integrated with an SBES in this study

Note: The control tank model is identical but not harboring the SBES components (graphite granules and graphite felt as the anode at the sediment, graphite rods, graphite felt cathode, external resistor, and wires).

Six cylindrical plexiglass columns, each of which is 150 cm high and has a 10 cm diameter, were used as aquaculture tank models (Figure 1A): three identical experimental columns with integrated SBESs (T_C1, T_C2, T_C3) for replication, three identical control columns without SBESs (C_C1, C_C2, C_C3) for replication. The installation of the SBES in each experimental tank was similar to that in previous studies by the group [16], [22]. An SBES generally consists of an anode electrode at the bottom of the column and a cathode electrode at the water's surface. The anode consists of a 10 cm thick layer of graphite particles (2 - 5 mm in diameter) (Xilong, China) evenly covering an underneath 8 cm diameter graphite cloth (0.5 cm thick) (Xilong, China) at the bottom of the tank. A 4 cm diameter graphite felt of the same type was used as the floating cathode (~50% in contact with water and ~50% with air as it floats). A graphite rod (5 mm diameter) was glued to the graphite felt of the electrode to collect the electrical current. The graphite rods of the anode and the cathode were connected by a titanium wire (having a negligible resistance) to an external circuit containing 10 Ω resistors to form a closed circuit. The graphite rod part of the anode and the titanium wire immersed in water were covered with inert acrylic tubing to prevent short circuits. Each test column (integrated with an SBES) has valves in positions corresponding to the anode and cathode for sampling and mounting the reference electrode (Figure 1A). Each model column was filled with 10 L of artificial brackish water (15‰ salinity) generated using Marinium Reef sea salt (Mariscience International Co. Ltd., Thailand).

To enrich electrochemically active bacteria in each SBES, the sediment of the respective test tank was inoculated with 300 g of a mixture of sediment mud samples collected in Quang Ninh province, Vietnam and from an operating SBES in the laboratory. Both experiment and control setups were operated under the conditions mimicking those when culturing 30-day-old white leg shrimp, with brackish water having a salinity ~15‰, and the amount of feed provided was 0.1 g/day (calculated as the equivalent of uneaten daily feed residue (~50% of total feed input) in actual aquaculture ponds, based on our previous calculations) [22]. The systems were operated at room temperature (27 ± 3°C) and pH = 7.

A real-time digital multimeter (model 2700, Keithley, USA) measured the voltage between the anode and the cathode of the SBES installed in each test setup. Electrical parameters (current I(A), voltage U(V)) were measured as previously reported [22]. pH of the water near the anode or the cathode was measured with a pen type digital pH meter (model pH-107, Total Meter Services Inc., Canada) during the operation.

2.3. Preparing bacterial suspensions for experiments, counting bacteria during experiments, and calculating their survival rates

Each *Vibrio* bacteria suspension was prepared by: (i) shaking incubation of the respective test strain in 3% NaCl LB broth for 4 hours, at 200 rpm (at 30°C for Vh or 37°C for Vp and Vp CED); (ii) harvesting the cells from that liquid culture by centrifugation (at ca. 4000 x g, 4°C, for 5 min.); and finally suspending the obtained cell pellet in an appropriate volume of artificial brackish water (having a 1.5% salinity, prepared as described above) so that the cell densities in the final suspension was approximately $1x10^7$ CFU/mL for Vp; approximately $1x10^9$ CFU/mL for both Vh and Vp CED.

The cell density of the tested *Vibrio* strain in any sample of interest was quantified by diluting the sample with sterile artificial brackish water (prepared as described above), plating on TCBS agar, incubating the plates (at 30°C for Vh and 37°C for Vp, Vp CED) and counting the colonies (usually after 24 hours). From the counting results, the cell density of each sample (in CFU/mL) was calculated using standard formulas [4].

2.4. Testing the inhibitory effect of the SBES aqueous filtrate on single *Vibrio* strains

A 100 µL volume of cell suspension containing approximately $1x10^7$ CFU/mL for Vp or approximately $1x10^9$ CFU/mL for both Vh and Vp

CED (prepared as described above) was added to 1.5 mL of a filtrate of interest. Therefore, the cell densities were reduced down to $6.25x10^5$ CFU/mL for Vp and $6.25x10^7$ CFU/mL for Vh, Vp CED. These are the densities that were actually tested. These cell densities are at the levels causing lethality for shrimp, which is at least 10^5 CFU/mL for Vp and 10^7 CFU/mL for Vh [23]. The filtrates of interest include those from the aqueous solutions at the cathode or the anode, in both the experimental and the control setups. For the control, the upper position near the water surface is considered to be equivalent to the cathode position, and the position at the bottom of the column sludge is considered to be equivalent to the anode position. To prepare a filtrate, the respective aquaeous solution was taken and filtered with a 0.22 µm filter (to remove microorganisms). We only tested the filtrates because our previous study results (at a scale of < 1 L) showed that the inhibitory effect on *Vibrio* was mainly due to the chemical components in the system fluid, rather than physical factors and direct biological contacts (Nhung, Phuong, *et al.*, manuscript to be accepted). Hereafter, the filtrates from the cathode and the anode of the experimental setups integrated with SBESs and the respective ones of the control are called the test/control cathode/anode filtrates, respectively. *Vibrio* survival in the samples was determined (as shown above) after 5 min, 30 min, 90 min, and 180 min upon the addition of the tested cell suspension.

2.5. Testing the inhibitory effect of the SBES aqueous filtrate on Vp and Vh when they were co-present

100 µL of cell suspension containing approximately $1x10^7$ CFU/mL of Vp and 100 µL of cell suspension containing approximately $1x10^9$ CFU/mL of Vh (prepared as described above) were added to 1.5 mL of a filtrate of interest. The survival of each strain in the samples was determined (as shown above) at 5 min, 30 min, 90 min, and 180 min.

3. RESULTS AND DISCUSSION

3.1. Installation and operation of SBES

Based on the design (Figure 1A), we successfully built six 10-litre column tanks, including three test models with an integrated SBES system and three control models without SBES.

The SBESs installed in the test column models (T_C1, T_C2, T_C3) started to generate electricity from the first days of operation (as described above). However, the currents gradually increased and only stabilized (about 2.5 mA) after 45 days (Figure 2). Regarding our previous studies on SBES, such electricity assures that electroactive bacteria were enriched in our SBESs and that the SBESs were functioning [22].

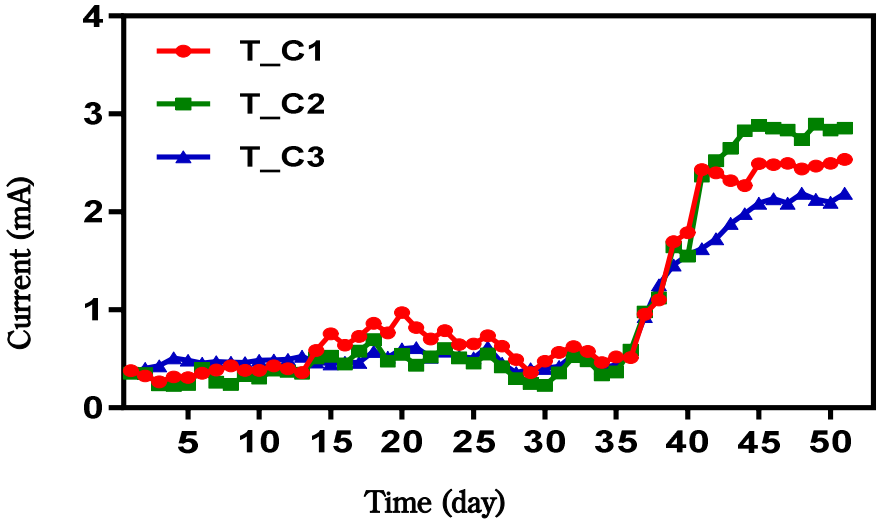

Figure 2. Electricity generation by the SBESs integrated in the test column models

3.2. Effect of the SBES cathode filtrate on the viability of Vh, Vp, and Vp CED

Arends *et al.* (2014) [17], Gajda *et al.* (2020) [18] reported that the pH change in a biochemical system has a certain inhibitory effect on pathogenic bacteria in the cathode compartment. In addition, the study of Lu *et al.* (2012) [19] showed that the electrochemical system is capable of generating H_2O_2, a potent oxidizing agent produced at the cathode, which is toxic to bacterial cells. Therefore, we tested the inhibition of the cathode aqueous filtrate on the *Vibrio* strains.

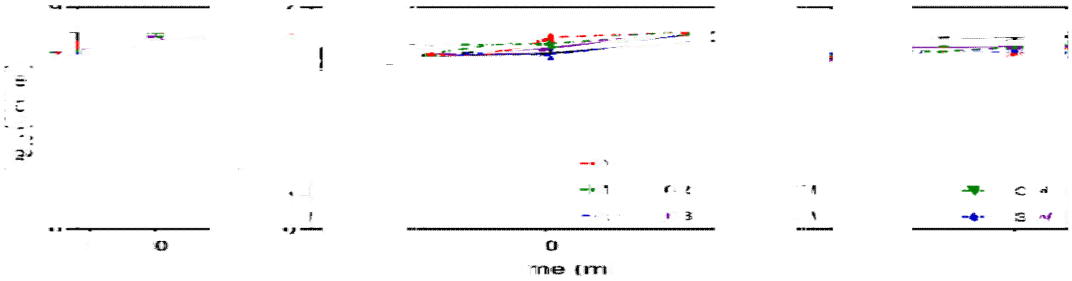

Figure 3. Effect of the SBES cathode filtrate on the viability of the *Vibrio* strains

Note: A: Vh, B: Vp, C: Vp CED; T_C1, T_C2, T_C3: filtrate samples from the 3 respective test models; CM: a mixture of the "cathode" filtrate from the 3 control models (C_C1, C_C2, C_C3); BW: artificial brackish water only (abiotic control).

The results (Figure 3) showed that, for all three tested strains, the bacterial density did not decrease after 180 min in direct contact with the cathode filtrates from both the test setups containing the SBESs and the controls. Even for Vh, the bacterial densities in all cases increased by more than 1 log (logarithm), which shows that Vh still can grow in the cathode filtrate. Thus, it can be seen that the cathode filtrate does not inhibit *Vibrio* bacteria.

Although the studies by Arend *el al.* (2014), Gajda *el al.* (2020), and Lu *el al.* (2012) showed an apparent bactericidal effect by H_2O_2 or high pH at the cathode, such an effect by the cathode was not strong [17 – 19]. In our study, we could not measure H_2O_2 so whether it is involved in the SBES effect is still questioned. Furthermore, the pH of the aqueous filtrates of both the test setup (even near the electrodes) and the controls did not change significantly during operation (Figure 4). Those phenomena may explain why in our study, the aqueous solutions at the cathode side of SBES could not inhibit *Vibro*. Probably, our SBES is at a much larger scale (compared to those in previous studies) and thus the amount of generated H^+ or H_2O_2 may be too small to have any significant effect on the bulk medium in the system.

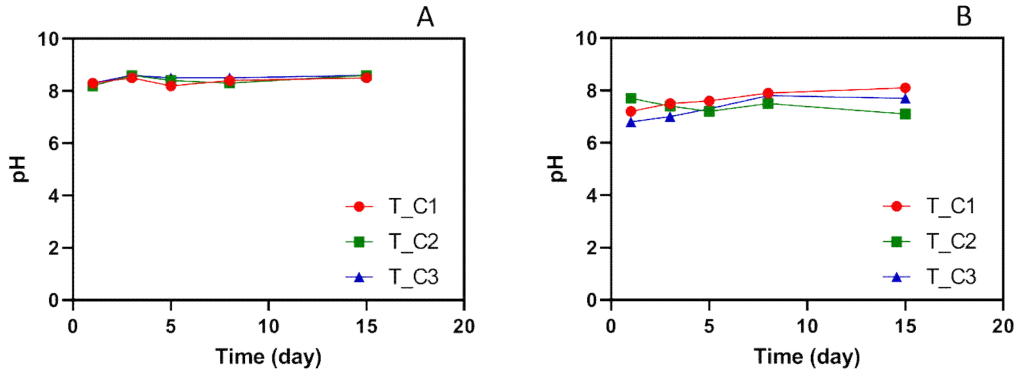

Figure 4. pH of SBES

Note: A: pH in cathode, B: pH in anode.

3.3. Effect of the SBES anode filtrate on the viability of Vh, Vp and Vp CED

Several research groups have previously reported that the reduced redox potential at the anode can induce the formation of redox agents capable of inhibiting the metabolic activity of pathogenic bacteria [23, 24]. Therefore, we tested the viability of *Vibrio* bacteria when exposed to the SBES anode filtrate.

The obtained results (Figure 5) showed that when exposed to the anode filtrates of the test setups having the SBESs, the cell densities of the *Vibrio* strains decreased over time, while they seemed to survive well in the "anode" filtrate (from the bottom position) of the control. Specifically, the Vh densities decreased after 180 min by 2.5 log, 1.6 log, and 2.6 log (equivalent to 99%), respectively, when exposed to the anode filtrates of T_C1, T_C2, and T_C3 (Figure 5A). The ability to inhibit Vp of T_C3 anode filtrate seemed to be weaker (with only 0.6 log reduction) compared to that of Vh; however, when Vp exposed to T_C1 and T_C2, this ability is strong, with the cell densities decreased by 1.6 log and 1.7 log (equivalent to 99%) (Figure 5B). For Vp CED, a wild-type strain isolated from actual ponds, which is generally more resistant, the cell density of this bacterium in the anode filtrate of T_C2 was not significantly reduced. However, it slightly decreased in T_C1 (0.8 log) and decreased by 90% in T_C3 (1.3 log) (Figure 5C). These results generally demonstrate an evident inhibition of the SBES aqueous environment against *Vibrio* bacteria.

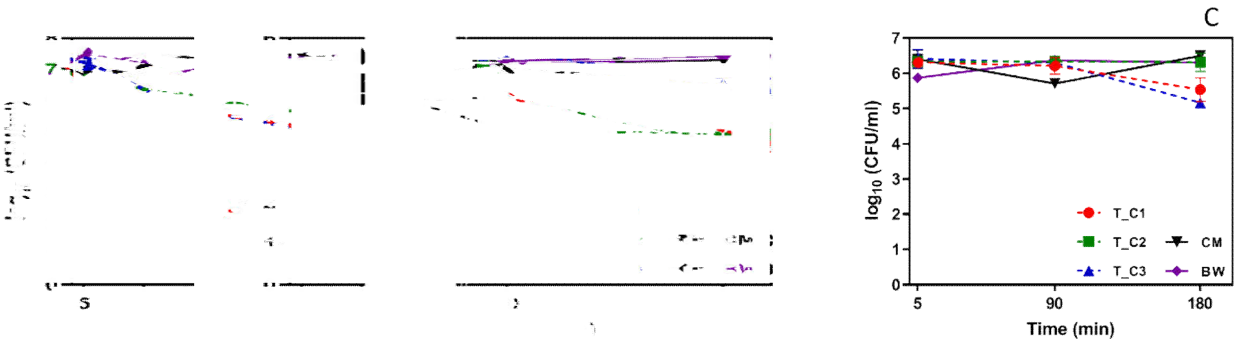

Figure 5. Effect of the SBES anode filtrates on the viability of the *Vibrio* strains

Note: A: Vh, B: Vp, C: Vp CED; T_C1, T_C2, T_C3: filtrate samples from the 3 respective test models; CM: a mixture of the "anode" filtrate from the 3 control models (C_C1, C_C2, C_C3); BW: artificial brackish water only (abiotic control)

In a previous study, Ieropoulos *et al.* (2019) [20], Ieropoulos *et al.* (2017) [24] hypothesized that the formation of redox agents reduces redox capacity at the anode and, at the same time, may create an inhospitable environment for pathogenic bacteria. Accordingly, the anode medium of their BES could strongly reduce the number of bacteria *Salmonella enteritidis* (up to 10 - 100 times) [24], *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (100 - 1000 times on average) [20]. This bactericidal ability is further demonstrated by the research results of Vasieva *et al.* (2019) [21], which showed a sharp decrease in the proportion of DNA of common pathogenic bacteria such as *Shigella*, *Yersinia*, *Vibrio*, *Haemophilus*, and *Pseudomonas* in the metagenome of all bacteria in the anode medium of the studied BESs. In addition, to create biofilms, electrochemical bacteria such as *Pseudomonas aeruginosa* are capable of producing mediators such as the antibiotic pyocyanin, which can kill other competing bacteria [25]. Those are the possible explanations for the inhibitory effect on *Vibrio* bacteria of the SBES anode filtrate.

One may notice that in some experiments, the three replicates of SBES-containing columns did not always display the same inhibitory activities against the *Vibrio* bacteria (sometimes one of

them did not show a clear inhibitory activity). We still can not explain this observation but according to previous studies, sometimes a slight change in the internal resistance (due to disconnection or micro-environments in such large volumes) may lead to unstable performance of BESs [15]. Such a change can even significantly change the redox potential of the system, which may severely affect the bactericidal effect of the SBES, as mentioned above [24]. In this study, we were not be able to check the internal resistances and the redox potentials of our SBESs, and thus further tests are needed to evaluate the stability of the system. Nevertheless, in general, at least 2 of 3 experimental models always displayed significant inhibitory activities against the *Vibrio* strains, which supports our hypothesis that the SBES does have bactericidal effects on *Vibrio* bacteria.

3.4. Effect of the SBES anode filtrate on the viability of Vh and Vp when they are co-present

Different species of *Vibrio* may be found to co-infect on the same host leading to more severe infections, or they may co-occur during vibriosis outbreaks [26]. The question is whether this coexistence makes them more resistant. Therefore, we tested the viability of Vh and Vp when they were present simultaneously and in contact with the SBES anode filtrate.

The results showed that the anode filtrates of all three test models could still inhibit Vh and Vp when they co-existed, while they seemed to live well in the control "anode" filtrate and the abiotic control solution (artificial brackish water) (Figure 6). Noticeably, in this experiment, the inhibition of Vh was more substantial than in the above-mentioned experiment when it was tested individually. Specifically, Vh cells were completely eliminated after 180 minutes in all cases when tested with the SBES anode filtrates. The inhibition was less for the Vp strain; however, it was still comparable to those in the individual strain test, with the bacterial densities significantly reduced by 1.2 log, 2.2 log, and 1.9 log when exposed to the T_C1, T_C2, and T_C3 filtrates, respectively.

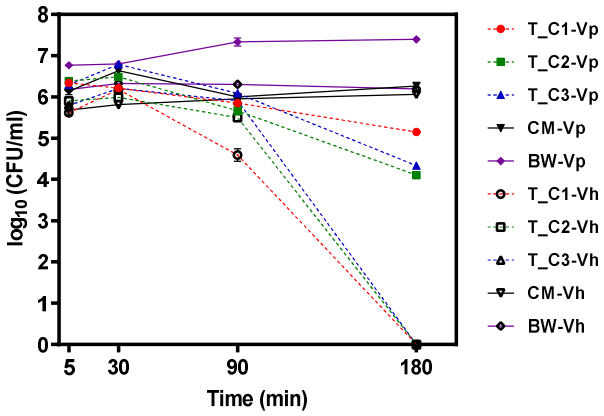

Figure 6. Effect of the SBES anode filtrate on the viability of Vh and Vp when they are co-present

Note: T_C1, T_C2, T_C3: filtrate samples from the 3 respective test models; CM: a mixture of the "anode" filtrate from the 3 control models (C_C1, C_C2, C_C3); BW: artificial brackish water only (abiotic control).

Most previous studies reported that different *Vibrio* species often occur together in habitats, such as *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *V. cholerae*, … [27]. However, there is little research interest in the co-infection of aquatic animals by different *Vibrio* species, although this is common. During co-infection, interactions between infectious agents lead to different outcomes: the virulence of one or both pathogens may be increased, one or both may be inhibited, or one can be favored while the other is inhibited [28]. Thus, interactions between concomitant pathogens can be supportive or antagonistic. The supportive interactions can lead to increased agent resistance [28]. In our study, the inhibition of the SBES to Vh in the co-presence of Vp was even stronger than that when the strains were tested alone. Thus, the interactions between the *Vibrio* species (if any), at least for the strains in this study, do not seem to affect the ability of SBES to inhibit these bacteria.

4. CONCLUSION

With the potential ability to inhibit some representative strains of *V. harveyi* and *V. parahaemolyticus* in our study, SBES can be a promising new technology in aquaculture to control pathogenic *Vibrio* bacteria. Integrating

SBESs in aquaculture ponds to combat *Vibrio* diseases would also be a potential alternative to using antibiotics and other chemicals, which are currently not supported in many countries. This offers the prospect of reducing the burden of aquaculture water treatment and enables longer operations of the ponds. Thus, the application of SBES should be considered in aquaculture to ensure more economical operation and sustainability. Obviously, further studies in actual conditions are needed to realize this fascinating prospect.

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CHARACTERIZATION AND ANTIBACTERIAL ABILITY OF THE *Bacillus* sp. STRAIN VNUA16 ISOLATED FROM FISH POND SLUDGE IN HAI PHONG

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ABSTRACT

Aquaculture industry, which plays an important role in Vietnam economy, has been oriented for effective and sustainable development. However, aquaculture is facing with the appearance of more serious water pollution and aquatic animal diseases. *Bacillus* species are capable of synthesizing antibacterial substances and degrading organic compounds, contributing to the control of aquatic diseases and improve water quality. Therefore, the study was carried out to determine biological characteristics and antibacterial ability of the VNUA16 strain isolated from fish pond sludge in Hai Phong province. The study of morphological, biochemical characteristics and 16S rRNA gene sequence showed that the strain VNUA16 belongs to the genus *Bacillus*. The antagonistic activity of this strain was evaluated by the diffusion plate method. According to the results, the *Bacillus* sp. strain VNUA16 was able to antagonize *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa* P2.1 and *Staphylococcus aureus* SA12, recording inhibition zones of 6.43; 8.53; 9.57 mm, respectively. At pH6 and 30°C, this strain exhibited the strongest antibacterial activity against *Pseudomonas aeruginosa* P2.1. The strain *Bacillus* sp. VNUA16 has a promising potential to be used as a biological agent for control of aquatic animal diseases.

Keywords: *Antibacterial activity, Bacillus, Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio parahaemolyticus.*

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1. INTRODUCTION

Aquaculture has been practiced in Vietnam for a long time, which plays an improtant role in developing the economy of the country [1]. According to General Statistics Office, in the first five months of 2023, total fisheries and aquaculture production reached a record about 3.42 million tonnes, increased by 1.4% compared to the same period last year, comprising around 2.59 million tonnes of fish, 0.386 million tonnes of shrimp and 0.526 million tonnes of other aquatic animals [2]. Three species dominate the aquaculture sector, including the freshwater Pangasius (*Pangasianodon hypophthalmus*), tilapia and shrimp [3].

Aquaculture production has been significantly affected by bacterial diseases such as motile aeromonad septicaemia, vibriosis, columnaris, edwardsiellosis and furunculosis, causing heavy mortalities and high economic losses [4]. Bacteria responsible for causing disease in aquatic animals belong to the genera *Vibrio*, *Aeromonas*, *Flavobacterium*, *Pseudomonas*, *Edwardsiella*, *Streptococcus* as well as *Staphylococcus* [4, 5, 6, 7, 8]. *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* are known as the most common opportunistic pathogens causing ulcerous disease [8]. While *Staphylococcus* species, namely *S. epidermidis*, *S. aureus*, *S. hominis*, *S. cohnii* and *S. warneri* generally induce hemorrhagic septicemia in fish [5]. *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. vulnificus* and *V. harveyi* are the major pathogenic species of *Vibrio* affecting fishes and shrimps in aquaculture [4].

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There are various methods to prevent diseases or reduce the losses caused by bacterial pathogens, including good farm management and the use of antibiotics. However, treatment by extensive feeding antibiotics may cause the rise of bacterial antibiotic resistance and antibiotic residues in cultured aquatic animals [9]. Therefore, the use of probiotics is an effectively alternative way to control bacterial diseases and improve farm production yields in aquaculture [9]. *Bacillus* species have been applied as potential probiotics in the aquaculture sector because of their rapid growth, ability to produce a wide range of extracellular enzymes as well as broad spectrum antimicrobial compounds [9, 10]. It was demonstrated that *Bacillus* spp., *Bacillus subtilis* BT23 had inhibitory effects against diseases caused by *Vibrio* spp. (*V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*) in tiger shrimp *Penaeus monodon* [11, 12]. Moreover, *B. subtilis* with a remarkable antimicrobial activity againts *Edwardsiella ictaluri* was found in the channel catfish intestine [13]. *Bacillus licheniformis* isolated from the gastrointestinal tracts of Nile tilapia (*Oreochromis niloticus*) revealed pathogen inhibition and potential probiotics characteristics [14]. Other *Bacillus* species, such as *B. pumilus*, *B. megaterium*, *B. tequilensis*, *B. amyloliquefaciens* also were reported by producing extracelluar enzymes and antagonistic ability against bacterial pathogens (*Aeromonas* spp., *Edwardsiella ictaluri*, *Streptococcus iniae)* in goldfish (*Carassius auratus*), Indian major carps (*Labeo rohita, Catla catla*, and *Cirrhinus mrigala*), catfish, Nile tilapia and grass carp [13, 15, 16, 17, 18].

The aim of the present study is to determine biological characteristics as well as antimicrobial activity of the bacterial strain *Bacillus* sp. VNUA16 from fish pond sludge in Hai Phong province. The effect of temperature and pH on inhibitory activity of this strain against bacterial pathogen *Pseudomonas aeruginosa* was also conducted.

2. MATERIALS AND METHODS

2.1. Materials

Bacterial strain VNUA16 was isolated from fish pond sludge in Hai Phong province, Kien Thuy district according to the method descr bed by Duong Nguyen Anh Tam *et al.* (2022) [19]. The strain was purified and preserved on agar slant tubes in a refrigerator and in LB medium containing 20% glycerol at -20°C at the Department of Microbial Biotechnology, Faculty of Biotechnology, Vietnam National University of Agriculture for further examination.

Pathogenic strains, including *Pseudomonas aeruginosa* P2.1, *Vibrio parahaemolyticus* and *Staphylococcus aeureus* SA12. These strains were provided by the Department of Microbial Biotechnology, Faculty of Biotechnology, Vietnam National University of Agriculture.

2.2. Methods

2.2.1. Biological characterization of the VNUA16 strain

The VNUA16 strain was streaked in Luria-Bertani (LB) agar medium, containing the following components (g/l): Peptone 10, yeast extract 5, NaCl 10, agar 20, pH7. After 24 hours of incubation at 30°C, the colony morphological characteristics of the strain such as size, form, elevation, margin, color, surface and shape were determined. This strain was also characterized by different biochemical tests, including the Gram staining, catalase, motility, citrate, indole, nitrate reduction, carbohydrate utilization, starch hydrolysis, cellulose hydrolysis, Methyl Red (MR) and Voges-Proskauer (VP) tests according to the method described by Barrow and Feltham (2004) [20].

The effect of temperature on the growth of the strain VNUA16 was also conducted. The strain was cultured at different temperature of 30, 35 and 40°C in a liquid LB medium at 160 rpm. After 48 hours of incubation, the growth of this strain was determined by measuring optical density (OD) at 620 nm wavelength. The experiment was tested three times and mean values were calculated [21].

2.2.2. Molecular identification based on 16S rRNA sequence analysis

Bacterial genomic DNA was extracted using a DNA isolation kit (iNtRON Biotechnology, Korea) described by the manufacturer's instruction and

stored at -20^{0}C for further analysis. The 16S rRNA gene of the bacterial strain was amplified by PCR using the specific primers sequences, including 27F:5'- AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5' TACGGYTACCTTGTTACGACTT-3'. The program for PCR amplification was carried out in a Thermal Cycler (BIO-RAD) using following conditions: An initial denaturation at 95^{0}C (5 min), 29 cycles of denaturation at 95^{0}C (30 sec), annealing at 53^{0}C (30 sec), then extension at 72^{0}C (1 min), and a final extension at 72^{0}C for 10 min. The PCR products were checked using gel electrophoresis on 1.5% agarose, then were sequenced by 1st BASE (Singapore). Subsequently, the obtained nucleotide sequences were aligned and compared with the sequences belonging to the other bacterial isolates published in GenBank database of NCBI using basic local alignment search tool (BLAST) program. A phylogenetic tree was created by the neighbour-joining method using MEGA 7.0 software with bootstrap replications (1000) [19, 22].

2.2.3. Determination of antimicrobial ability of the VNUA16 strain

An antimicrobial activity test was performed using the inhibition zone assay according to the procedures of Chen *et al.* (2016) with some modifications [23]. Bacterial pathogens, namely *Pseudomonas aeruginosa* P2.1, *Vibrio parahaemolyticus* and *Staphylococcus aureus* SA12 were incubated for 24 hours at 30^{o}C and 160 rpm in proper media, including LB broth (for *Pseudomonas aeruginosa* P2.1 and *Staphylococcus aureus* SA12) and Tryptic Soy Broth (TSB), containing 17.0 g/l tryptone, 3.0 g/l soytone, 2.5 g/l dextrose, 2.5 g/l K_2HPO_4 with 2% NaCl (for *Vibrio parahaemolyticus*). After that, 50 µl of culture broth were thoroughly spread on the agar media and wells were created on plates.

Regarding the VNUA16 strain, it was cultured in LB broth at 30^{0}C in a incubator shaker (160 rpm). After 48 hours of incubation, the obtained culture broth was centrifuged at 10,000 rpm at 10^{0}C for 10 min to collect supernatants. 100 µl of cell - free supernatant was added into each well in the plates. The plates were placed at 4^{0}C for 2 - 3 hours, then incubated at 30^{0}C for 12 hours. The presence of antimicrobial activity is indicated by the formation of a clear zone around the well. Diameter of inhibition zone was measured and recorded. Each experiment was tested in three replications and the diameter of the clear zone was measured.

2.2.4. The effect of temperature and pH on antibacterial activity of the VNUA16 strain

The strain was incubated in LB broth at 160 rpm at different temperature and pH conditions. The initial pH of culture media were adjusted at 2, 4, 6, 8, 10 and 12. In terms of temperature, the strain was cultured at temperature ranging from 30^{0}C to 40^{0}C at an interval of 5. Antimicrobial activity against *Pseudomonas aeruginosa* P1.2 was determined as described in 2.2.3. The experiment was performed in triplicate [24].

2.2.5. Data analysis

All experiments were performed in triplicate and results represented mean values. Based on GraphPad Prism sofware version 9.0, the data of these experiments were analyzed with one-way ANOVA ($p<0.05$) and Tukey's test. A value of $p<0.05$ was considered statistically significant.

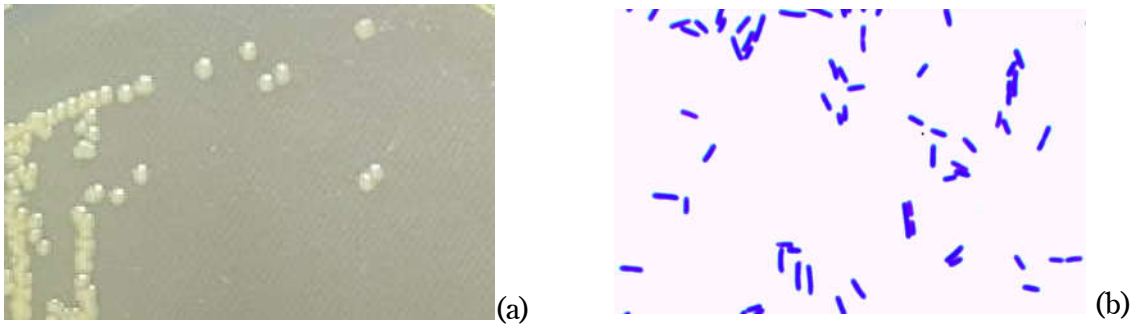
3. RESULTS AND DISCUSSION

3.1. Characterization of isolated bacterial strain

The morphological and biochemical characteristics of VNUA16 strains were conducted according to the method described above, and the results were presented in figure 1 and table 1. As can be seen from the figure 1, the colony morphology of this strain cultured on LB medium was circular form with small diameters ranging from 1 to 2 mm, slightly raised elevation, entire margin, moist in texture, opaque in opacity and cream-yellow in color.

Observed under light microscope, cells of this strain were rod-shaped, arranging in singles or in pairs with rounded or square ends. It was also found that the VNUA16 strain was a Gram-positive and spore-forming bacterium, exhibiting positive for catalase, nitrate reduction, Methyl Red and Voges-Proskauer tests, but negative for indole and citrate tests. This strain produced acid from different carbohydrate sources such as D-glucose,

D-galactose and maltose. However, it was unable to utilize lactose, starch, sorbitol, sucrose and D-inositol. In terms of extracellular hydrolytic enzymes, the *Bacillus* sp. strain VNUA16 had the capacity to produce amylase and cellulase. Similarly, Ghosh *et al.* (2017) indicated that *Bacillus licheniformis* strain ONF1P isolated from the gut of Nile Tilapia (*Oreochromis niloticus*) exhibited amylase and cellulase activities [14]. According to Pham Thi Tuyet Ngan *et al.* (2021), five strains of *Bacillus* spp., namely CM3.1, CM2.2, TV1.2, TV3.1 and BT1.2 isolated from shrimp pond sludge were also able to hydrolyze starch and cellulose [25].



(a) (b)

Figure 1. Morphological characteristics of the VNUA16 strain

a. Morphological colony of the VNUA16 strain; b. Cellular morphology of the VNUA16 strain

Based on the description in Bergey's Manual of Determinative Bacteriology, it is indicated that the VNUA16 strain has morphological and biochemical characteristics similar to the genus *Bacillus* [26].

Table 1. Phenotypic and biochemical characteristics of the VNUA16 strain

Characteristics	VNUA16	Characteristics	VNUA16
Gram staining	+	Starch hydrolysis	-
Shape	Rod	Cellulose hydrolysis	-
Spore formation	+	Fermentation for carbon sources: Lactose	-
Motility	+	D-glucose	+
Catalase	+	D-galactose	+
Citrate utilization	-	Maltose	+
Indole production	-	D-inositol	-
Nitrate reduction	+	Starch	-
Voges-Proskauer (VP)	+	Sorbitol	-
Methyl Red (MR)	+	Sucrose	-

The bacterial growth was determined by measuring its absorbance at a wavelength of 620 nm after 48 hours of incubation in LB broth. The result was shown in figure 2. As indicated in this figure, there was a varied growth of the VNUA16 at different temperature. The VNUA16 strain had the highest growth at 30^{0}C, recording optical density (OD) of 2.48. Moreover, the growth of this strain decreased dramatically at 40^{0}C (OD 620 nm = 0.73). As a result, the VNUA16 strain was considered as a mesophilic bacterium.

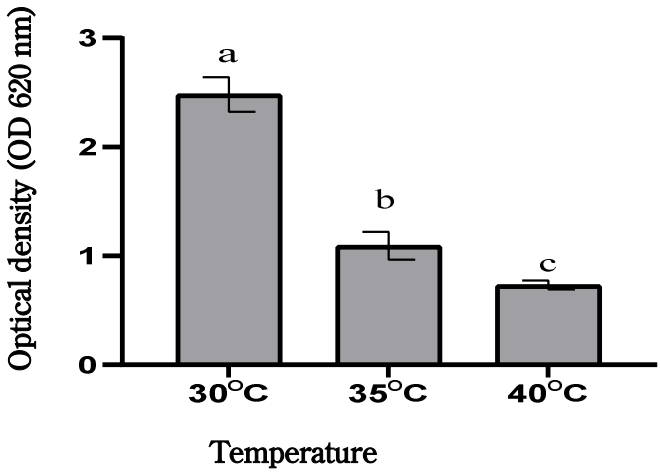


Figure 2. The effect of temperature on the growth of VNUA16 strain

Note: Different letters means significant differences according to Tukey's test at p <0.05

Similarly, nine novel species of the *Bacillus cereus* group, including *Bacillus paranthracis* Mn5, *Bacillus pacificus* EB422, *Bacillus tropicus* N24, *Bacillus albus* N35-10-2, *Bacillus mobilis* 0711P9-1, *Bacillus luti* TD41, *Bacillus proteolyticus* TD42, *Bacillus nitratireducens* 4049 and *Bacillus paramycoides* NH24A2 isolated from sediments and seawater had an optimum temperature for the growth at 30^{0}C [27]. Wekesa *et al.* (2022) recorded that *Bacillus velezensis* isolated from Lake Bogoria grew optimally at temperature of 30-35^{0}C [28]. Various *Bacillus* species, namely *Bacillus subtilis*, *Bacillus firmus*, *Bacillus flexus*, *Bacillus safensis* also had optimal growth at 30^{0}C [29, 30]. However, *Bacillus* sp. Bsa 1 and *Bacillus flexus* APGI had maximal growth at 40^{0}C [24, 31].

3.2. Identification of the VNUA16 strain by 16S rRNA sequence analysis

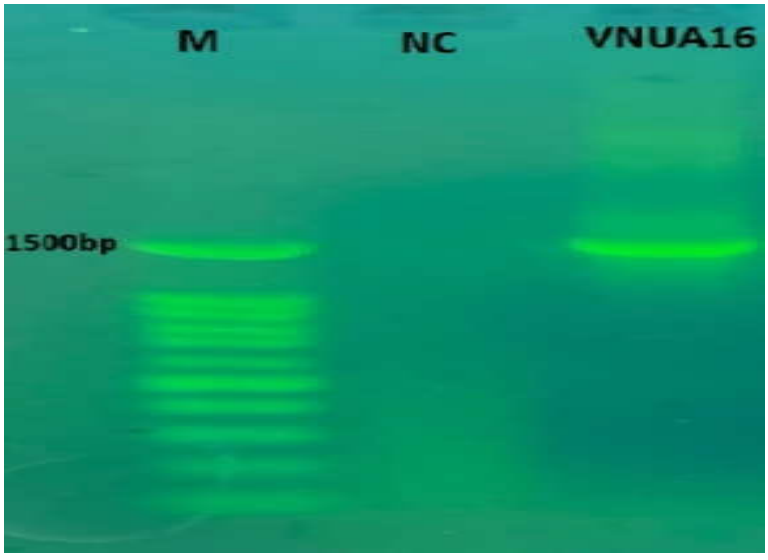


Figure 3. PCR product of 16S rRNA gene from the *Bacillus* sp. strain VNUA16

NC. Negative control (no template DNA added); M. DNA ladder marker 1,500 bp

PCR amplification of 16S rRNA revealed the possibility of amplifying PCR products with size of 1,500 bp. As can be seen from the figure 3, the PCR product of the VNUA16 strain was around 1,500 bp in length. After comparing for homology of 16S rRNA sequence of the VNUA16 strain with other reference sequences in GenBank database, it is indicated that the highest genetic similarity 97.61% was with *Bacillus paramycoides* MCCC 1A04098.

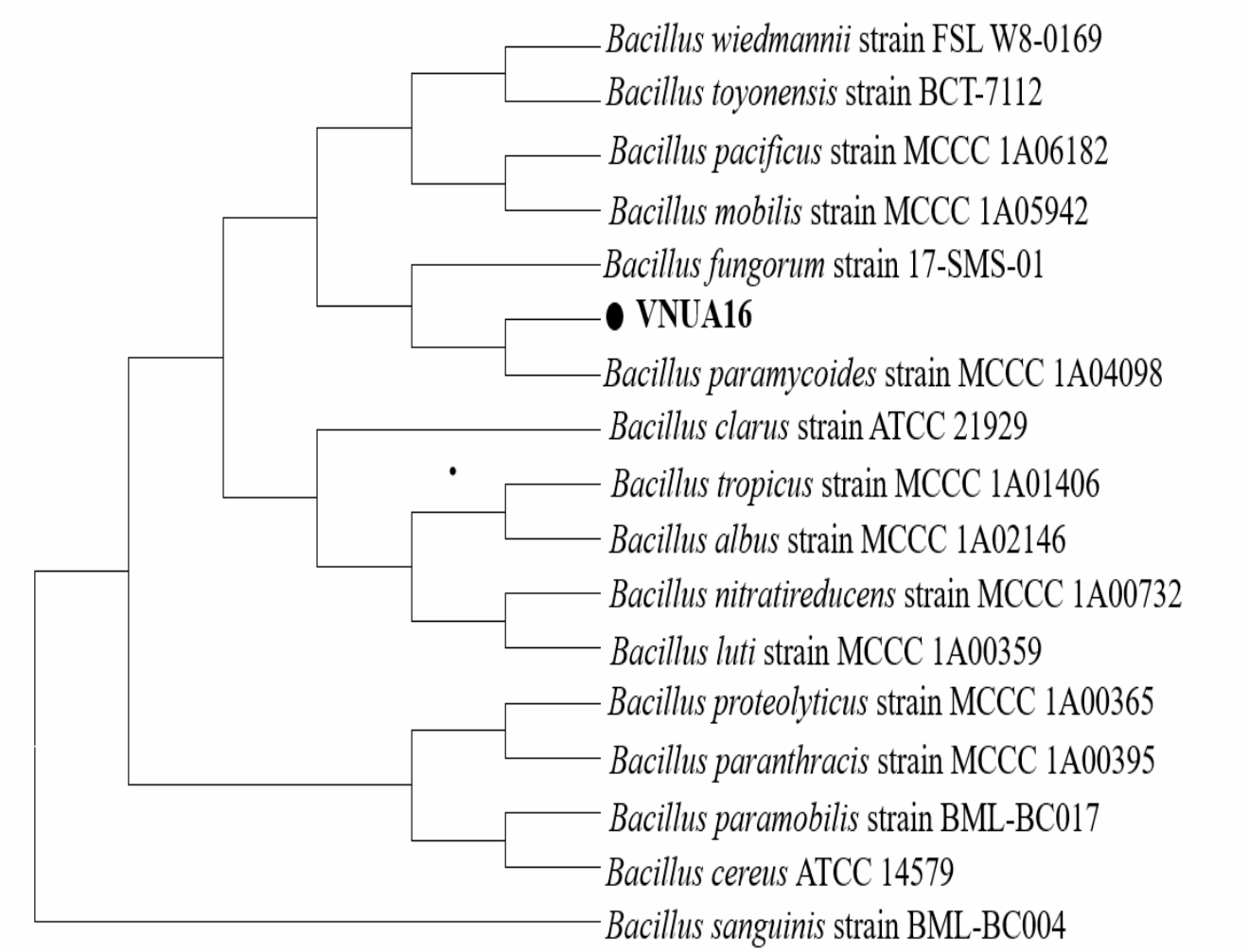


Figure 4. Phylogenetic tree of the VNUA16 and representatives of other related strains within *Bacillus* genus based on 16S rRNA sequence analysis

Based on the phylogenetic tree construction, it is apparent that the VNUA16 strain formed a clade with *Bacillus paramycoides* (Figure 4). The formation of clade in VNUA16 strain with *Bacillus paramycoides* showed that two bacterial strains in the taxonomic group has the same ancestor. The phylogenetic analysis of 16S rRNA gene sequences suggested that this strain was clustered within the taxonomic groups of *Bacillus* genus. Morphological and biochemical features also corresponded to those of this genus.

3.3. Determination of antimicrobial ability of the VNUA16 strain

The *Bacillus* sp. strain VNUA16 displayed moderate antibacterial activity against three bacterial pathogens, namely *Pseudomonas aeruginosa* P2.1, *Vibrio parahaemolyticus* and *Staphylococcus aureus* SA12 (Figure 5). The results showed that the VNUA16 strain had the highest antagonistic activity against *Staphylococcus aureus* SA12 with zone inhibition diameter of 9.57 ± 0.60 mm, followed by *Pseudomonas aeruginosa* P2.1 (8.53 ± 0.50 mm) and *Vibrio parahaemolyticus* (6.43 ± 0.49 mm). While among tested pathogenic bacteria, *Bacillus subtilis* 11A exhibited the lowest activity to

Staphylococcus aureus SA12 [32]. It is apparent that *Bacillus* sp. strain VNUA16 exhibited broad spectrum antibacterial activities because of inhibiting both Gram-positive and Gram-negative bacterial pathogens.

The genus *Bacillus* is known to be able to secret different proteinase and antimicrobial secondary metabolites resulting in inhibiting a lot of pathogenic bacteria [22]. The results obtained by Sarjito *et al.* (2022) indicated that seven bacterial strains, belonging to *Bacillus* genus such as *Bacillus cereus*, *B. aerius*, *B. paramycoides*, *B. thuringiensis*, *B. altitudinis, B. salarius and B. wiedmanni* had antibacterial activity against three species of *Vibrio* from vannamei shrimp (*Litopenaeus vannamei*) such as *Vibrio vulnificus*, *V. anguilarum*, *V. alginolyticus*. All these strains had no zone of inhibition against *V. parahaemolyticus*. Among them, *Bacillus paramycoides* was capable of inhibiting *V. anguiliarum* with a clear zone of 9.73 mm [22].

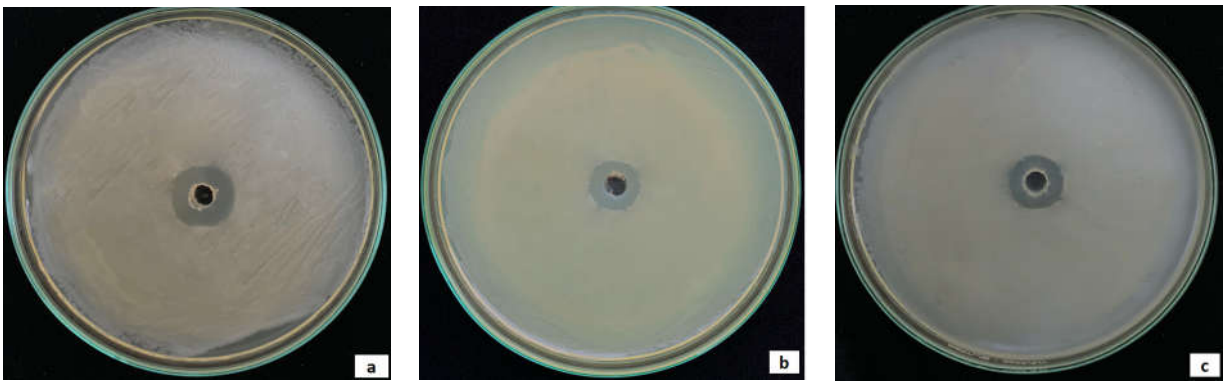


Figure 5. Antagonistic activity of *Bacillus* sp. VNUA 16 against bacterial pathogens

(a) *Pseudomonas aeruginosa* P2.1; (b) *Vibrio parahaemolyticus*; (c) *Staphylococcus aureus* SA12

According to Pham Thi Tuyet Ngan *et al.* (2021), 13 strains of *Bacillus* spp. isolated from sludge of extensive shrimp ponds were capable of antagonizing to *V. parahaemolyticus* with zone of inhibition ranging from 2.05 to 13.05 mm. Among them, there were less than half of isolates having lower inhibition activities compared to the VNUA16 strain [25]. Setiaji *et al.* (2020) explored the inhibitory effects of different *Bacillus* species on the growth of *Vibrio alginolyticus*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* because they could produce terpenoid compounds. *Bacillus cereus* strain JS22 had antimicrobial activity against *Pseudomonas aeruginosa* similar to the *Bacillus* sp. strain VNUA16 [33].

3.4. The effect of temperature and pH on antagonistic activity of *Bacillus* sp. VNUA16

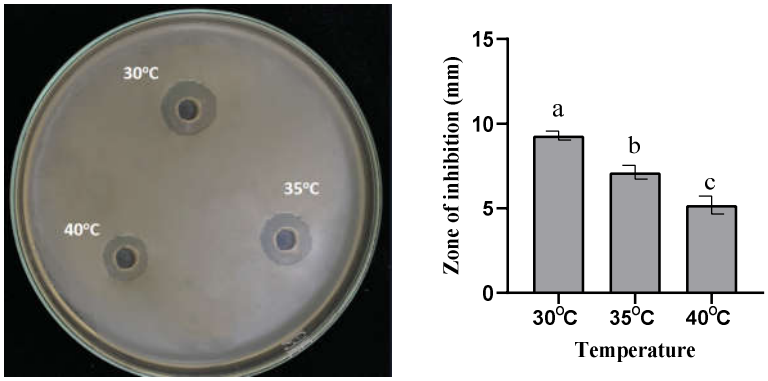


Figure 6. Effect of temperature on antagonistic activity of *Bacillus* sp. VNUA16 against *P. aeruginosa* P2.1 after 48 hours of incubation

Note: Different letters means significant differences according to Tukey's test at p <0.05

The inhibitory effect of *Bacillus* sp. VNUA16 strain agaisnt *Pseudomonas aeruginosa* was conducted in different temperature and pH. The influence of different pH and temperature on the inhibitory activity against *P. aeruginosa* P2.1 of *Bacillus* sp. strain VNUA16 is presented in figure 6 and figure 7. As can be seen from figure 6, the optimum temperature for antagonistic effect of the *Bacillus* sp. VNUA16 against *P. aeruginosa* was at 30^0C with a clear zone of inhibition of 9.3 mm, followed by 35^0C with an inhibitory zone of 7.13 mm. Meanwhile, the antibacterial activity of this strain against *P. aeruginosa* considerably decreased at 40^0C. The results were in agreement with the study of Iqbal *et al.* (2018). According to Iqbal *et al.* (2018), *Bacillus safensis* MK-12 isolated from waste dump soil revealed maximum antibacterial activity againts different pathogens, including *Staphylococcus aureus*, *P. aeruginosa*, *Vibrio cholera*, *E. coli* at 30^0C. However, the antibacterial activity of *B. safensis* MK-12 against *P. aeruginosa* was significantly higher than that of the VNUA 16 strain, recording zone of inhibition of around 17 mm [30]. Besides, unlike to the *Bacillus* sp. strain VNUA16, *Bacillus flexus* APGI and *Bacillus* BSA1 strains isolated from the gut of common carp *Cyprinus carpio* had better level of zone of inhibition against the test bacterial pathogen at 40^0C [24, 31].

With regard to pH, the VNUA16 strain recorded highest inhibitory effect against *P. aeruginosa* at pH6, indicating zone of inhibition of 9.53 mm. There was no significant differences in the antibacterial activity of the VNUA16 between pH 6 and pH 8, while zone inhibition diameters of this strain against test bacterial pathogen were lower at pH 2, 4 and 10, ranging from 5.43 mm to 8.17 mm. The bacterial pathogen *P. aeruginosa* was not inhibited by the strain VNUA16 at pH 12. According to Ramasubburayan *et al.* (2014) [24], *Bacillus flexus* APGI strain could not inhibit the growth of *P. aeruginosa* not only at pH 12 but also at pH 4. However, this strain had maxium antagonistic activity against *P. aeruginosa* at pH 8, which was still higher than that of the *Bacillus* sp. strain VNUA16. The study of Iqbal *et al.* (2018) [30] reported that *Bacillus safensis* strain MK-12 also exhibited strongest antagonistic effect against bacterial pathogen at pH 8.

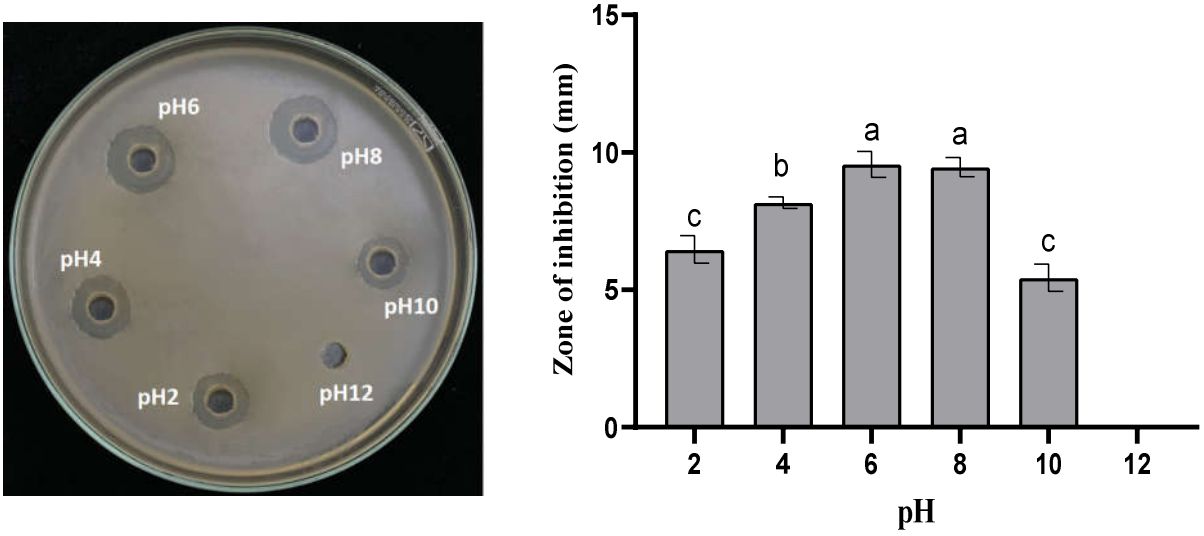


Figure 7. Effect of pH on antagonistic activity of *Bacillus* sp. VNUA16 against *P. aeruginosa* P2.1 after 48 hours of incubation

Note: Different letters means significant differences according to Tukey's test at p <0.05

4. CONCLUSION

The present study demonstrated that the VNUA16 strain belongs to the *Bacillus* genus based on its morphological, biochemical characteristics and the analysis of 16S rRNA gene sequences. The optimal growth temperature of the strain was at 30°C. This strain possed a broad spectrum antibacterial activity because of suppressing both Gram-negative bacteria (*P. aeruginosa*, *V. parahaemolyticus*) and Gram-positive bacterium (*S. aureus*). The antagonistic activity of the strain against *P. aeruginosa* was highest at pH6 and 30°C. The *Bacillus* sp. strain VNUA16 is considered as an important biological control agent against aquatic pathogenic bacteria, maintaining environmentally friendly and sutainable aquaculuture.

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SURVIVAL LONGEVITY OF *Clonorchis sinensis* METACERCARIAE COLLECTED IN RAY - FINNED FISH (*Toxobramis houdemeri*) *IN VITRO* CONDITION

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ABSTRACT

Clonorchis sinensis is a biological carcinogen inducing human cholangiocarcinoma (CCA). Clonorchiasis is one of the important endemic infectious diseases in Vietnam. The present study investigated the survival longevity of *C. sinensis* metacercariae in various *in vitro* conditions to find the best way of inactivating metacercariae in fish muscle. The fish (n=910; 6.74 - 9.85 cm in length, and body weight 4.52 - 7.42 g/fish) were randomly sampled from Thac Ba reservoir, Yen Bai province. A total of 2358 *C. sinensis* metacercariae were detached from soaked fish individuals with different solutions of Lemon juice, Vinegar, Alcohol 55%, and NaCl 10% at different immersion times (1, 6, 12, 24, 48, 72 hours) to evaluate the inactivated rate of metacercariae. The results showed that the metacercariae did not die after 1 and 6 hours in 4 different solutions. However, a proportion of *C. sinensis* metacercariae (40%) immersed in Alcohol 55% became inactivated after 12 hours. The mortality rate of *C. sinensis* metacercariae in the Alcohol was higher than in Vinegar, Lemon juice, and NaCl during all the time of the experiment. These data suggest that Alcohol 55% are effective for inactivating metacercariae of *C. sinensis* after 60 hours.

Keywords: *Clonorchis sinensis*, *metacercariae*, *survival*, *fish, Thac Ba reservoir, Yen Bai, Vietnam.*

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1. INTRODUCTION

Small liver flukes (clonorchiasis, opisthorchiasis) are of important significance for public health in Asia [1]. It occurs primarily in East Asia, and it is currently endemic in South Korea, China, Taiwan, and Vietnam [1, 2, 3, 4]. The number of people infected in this region is estimated at 7 - 15 million [5, 6] and prevalence varies widely, from less than 1.0% in Guang Xi, China, more than 40% in North Vietnam, and more than 70% in Guangdong Pr., China, and 1.5 - 2.0 million people show symptoms or complications [7, 8, 9, 10]. Importantly, infected people had heavy infections up to 1.1 million with more than 1.000 eggs/g feces [5]. Currently, it is estimated that more than 200 million people are at risk of *C. sinensis* infection, and over 15 million are infected worldwide [11]. In Vietnam, at least 1 million people are infected with liver flukes with *C. sinensis* in the North and *Opisthorchis viverrini* in the Central and South regions [12, 13]. *C. sinensis* is endemic in 21 northern provinces, whereas *Opisthorchis* spp. in 11 central and southern provinces [14]. In general, all the liver fluke (*C. sinensis*, *O. viverrini*, and *O. felineus*) infections induce chronic inflammatory diseases of the hepatobiliary system, and in chronic high worm burden infections this may lead to bile duct cancer termed cholangiocarcinoma (CCA) [15, 16, 17]. Most of these manifestations are mild and

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asymptomatic. However, once advanced CCA develops, clinical manifestations such as jaundice occur in approximately half of the cases, while the other half may have no specific symptoms [1]. People infected with less than 100 worms have no symptomatic [16, 17, 18, 19]. In which, people infected with one-hundred to thousands of worms may cause jaundice, indigestion, epigastric discomfort, anorexia, general malaise, diarrhea, and mild fever [5]. Over time, infected people without treatment may have serious symptom such as liver enlargement, allergic lesions, congestion of the spleen, bile stone development, cholecystitis, and liver cirrhosis. The most serious possible outcome is the development of cholangiocarcinoma. Benign hepatobiliary diseases are characterized by cholangitis, obstructive jaundice hepatomegaly, periductal fibrosis, cholecystitis, and cholelithiasis [1, 15]. Clonorchiasis is a major health problem in endemic areas, it is estimated global loss of 275,370 disability–adjusted life years (DALYs) on health status in endemic areas [17]. In Western Siberia where in highly endemic foci of *O. felineus*, CCA was detected in 77% of patients infected with *Opisthorchis* and in 34.2% of patients without *Opisthorchis* [16]. In Vietnam, a new endemic area of *C. sinensis* has been identified in residents living around Thac Ba Lake, Yen Bai province with the prevalence of 35% [20]. Recently, wild - caught fish from this lake were found infected with high prevalence and intensity of *C. sinensis* metacercariae [21]. In these mountainous regions, the risk for human infection is closely related to social and cultural traits that determine food behaviors [22]. Eating habit of raw or undercooked fish is common in localities near lakes, reservoirs, streams and ponds where fresh fish are readily available which can lead to infection of *C. sinensis* in the fish muscle.

However, there is little information and big gaps of knowledge on the viability of metacercariae of *C. sinensis* in different treatment methods. The previous study conducted by Fan (1998) revealved that metacercariae of Clonorchis were not effective to inactivate in frozen freshwater fish [23]. The viability of *C. sinensis* metacercariae obtained from cyprinid fish-*Pseudorasbora parva* in different temperatures and saline solutions showed that, metacercariae had lived for 10 - 20 days at -12°C, but died after 3 - 7 days at -20°C. With saline solution (3 g salt /10 g fish) metacercariae can survived for 5 - 15 days at 26°C [23]. At room temperature, metacercariae of *C. sinensis* lived for 5 - 12 days (under water conditions) and for 3 - 7 hours (without water). At 65°C, metacercariae of *C. sinensis* died after 3 hours but at 40^{0}C metacercariae live within 2.5 hours. Metacercariae isolated from dead fishes are alive for 3 - 7 days at room temperature and for 30 days at 4°C. There were over 50% of metacercariae alive up to 60 days after being isolated. Under freezing conditions, metacercariae died after 35 hours at -12°C and after 30 seconds at -196°C [1]. This study investigates the survival longevity of *Clonorchis sinensis* metacercariae *detached from soaked fish* in different sollutions.

2. METHODOLOGY

Fish collection: A total of 910 the ray-finned fish (*Toxobramis houdemeri*) with average length from 6.74 to 9.85 cm, bodyweight from 4.52 - 7.42 g/fish were collected from Thac Ba reservoir, Yen Bai province.

Experimental design

We used Lemon juice, Vinegar, Alcohol 55%, and NaCl 10% to inactivate metacercariae in fish because they are the common ingredients used in food processing and the making of raw fish food "Gỏi cá". In the habit of eating raw fish food is sliced thin and then soaked with these solutions.

Each fish will be measured the total length by a ruler, and weighted by analytical scales. After that, each of 10 fish was grouped together in one glass (a total of 30 fish for one experiment). Fish samples were kept in 4 different solutions (NaCl 10%, Alcohol 55%, Vinegar and Lemon juice) in different times (1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 60 hours, 72 hours) in room temperature 37^{0}C. Fish samples were stored in NaCl 0.85% in 4° as a control group. And then, fish samples from each experiment were crushed and

incubated for 3 hours at 37°C and then digested and examined the mortality of isolated metacercariae by observing the ability of movement of small live fluke under Olympus-CX31 (Japan).

Metacercariae from the soaked fish in 4 different solutions were detached by using the pepsin tissue digestion method and morphologically identified according to the key published by Sohn (2009) [24].

Statistic

Data were inserted into an Excel and converted to the R software for statistical analysis. The effect of different solutions for inactivating metacercariae infected in fish was compared by using prop.test in R software. All statistical tests with p value < 0.05 were considered statically significant.

3. RESULTS

Table 1 shows the average length and body weight of the ray-finned fish (*Toxobramis houdemeri*). The length and body weight of *T. houdemeri* in Alcohol solution were statically different compared with NaCl, Lemon juice, and Vinegar ($p < 0.01$).

Table 1. Fish length and body weight

Solutions treatment	No. Fish	Mean length (±SE)	95% CI	Mean body weight (±SE)	95% CI
NaCl	230	$9.73^{b} \pm 0,61$	9.48 – 10.00	$7.42^{b} \pm 1.64$	6.71 - 8.13
Lemon juice	240	$9.57^{b} \pm 0.75$	9.25 - 9.89	$7.08^{b} \pm 1.86$	6.29 - 7.87
Alcohol	230	$6.74^{a} \pm 2.09$	5.82 - 7.64	$4.52^{a} \pm 1.64$	3.81 - 5.24
Vinegar	210	$9.85^{b} \pm 0.85$	9.46 - 10.23	$6.83^{b} \pm 1.67$	6.07 - 7.59

Superscript letters in the column indicate significant ($p \leq 0.05$) differences in length and body weight intensity; the same letters indicate no significant difference, SE standard error, CI confident interval

Table 2. The mortality rates of *C. sinensis* metacercariae of infected fish after being immersed with different solutions

Time post immersion (hours)	NaCl 10%	Alcohol 55%	Vinegar 100%	Lemon juice 100%
1	0% (0/44)	0% (0/243)	0% (0/48)	0% (0/48)
6	0% (0/67)	0% (0/106)	0% (0/47)	0% (0/50)
12	6.85% (5/73)	40% (10/25)	0% (0/217)	0% (0/210)
24	16.28% (7/43)	54.05% (20/37)	10.29% (7/68)	10.38% (11/106)
48	37.5% (12/32)	60.61% (20/33)	10% (5/50)	11.59% (8/69)
60	98.48% (65/66)	100% (28/28)	54.55% (36/66)	25.21% (61/242)
72	97.83% (45/46)	100% (47/47)	35.68% (66/185)	58.06% (36/62)

A total of 2358 *C. sinensis* metacercariae from 910 soaked fish individuals in 4 different solutions experiments were detached including 371 metacercariae from fish fixed in NaCl 10%, 787 metacercariae in Lemon juice, 519 metacercariae in Alcohol 55% and 681 metacercariae in Vinegar. The mortality rate and live rate of metacercariae were calculated after 1, 6, 12, 24, 48, 60, and 72 hours (Table 2). After 1 and 6 hours, no metacercariae died. However, *C. sinensis* metacercariae in fish fixed in alcohol became inactivated after 12 hours 40% (10/25). The mortality rate of *C. sinensis* metacercariae which detached from soaked fish in the Alcohol was higher than in Vinegar, Lemon juice and NaCl in all most of the time at 12, 48, 60, and 72 hours with the following values 40, 17.16, 5.84; 5.25, 5.20, 3.32; 6.06, 5.23 1.61; 2.80, 3.97, 1.01; 1.83, 1.72, 1.02 times respectively. The mortality of *C. sinensis* metacercariae in 4 different solutions after 12, 24, 48, 60, and 72 hours were statistically significant ($p < 2.2e\text{-}16$).

There has been little doubt about the mortality of *C. sinensis* metacercariae in the infected fish in different solutions. The present study addressed a small part of the problem by using common ingredients used in food processing and the making of raw fish food every day. We found that (1) all 4 different solutions were associated with the mortality of *C. sinensis* metacercariae, (2) NaCl 10% and alcohol 55% can be inactivated *C. sinensis* metacercariae after 12 hours and (3) the Alcohol 55% was the best solution to inactivated *C. sinensis* metacercariae. Our finding is consistent with previous studies. In previous study, the metacercariae isolated from fish were exposed to Lemon juice 100% and Ethanol 30%, 40%, and 50% directly. The survival rates were 100% within 1 hour, 95.3% within 3 hours, and about 70% after 6 and 12 hours in Lemon juice. In Alcohol, the survival rates of the metacercariae varied on different concentrations in the range of 83.3 - 98.5% after the first 30 minutes and 85.4 - 96.6% after 60 minutes and 62.1 - 83.3% after 90 minutes by Phan *et al.* (2016) [25]. However, in the present study, the survival rate is 100% in Lemon juice and Vinegar, 60% in Alcohol, and 93.15% in NaCl 10% after 12 hours, suggesting that *C. sinensis* metacercariae in infected fish can live longer than those isolated when are soaked in solutions.

The other studies detected metacercariae remained viable and ineffective after storing at a heavy salt concentration (30%) for 7 days by Fan (1998) [23] and these findings gave results similar to a study by Wongsawad *et al.* (2005) [26], which showed that Lemon juice (pH = 3) had no killing effect on metacercariae of *Stellantchasmus falcatus* and also agreed with that by Prasongwatana *et al.* (2013) [27], which showed that metacercariae of *O. viverrini* could survive in fermented fish, pla-ra and plasom. However, there is little difference with time for inactive metacercariae in salt, our findings indicate that no metacercariae were viable after 72 hours but Fan (1998) [23] observed metacercariae still alive after 7 days. Among 4 different solutions, our results also show that using NaCl 10% and Alcohol can inactivate metacercariae better than using Lemon juice and Vinegar for treatment. The potential mechanisms for causing the higher death of metacercariae remain to be elucidated, but there are several reasonable hypotheses: Alkalosis had an effect on the metacercariae wall. The mortalily rate of *C. sinensis* in Alcohol solution treatment is highest after 12 hours experiment. The reason may be due to the mean body weight of fish in Alcohol treatment is smaller than NaCl, Lemon juice, and Vinegar that make alkalosis, dehyration process is faster than those. The mortality rate of *C. sinensis* in Lemon juice and Alcohol and NaCl solutions treatment increased with time, while in Vinegar changed at 72 hours. The reason may be due to fish being infected with a number of *C. sinensis* metacercariae are larger than in NaCl, Lemon juice, and Alcohol solutions which cause the different.

These results have taken together suggest that keeping the fish in NaCl 10%, Lemon juice, Alcohol 55% and, Vinegar in a short time are ineffective for inactivating metacercariae. The present findings must be interpreted in the context of a number of potential limitations such as

increased sampling to increase the fish samples for analysis in the long time with another treatment, and collect the same fish size for all solutions treatment. In conclusion, these data suggest that we should not eat raw fish or undercooked fish.

4. CONCLUSION

The mortality rate of *C. sinensis* metacercariae detached from fish immersed in the Alcohol 55% was higher than those in Vinegar, Lemon juice and NaCl. Alcohol 55% are effective for inactivating *Clonochis sinensis* metacercariae recollected from fish after 60 hours. The result of this study provides important information on the Survival longevity of *Clonorchis sinensis* metacercariae detached from soaked fish in NaCl, Alcohol, Lemon juice, and Vinegar, which can be used to warn people like eating uncooked fish, and raising awareness for everyone to prevent infection of *C. sinensis* from fish.

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PARTIAL REPLACEMENT OF MARINE FISHMEAL BY BLACK SOLDIER FLY LARVAE MEAL IN DIET FOR SNAKEHEAD FISH (*Channa striata*)

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ABSTRACT

This study aims to evaluate the replacement effect of marine fish meal (FM) protein with black soldier fly (BSF, *Hermetia illucens*) larvae meal protein on the growth, survival rate, and feed utilization of snakehead fish (*Channa striata*). BSF larvae were produced by feeding with the mixture of soybean milk by-product and ensiled elephant grass (*Pennisetum purpureum*) (ratio of 1: 1). Five iso-nitrogenous (44%) and iso-lipidic (8%) experimental diets were formulated to replace FM using BSF larvae meal at 0% (control), 25% (BSF_{25}), 50% (BSF_{50}), 75% (BSF_{75}) and 100% (BSF_{100}). There were three replicates for each treatment. Juvenile fish (19.75 g/fish) were randomly distributed into 15 hapas at 50 fish/hapa. At the end of 6 weeks of a feeding trial, the results showed that final weight, weight gain (WG), daily WG, and the specific growth rate of fish fed the control diet were statistically higher than those of fish fed the BSF_{50}, BSF_{75}, and BSF_{100} diets ($p < 0.05$), but not statistically different from those fed the BSF_{25} diet ($p > 0.05$). Compared to the control group, replacement of 25 - 75% of FM by BSF meal did not significantly affect feed conversion ratio (FCR), but the FCR of fish fed BSF_{100} treatment was statistically higher than that fed the control, BSF_{25}, and BSF_{50} diets ($p < 0.05$). There were no significant differences in survival rate among treatments ($p > 0.05$). This study suggests that BSF larvae meal protein could substitute 25% of FM protein without any negative effects on growth performance, survival rate, and feed efficiency of the snakehead fish.

Keywords: *Black soldier fly, fish meal, growth performance, snakehead fish.*

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1. INTRODUCTION

Larvae and prepupae of many insects are rich in protein, lipid, vitamins, minerals, etc. and are becoming a great source of nutrients for both humans and animals [1 - 2]. Compared to other protein sources such as fish meal (FM), soybean meal and animal and seafood by-products (blood meal, meat and bone meal, and other rendered products), insect meals are efficient and sustainable sources of protein for aquaculture [1 - 2]. In addition to being a source of food materials, insects also contain various bioactive substances with several pharmacological functions, such as antiviral and microbial activity as well as the immune response [3 - 4]. Insects such as the black soldier fly (BSF) (*Hermetica illucens*) have been studied more extensively, not only for aquaculture but also for livestock feed industry. In aquaculture, BSF meal was used as a replacement for FM for many species such as rainbow trout (*Oncorhynchus mykiss*) [5], whiteleg shrimp (*Litopenaeus vannamei*) [6], European seabass (*Dicentrarchus labrax*) [7], climbing perch (*Anabas testudineus*) [8],…

Snakehead fish (*Channa striata*) is an increasingly important fish for freshwater aquaculture in Vietnam. As snakehead fish farming production increases, there will be

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increasing demand for high quality feed. The growth of snakehead fish is highly affected by the protein level. Previous study has determined that the dietary protein level for maximum specific growth rate of snakehead fish should contain 46.5 - 50.8% [9]. The main protein source in snakehead fish feed is FM, due to its high protein content with balanced essential amino acid profile [10]. However, the price of FM has been increased by more than twofold in recent years, leading to FM reduction trend in aquafeed [1]. Recent studies have shown that the larvae/prepupae meal of insects, including BSF, can be used as a substitute for FM in snakehead fish feed [11 - 14]. The advantage of BSF larvae is effectively convert a wide range of organic materials (domestic and livestock waste, agricultural by-products) into edible biomass. However, utilizing these rearing substrates for BSF production often resulted in accumulating a high heavy metal level of BSF meal [15 - 16]. Considering the heavy metal accumulation in larvae, and substrate availability and costs, several studies have focused on the use of plant leaves, such as sesbania (*Sesbania grandiflora*), as growing media for BSF larvae. Compared to utilizing kitchen and agricultural wastes, the sesbania-reared BSF larvae had the same protein content (43.5%), but lower content of fat (16.7%) and heavy metals [17]. Katya *et al.* (2017) [18] reported that the sesbania-reared BSF larvae meal could be used as a protein replacement for FM for Asian seabass (*Lates calcarifer*) juvenile. Napier or elephant grass (*Pennisetum purpureum*) is one of the highest yielding tropical forage grasses and has a high content of protein, lipid, minerals, and vitamins [19]. Elephant grass is often ensiled to improve its nutritional value (protein or energy). It has previously been utilized as a diet for tilapia and grass carp, and it has demonstrated good growth, survival, and feed utilization [20 - 21]. Therefore, this study was carried out to evaluate the effect of the partial or total replacement of dietary marine FM protein by protein meal of Napier grass reared BSF larvae on the growth, survival rate, and feed utilization of snakehead fish.

2. MATERIALS AND METHODS

2.1. Materials

Black soldier fly colony was established at the Research Institute for Biotechnology and Environment (RIBE), Nong Lam University - Ho Chi Minh city. Soymilk by-product (soy pulp) was obtained from the Vietnam Soya Products Company (Vinasoy), Tan Uyen district, Binh Duong province and transported to the BSF rearing experimental facility at the RIBE. Elephant grass was taken from a farmer in Trang Bang district, Tay Ninh province and delivered to the RIBE. Grass was ensiled as guided by Moran (2005) [22] with some modifications. Grass was chopped into 3 - 5 cm pieces by using a chaff cutter, wilted for 6 - 8 hours, and mixed with 3 - 5% molasses (on a fresh weight basis) as additive just prior to ensiling. The ensiling was conducted by packing the chopped grass materials in the ensiling plastic bags. The bag was squeezed, and the neck of the bag was twisted, tied with a rubber band, and stored in a roofed house. The silage was kept until pH dropped to 3.5 to 4.5, and silage was used to grow BSF larvae. Nutritional composition of elephant grass silage and soy pulp for rearing BSF larvae was presented in table 1.

Table 1. Nutritional composition (%, dry weight basis) of elephant grass silage and soy pulp for rearing BSF larvae

Parameters	Elephant grass silage	Soy pulp
Moisture	77.03	83.23
Protein	10.19	19.33
Fat	2.37	9.31
Fiber	35.45	23.13
Ash	7.65	6.10

BSFL were reared vertically in plastic containers (61 × 42 × 15 cm) in a greenhouse at 28°C with 90% air moisture. Based upon initial trials, about 1 g of BSF egg was put into each container and the larvae were grown on media containing grass silage mixed with soy pulp (1: 1). When about half of the larvae metamorphosed into prepupae, the larvae were harvested, gently

cleaned, frozen at – 20°C, dried at 60°C to a constant weight and ground into a fine particle consistency (approx. 0.5 - 1 mm). Each container was added 1 kg of mixture daily for 15 days. After a 15 day growing period, the larvae were picked from the mixture residue, washed with distilled water, dried at 60°C to constant weight, and then ground into homogenous powder for determination of the chemical composition. Contents (%, dry weight basis) of crude protein, fat, fiber, and ash of the BSF larvae were 50.17 ± 2.46, 20.39 ± 4.2, 11.8 ± 0.8, and 15.1 ± 0.9, respectively.

Snakehead fish juvenile (2.5 g/fish) was obtained from a hatchery in An Giang province, shipped to the experimental farm, Faculty of Fisheries, Nong Lam University - Ho Chi Minh city, and then acclimated to the experimental conditions for three weeks before the feeding trial began. Fish were fed commercial pellets (Cargill Company) during the first week, and a control diet in the following two weeks to apparent satiation twice daily (7:00 and 15:00).

Feed ingredients were taken from the Lai Thieu Feed Mill Co., Ltd. The main protein sources were marine fish meal (60% protein, 7.12% lipid, Kien Giang province), soybean meal (Argentina), poultry by-product meal (65% protein, Brazil), and blood meal (Hungary); the lipid sources were marine fish oil (Peru) and soybean oil, while the carbohydrate sources were rice bran, and cassava meal.

2.2. Experimental diets

Five iso-nitrogenous (44%) and iso-lipidic (8%) experimental diets were formulated to replace FM using BSF larvae meal at 0% (control), 25% (BSF_{25}), 50% (BSF_{50}), 75% (BSF_{75}), and 100% (BSF_{100}). There were three replicates for each treatment. The formulation and the analyzed composition of the experimental diets are shown in table 2.

Table 2. Formulation and proximate composition of the five experimental diets

	Control	BSF_{25}	BSF_{50}	BSF_{75}	BSF_{100}
Ingredients (%)					
Marine fish meal 60% protein	12.00	9.00	6.00	3.00	0.00
Soybean meal	30.00	30.00	30.00	30.00	30.00
Cassava meal	14.50	14.22	14.43	14.26	13.98
Marine fish oil	1.00	1.00	1.00	1.00	0.80
Soybean oil	1.60	1.10	0.60	0.10	0.00
Rice bran	10.00	10.00	10.00	10.00	10.00
BSF larvae meal	0.00	3.50	7.00	10.50	14.00
Monocalcium phosphate	0.50	0.70	0.70	1.00	1.20
Dicalcium phosphate	2.00	2.00	2.00	2.00	2.00
Poultry by-product meal	24.30	24.30	24.00	23.80	23.60
Blood meal	2.00	2.00	2.00	2.00	2.00
Vitamin and mineral premix	0.35	0.35	0.35	0.35	0.35
Stay C 35%	0.03	0.03	0.03	0.03	0.03
Binder (Carboxymethylcellulose)	1.00	1.00	1.00	1.00	1.00
Methionine	0.20	0.21	0.23	0.24	0.25
Threonine	0.27	0.29	0.30	0.31	0.32
Lysine	0.02	0.09	0.17	0.24	0.31

Tryptophan	0.06	0.05	0.03	0.01	0.00
Choline chloride	0.10	0.10	0.10	0.10	0.10
Antioxidant	0.03	0.03	0.03	0.03	0.03
Mold inhibitor	0.03	0.03	0.03	0.03	0.03
Proximate composition (%, dry weight basis)					
Crude protein	44.08	43.97	43.45	42.83	42.60
Crude fat	8.38	7.88	8.49	8.54	7.99
Ash	11.52	11.32	10.71	10.18	10.32
Crude fiber	3.02	3.04	3.66	3.72	5.01
Gross energy (Kcal/g, calculated values)	3.90	3.90	3.90	3.90	3.90

The ingredients were thoroughly mixed (about 30 min) using a Hobart mixer (Hobart Food Equipment Co. Ltd., Tianjin, China), then hot distilled water was added to produce a dough and pelleted by a 1.5 mm-die meat grinder. Pellets were dried at 60°C for 24 hours and kept in plastic bags in a refrigerator (4°C) until feeding.

2.3. Experimental design and management

Before the trial, fish were fasted for 24 hours for gut evacuation and weighed (initial mean body weight of 19.75 g/fish). A total of 750 snakehead juveniles were randomly allocated into 15 hapa at 50 fish/hapa (1 x 1 x 1.5 m) placed in an earthen pond. Fish were fed one of the trial diets *ad libitum* twice daily (7:00 and 15:00) using feeding trays (ϕ 40 cm) for six weeks. The sinking feed with known weight was placed in the feeding tray, then it was immersed into each hapa. One hour after feeding, left-over feed was collected and stored in a plastic container, then stored in a freezer. At the end of the experiment, feed in each container was dried in an oven to calculate total weight of left-over feed in 100% dry matter basis.

Water temperature, pH, and dissolved oxygen (DO) were measured twice daily at 7:00 and 14:00 using Digital thermometer WT - 1, Denver pH meter UP- 10, and Milwaukee MW 600 Dissolved Oxygen, respectively. Total ammonia nitrogen (TAN) and nitrite (NO_2^-) were determined twice weekly using the phenate method (4500-NH_3 F) and colorimetric method (4500-NO_2^- B), respectively; both analytical methods were performed according to the APHA standards [23]. Chemical composition of the experimental diets (moisture, crude protein, crude fat, crude fiber, and ash) was analyzed using the standard methods of AOAC (1995) [24]. All analyzed chemical parameters were conducted at the RIBE.

2.4. Growth performance and feed efficiency

Fish were counted at the beginning and the end of the trial to calculate the survival rate (SR, %). At the end of the feeding trial, all the fish were weighed for the estimation of weight gain (WG), daily WG (DWG), specific growth rate (SGR), and feed conversion ratio (FCR). Growth performance parameters were estimated using the following equations:

Weight gain (g/fish) = $W_f - W_i$

Daily weight gain (g/day) = $(W_f - W_i) / t$

Specific growth rate (%/day) = $100 \times (\ln W_f - \ln W_i) / t$

Where: *t*: duration of the trial (42 days);

W_i: initial mean body weight (g/fish);

W_f: final mean body weight (g/fish).

Feed conversion ratio = Feed intake (dry weight basis)/Wet weight gain

2.5. Statistical analyses

Statistical procedures were carried out as recommended by Gomez and Gomez (1984) [25] and Bhujel (2008) [26]. Arcsine square-root transformation was applied for survival rate data. After verification of normality and homogeneity of variance using Kolmogorov -Smirnov's one sample test and Levene test, respectively, data were

analyzed by one-way ANOVA with Duncan multiple range test as pairwise comparisons. Differences among treatment means were considered at $p < 0.05$ level of significance. All statistical analyses were conducted using the IBM SPSS Statistics for Windows, Version 19.0 (Armonk, NY: IBM Corp). All data were presented as the mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1. Water quality parameters

Table 3. Water quality parameters monitored (mean ± SD) in earthen pond during the feeding trial of snakehead fish

Parameters	Morning		Afternoon
Temperature	29.5 ± 0.4		30.4 ± 0.7
pH	7.9 ± 0.2		8.3 ± 0.2
DO (mg/L)	3.7 ± 0.3		4.7 ± 0.4
TAN (mg/L)		0.87 ± 0.96	
NO_2^- (mg/L)		0.52 ± 0.60	

The water quality parameters taken during the feeding trial were shown in Table 3. Mean values of temperature, pH, and DO were ranged from 29.5 - 30.4°C, 7.9 - 8.3, and 3.7 - 4.7 mg/L, respectively, while TAN and nitrite levels were 0.87 ± 0.96 mg/L and 0.52 ± 0.60 mg/L, respectively. Optimal values of temperature, pH, DO, TAN, and nitrite for snakehead fish were in ranges of 20 – 30°C, 6.5 - 8.5, 3.0 - 6.5 mg/L, 0.25 - 5.20 mg/L, and 0.01 - 0.56 mg/L [27 - 28], respectively. The results of this study showed that these obtained values were maintained within acceptable levels for *C. striata* in earthen pond.

3.2. Growth performance, survival rate and feed efficiency

3.2.1. Growth performance

The growth performance, survival rate, and feed efficiency of snakehead fish fed five trial diets for six weeks were shown in table 4. At the end of the trial, there were no significant differences in the average W_f, WG, and DWG between the control and BSF_{25} diets ($p > 0.05$). However, fish fed a diet with 100% BSF larvae meal had a significantly lower SGR ($p < 0.05$) than those fed with the control and BSF_{25} diets. No significant difference in growth performance of fish fed 50%, 75%, and 100% substitution level diets were observed ($p > 0.05$). These results indicated that BSF larvae meal could substitute 25% of FM without any negative effects on the growth performance of *C. striata*.

Table 4. Growth performance, survival rate, and feed efficiency of fish fed five trial diets after 6 weeks

	Control	BSF_{25}	BSF_{50}	BSF_{75}	BSF_{100}
W_i (g/fish)	19.7 ± 0.0^a	19.8 ± 0.0^a	19.7 ± 0.0^a	19.8 ± 0.0^a	19.7 ± 0.0^a
W_f (g/fish)	122 ± 11^a	109 ± 12^{ab}	98.4 ± 10.4^{bc}	98.2 ± 11.4^{bc}	80.4 ± 10.4^c
WG (g/fish)	102 ± 11^a	89.7 ± 11.6^{ab}	78.6 ± 10.3^{bc}	78.5 ± 11.4^{bc}	60.7 ± 10.4^c
DWG (g/day)	2.43 ± 0.26^a	2.13 ± 0.28^{ab}	1.87 ± 0.25^{bc}	1.87 ± 0.27^{bc}	1.45 ± 0.25^c
SGR (%/day)	4.33 ± 0.21^a	4.07 ± 0.25^a	3.82 ± 0.26^{ab}	3.81 ± 0.29^{ab}	3.33 ± 0.30^b
SR (%)	96.0 ± 2.0^a	96.0 ± 2.0^a	96.7 ± 2.3^a	96.0 ± 6.9^a	93.3 ± 3.1^a
FCR	1.48 ± 0.10^a	1.52 ± 0.12^a	1.78 ± 0.28^a	1.84 ± 0.34^{ab}	2.32 ± 0.36^b

Note: Data represents as mean ± SD (n = 3). Mean values in the same row with different superscript letters differ significantly (one-way ANOVA with Duncan test p < 0.05).

Similar to the findings of this study, most studies found that an optimal FM substitution level of 20 - 50% using BSF larvae meal would be possible without adverse effects on the growth performance of various fish species. Hoa and Dung (2016) [14] reported that snakehead fish (*C. micropeltes*) fed a diet including 40 and 50% BSF as substitution for the FM had a statistically lower growth rate compared to the other diets (containing 0, 10, 20 and 30% BSF). For barramundi juvenile (*L. calcarifer*), the growth rate including WG and SGR was significantly lower when BSF meal replacement levels were > 50% and an optimal substitution level of FM by BSF larvae meal in the diet was 28.4% [18]. In a feeding trial on rainbow trout (*O. mykiss*), fish fed a diet including 50% BSF as replacement for the FM obtained statistically lower W_f and WG as compared to the control diet [5]. Similarly, no significant change in the growth rate was observed in European sea bass (*D. labrax*) fed BSF meal at levels of up to 50% [7].

The decrease in growth rate responses to BSF larvae meal supplementation among carnivorous fish can be due to the substantial rise in chitin content besides the deficiency in essential amino acids such as cysteine, methionine and threonine [29]. Many studies stated that increasing inclusion level of BSF increased chitin content in the feed, and this may have affected its digestibility. The chitin levels in BSF biomass were in range of 8 - 24%, with shedding and cocoons being most rich in chitin [30]. Moreover, the growth decline in fish fed BSF could be due to reduced nutrient digestibility since protein digestibility for BSF was markedly lower than that of FM [29].

3.2.2. Survival rate

Survival rates ranged from 93.3% to 96.7% without significant differences among diets ($p > 0.05$) (Table 4). As stated above, all monitored water quality parameters were maintained at suitable levels for adequate growth and survival of snakehead fish. Furthermore, this result duplicates recent published studies, in which diets containing high inclusion levels of BSF, did not affect the SR of many fish species. Study of Abdel-Tawwab *et al.* (2020) [7] revealed that up to 50% of FM replacement would be possible without negative effects on the SR of European sea bass. Similarly, no significant difference was found on the SR when BSF larvae meal incorporated up to 100% in the climbing perch (*A. testudineus*) diet [8]. In contrast, Katya *et al.* (2017) [18] reported that there was significant difference in the SR among the treatments of barramundi fed different trial diets, but no clear trend in the effects of dietary treatments on fish SR could be taken.

3.2.3. Feed efficiency

Compared to control group, replacement of 25 - 75% of FM by BSF meal resulted in no significant effect on FCR ($p > 0.05$), but the FCR of fish fed BSF_{100} treatment was statistically higher than those fed the control, BSF_{25}, and BSF_{50} diets ($p < 0.05$) (Table 4). This result is in agreement with earlier studies on the inclusion of BSF larvae in feed of other farmed fish species. Hoa and Dung (2016) [14] recorded that FCR of the treatments with inclusion levels of BSF meal less than 30% in snakehead fish feed were not significantly different compared to the control. Similarly, no significant difference was found on the FCR when replacing 50% or 100% of BSF larvae meal in diets of the European sea bass [7], climbing perch [8], and Atlantic salmon (*Salmo salar*) [31]. In contrast, compared to the other diets (inclusion of 0 and 25% BSF meal), St-Hilaire *et al.* (2007) [5] discovered that rainbow trout fed a diet containing 50% BSF meal as substitution for the FM had a statistically higher FCR. Moreover, Nile tilapia *Oreochromis niloticus* fed housefly maggot meal exhibited a higher FCR compared to those fed a diet including 52% of FM [32]. In case of juvenile turbot *Psetta maxima*, the FCR was significantly higher when BSF meal replacement levels were > 33% [33].

4. CONCLUSION

This study is the first research work to offer a new technique for rearing BSF larvae, using silage of Napier grass (*P. purpureum*) as the substrate. Results revealed that substitution of FM by BSF meal at levels less than 50% had negative effects on

growth performance, survival rate, and feed efficiency of snakehead fish as compared to control group. The acceptable level of FM protein replacement for the performances of snakehead fish was 25%. Our observations from the present study suggested that snakehead fish might have limited efficacy to consume chitin from BSF larvae. Further investigation on this matter is recommended to examine the effect of dietary chitin on predatory fish species.

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ANTIMICROBIAL RESIDUES AND FISH - BORNE ZOONOTIC TREMATODES (FZT) IN RAINBOW TROUT FARMING IN LAO CAI PROVINCE, VIETNAM

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ABSTRACT

The rainbow trout farming industry began to develop in Vietnam in 2004. Since then, this industry has grown rapidly and made a positive contribution to local economic development. The presence of parasitic flukes and residues of drugs and chemicals is quite common in aquaculture. In this study, we determined the residues of Chloramphenicol, furazolidone (AOZ), furaltadone (AMOZ), Ciprofloxacin, Oxytetracycline, Malachite green (MG), and Leucomalachite green (LMG), as well as fish-borne zoonotic trematodes (FZT) in farmed rainbow trout at several farms and restaurants in the three largest rainbow trout farming areas in Lao Cai province, including Ngu Chi Son, Lao Chai, and San Sa Ho communes, from 2019 to 2022. The results from 173 collected samples showed no detection of FZT metacercariae, AOZ, AMOZ, and Oxytetracycline residues in the fish meat. Chloramphenicol and Ciprofloxacin were detected only once in 2019, with levels of 0.33 and 1.84 µg/kg, respectively. The residues of LMG and MG significantly decreased from 2019 to 2022. The levels of MG and MLG in these four years were 304.6, 343.9, 0.49, and 3.94 µg/kg, respectively. These positive results were obtained through the dissemination and training activities in Lao Cai province under the frame of the ICI Project Phase III, funded by Finland. However, it is necessary to continue implementing appropriate measures to minimize the residues of prohibited substances in farmed rainbow trout meat.

Keywords: *Antimicrobial, residue, zoonotic trematodes, rainbow trout.*

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1. INTRODUCTION

Global aquaculture production has grown significantly in recent years. The share of rainbow trout (*Oncorhynchus mykiss*) trade, a freshwater salmonid, has also intensely increased. Despite the availability of several vaccines, bacterial disease's rates in fish farms have increased due to intensive aquaculture. It led to widespread use of antibiotics. Since trout farming is mainly in open environments, antibiotic use may have a strong impact on the emergence, selection and/or dissemination of resistant microorganisms.

Since 2004, the Research Institute for Aquaculture No.1 (RIA1) has cooperated with Finland for the first time to successfully bring rainbow trout eyed eggs to trial farming in Sapa town, Lao Cai province. Up to now, cold-water fish farming has developed strongly in 25 provinces/cities across the country, typically in Lam Dong, Lao Cai, Son La, and Lai Chau provinces [1]. Lao Cai province currently has more than 300 cold water fish farming establishments, mainly in Sapa town, Van Ban, and Bat Xat districts, mainly rainbow trout, and sturgeon. By the end of 2020, the total production of cold water fish in the province reached over 670 tons, an increase of nearly 300 tons compared to 2015. Farming facilities create jobs for thousands of local workers. However, extreme weather events with temperature increases, prolonged drought, frequent rains, and floods have, and will, cause unpredictable material losses to cold-water fish farmers in mountainous areas. The problem of

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disease and quality management of aquatic products is a dilemma and uses drugs to prevent and treat diseases outside the permitted list, which will affect the quality of fish [2]. Twenty different antimicrobial products were used for disease prevention and treatment in shrimp and marine fish cultures in three provinces in Northern Vietnam, including banned products such as Chloramphenicol, Enrofloxacin, and malachite green [3]. A survey using questionnaires in 7 provinces of Vietnam on the use of antibiotics in fish and shrimp farming reported that antibiotics were widely used in fish farms (64%) and shrimp farming (24%) [4].

Malachite green (MG) is a dye that has been used to make colors such as silk, wool, leather, cotton, and paper products [5, 6]. MG is also used to treat fungal and protozoal infections in fish and fish eggs farming industries around the world [7, 8, 9, 10, 11]. MG causes damage to liver, spleen, and kidney, and produces teratogenic, genotoxic, and carcinogenic effects. In waterborne exposure, MG is absorbed into fish tissues and transformed into the metabolite, colourless compound, leucomalachitegreen (LMG). This metabolite remains in fatty tissues for extended periods [7, 9, 12, 13]. The high temperature does not guarantee a full breakdown of the residue of MG and LMG which may occur in fish muscles [14]. Therefore, the use of MG in food-producing animals is not authorized in many countries including Vietnam [15, 16]. However, MG is still illegal to use in fish farms due to its low cost, easy availability, and effectiveness. The usage of MG in aquaculture in Vietnam for parasite and fungal disease treatments in brackish and marine aquaculture was reported [3]. However, the use and residue of MG and LMG for rainbow trout farming in Vietnam have not been reported yet.

Raw fish (e.g. sashimi) is a favorite dish widely served in restaurants around the world. In Vietnam, rainbow trout is also favored by fresh raw fish dishes. Eating raw or otherwise inadequately cooked fish can be associated with risks of acquiring fishborne zoonotic trematode (FZT) infection. An investigation of 483 specimens of nine freshwater fish species for FZT metacercariae in Ninh Binh province showed that all 9 fish species were infected with FZT, with a mean prevalence of 56.1% and a mean density of 33,245 [17]. A study on the prevalence of FZT infection in 180 fish farmers in Nam Dinh province reported that 32.2% of fish farms were infected with FZT [18]. A high prevalence of zoonotic trematodes was identified in more than half of the fish species investigated in Yen Bai province [19]. Similar to residues of antibacterial and banned substances e.g. MG, until now, there are no reports on the existence of FZT metacercaria in rainbow trout cultured in Vietnam.

2. MATERIAL AND METHODS

2.1. Study areas and sample collection

Rainbow trout samples were collected in three villages including Ngu Chi Son, Lao Chai, and San Sa Ho communes in Sapa town, Lao Cai province. These areas are the most concentrated cold water farming households of Sapa town in particular and Lao Cai province in general. In addition, rainbow trout samples were also collected at several restaurants in Sapa town for analysis of antimicrobial residue and FZT metacercaria presence. A total of 173 samples were collected from April 2019 to October 2022, divided into two periods:

- 1st period from 2019 - 2020: 79 fish samples from 15 farms and 6 restaurants were collected in seven investigations.

- 2nd period from 2021 - 2022: 116 fish samples from 21 farms and 4 restaurants were collected in 10 investigations.

The collected samples were stored in an icebox and transported to the National Agro-Forestry-Fisheries Quality Assurance Department (NAFIQAD) – Branch 1 in Hai Phong province for chemical residue analysis. FZT metacercaria samples were transferred to Research Institute for Aquaculture No. 1 (RIA1) for analysis.

2.2. Sample analysis

Antimicrobial residues including Chloramphenicol, Furazolidone (AOZ), Furaltadone (AMOZ), Ciprofloxacin, Oxytetracycline, Malachite green (MG), and Leucomalachite green (LMG) in fish were

analyzed by a quantitative liquid chromatography–tandem mass spectrometric (LC–MS/MS) followed the NAFIQAD internal ISO-accredited methods: 05.2/CL1/ST 03.69 (AOZ, AMOZ), 05.2/CL1/ST 03.71 (Ciprofloxacin, Oxytetracycline) 05.2/CL1/ST 03.73 (MG, LMG).

FZT metacercaria was analyzed following the instruction of Phan T. V. and T. N. Bui (2013) [20]. Briefly:

Sample preparation: Fish was weighed before sample processing. Each fish was processed separately. The market size of fish ≥ 300 g was sliced vertically from head to tail, taking representative slices from different parts to make up a total of 100 g. Samples were ground in Pepsin solution (MERCK) in a preheated incubator at a temperature of 37^{0}C for 2 to 3 hours. The ratio of sample to pepsin solution was 2/3 (dissolve 6 g of pepsin in 1000 ml of distilled water, then add 8 ml of HCl). Samples were stirred with a glass rod for 15 - 20 minutes to ensure a uniform and complete digestion process. After complete digestion, samples were filtered by a metal sieve with a mesh size of 1 x 1 mm and physiological saline solution (0.85% saline). Allow the sample to settle after filtration and discard the supernatant from the glass beaker (approximately 1/2 of the volume). Continue adding physiological saline solution, allow it to settle, and discard the supernatant until the liquid in the glass beaker becomes clear.

Classification: Each portion of the sample liquid from the glass beaker was poured onto a Petri dish (12 x 12 mm) for examination under a dissecting microscope (Olympus CZ21). The FZT metacercaria species were classified directly based on their morphological characteristics using the classification key described by Phan T. V. and T. N. Bui (2013) [20].

2.3. Data analysis

Differences in antimicrobial residue contents between years were analyzed using SPSS statistical software (version 23, IBM Corp). First, the data were tested for normality using the Shapiro – Wilk test. If the normality condition was satisfied, ANOVA was used to determine whether there were any significant differences between the groups. A nonparametric Kruskal – Wallis was used if the normality condition was not satisfied. A significant difference was recognized when $p < 0.05$. Principal Component Analysis (PCA) was performed by Statistica 12 (StatSoft). The mean values of each chemical residue content in each investigated event were used for PCA analysis.

3. RESULTS AND DISCUSSION

3.1. Occurrence frequency of FZT metacercaria and antimicrobial residue in fish

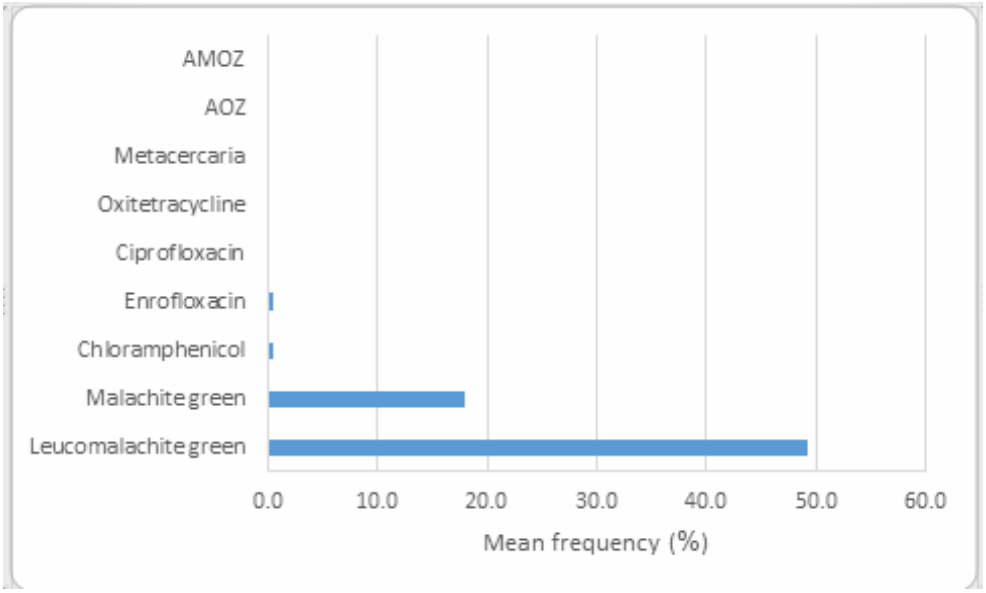

Figure 1a. Mean frequency of metacercaria and antimicrobial residues detection in fish in the period 2019 - 2022

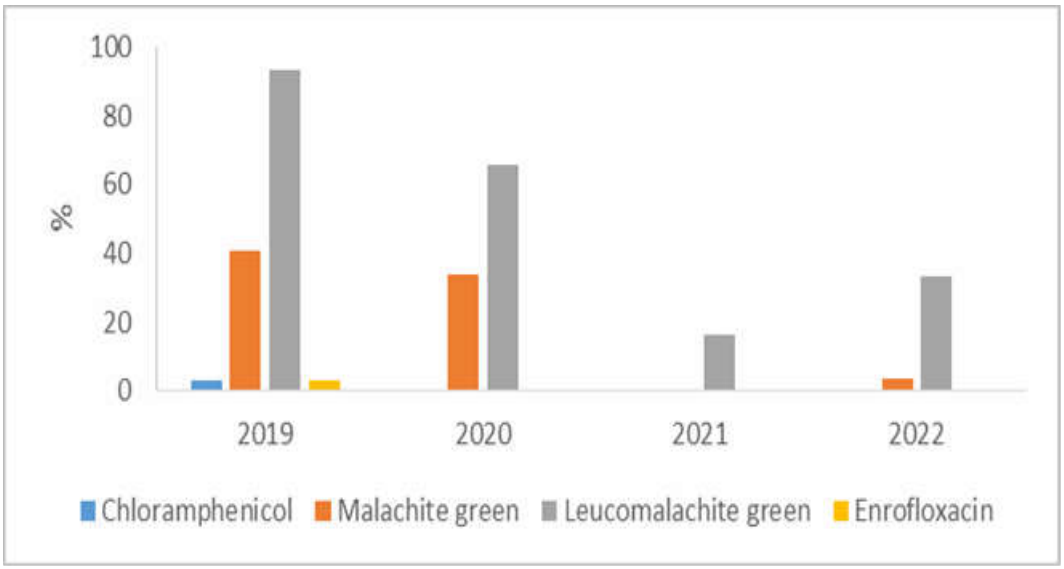

Figure 1b. Occurrence frequency of antimicrobial residues detection in fish from 2019 to 2022

Figure 1. Frequency of chemical residues detection in fish

From April 2019 to October 2022, we conducted 20 investigations, collecting a total of 173 fish samples. No AMOZ, ciprofloxacin, and FZT metacercaria were detected in fish tissue during the whole investigation. The analysis results showed that the residue of LMG was the highest, reaching 49.13% in fish meat, followed by MG at 17.92% (Figure 1a). Enrofloxacin and Chloramphelicon were only detected once in August 2019. Figure 1b demonstrates a obvlously decreasing trend in the chemical residue in fish meat. In 2019, the detected ratio of LMG was 93.75%, which decreased to 65.96%, 16.1%, and 33.33% in 2020, 2021, and 2022, respectively. Similarly, the detection rate of MG decreased from 40.63% in 2019 to 34.04%, 0%, and 3.51% in 2020, 2021, and 2022, respectively (Figure 1b). Previous works on FZT metacercarial infections have shown that many species of freshwater and brackish water fish play the roles as the source of FZT infections in humans [17 - 22]. The intermediate host, the snail, can play an important role in the transmission of these zoonotic diseases [23]. Farmed fish exposed to FZT cercariae released by snails are common in fish ponds [24]. The route of FZT transmission is more complex than in the presence of intermediate hosts, snails, in aquaculture ponds because the exchange of pond water with surrounding habitats such as rice fields and irrigation canals and these surrounding habitats can be a source of snails and free-swimming FZT cercariae, and contributes to FZT infection in farmed fish. However, FZT Cercariae emergence from snails was significantly reduced or stopped during the winter months [25]. One possible explanation for the absence of FZT in this study is that the rainbow trout culture environment is cold water, so there is little existence of snail species as an intermediate host. Moreover, rainbow trout are mainly raised in cement tanks or composite tanks, water is taken from the stream into the culture tank and flowed out, so the existence of snails in the tank is absent.

The presence of MG/LMG residues in fish samples was reported in several other studies. In Croatia from 2009 to 2011, MG residues were found in muscle tissues of 42 carp (*Cyprinus carpio*) and 30 rainbow trout (*Oncorhynchus mykiss*) samples collected from fish farms with a detected frequency of 68.1% [26]. In Malaysia MG and LMG were detected in muscle tissue of tilapia (*Oreochromis* hybrid), African catfish (*Clarias gariepinus*), barramundi (*Lates calcarifer*), hybrid grouper (*Epinephelus fuscoguttatus*% × %*Epinephelus lanceolatus*) and striped catfish (*Pangasius hypophthalmus*) with detected frequencies of 20 - 60% [27]. The Czech Republic reported 1.3% and 16.3% non-compliant results for MG and LMG, respectively, out of 80 aquaculture product samples. According to EFSA

2.5% and 8.4% MG/LMG samples were over the thresholds in Austria and Poland, respectively. Germany, Lithuania, and Spain reported 12.6% (out of 269 samples), 80% (out of 5 samples), and 100% (out of 2 samples) of non-compliant results for LMG in suspect samples, respectively. Lithuania and Spain reported 40% (out of 5 samples) and 100% (out of 2 samples) of non-compliant results for MG, respectively [28]. From 2017–2019, the Rapid Alert System for Food and Feed in the European Union (RASFF) recorded 11 cases of elevated concentrations of MG and LMG in fish and fish products. Among them, in 6 cases the products originating from Vietnam had exceeded the allowable amount, with the range of MG and LMG from 2 - 40.44 µg/kg in catfish and barramundi.

3.2. Chemical residues variation in rainbow trout tissue in Lao Cai province

10 investigations were conducted in 4 years from 2019 to 2022, with a total of 195 collected fish samples. Similarly to the trend in frequency of occurrence, the content of chemical residues also shows a decreasing trend during the period of 2019 to 2022 (Figure 2). The level of LMG tended to increase from April 2019 (169.0 µg/kg) to June 2020 (630 µg/kg) (Table 1). However, the level of LMG decreased significantly in 2021, with the highest detected level being only 0.7 µg/kg. In 2022, LMG slightly increased, ranging from 0.18 to 13.69 µg/kg. Malachite green fluctuated between 1.8 and 58.2 µg/kg from 2019 to 2020. In 2021, no LMG residue was detected in fish meat. In 2022, it was detected only twice, with the highest concentration being 0.45 µg/kg (Figure 2a). Table 1 shows that chloramphenicol and enrofloxacin were detected only in August 2019, with levels of 0.33 and 1.84 µg/kg, respectively. There were no detections of these two chemicals in the following years.

Table 1. The chemical residues in rainbow trout flesh investigated from 2019 - 2022

Sampling date	Malachite green (µg/kg)	Leucomalachite green (µg/kg)	Chloramphenicol (µg/kg)	Enrofloxacin (µg/kg)
April 10th 2019	4.62±9.71	169.04±287.89	ND	ND
May 30th 2019	11.20±29.07	357.23±677.63	ND	ND
August 12th 2019	6.42±11.0	394.07±723.65	0.33±087	1.84±4.88
March 18th 2020	58.16±101.29	580.87±1001.50	ND	ND
May 4th 2020	0.18±0.72	16.08±56.51	ND	ND
June 1st 2020	2.97±4.62	630.47±982	ND	ND
December 25th 2020	1.83±4.87	155.87±421.59	ND	ND
July 20th 2021	ND	1.11±1.88	ND	ND
September 24th 2021	ND	0.73±10.8	ND	ND

October 22nd 2021	ND	0.68±1.81	ND	ND
November 23rd 2021	ND	ND	ND	ND
December 20th 2021	ND	ND	ND	ND
January 19th 2022	ND	ND	ND	ND
February 15th 2022	ND	ND	ND	ND
March 15th 2022	ND	ND	ND	ND
April 18th 2022	0.45±1.18	3.74±1.87	ND	ND
May 17th 2022	ND	0.18±0.47	ND	ND
June 29th 2022	ND	0.33±0.76	ND	ND
September 7th 2022	ND	13.69±26.97	ND	ND
October 27th 2022	0.09±0.32	6.38±14.61	ND	ND

Note: ND: Not detected

According to EFSA (2020), exposure to food contaminated with MG/LMG at or below the Minimum Required Performance Limit (MRPL) of 2 µg/kg does not represent a health concern [29]. In our study, the mean content of MG and LMG was below the MRPL in 2021, with a content of 0.49 µg/kg. However, the mean contents of MG/LMG were higher than MRPL in 2019, 2020, and 2022, with levels of 304.6, 343.9, and 3.94 µg/kg, respectively. Although the malachite green and its metabolites significantly reduced in 2021 and 2022 compared to 2019 and 2020 ($p<0.05$). However, the contents of MG and LMG were still above the threshold in 2022. The results indicated that the content of MG and LMG has been well controlled recently. However, the situation is still a potential hazard to public health. The excessed MG residue in fish meat is widely observed in the world. Pipoyan *et al.* (2020) reported that 34.5% of investigated samples exceeded the MRPL in Armenia [30]. The investigation in Malaysia illustrated that residues of MG and LMG were highest in domestic striped catfish and lowest in red tilapia, with 8.1% of 149 samples above 2 µg/kg. The Czech Republic reported the LMG exceeded resides at levels of 7.73 - 13.4 µg/kg in rainbow trout originating from this country and Italy. Barani and Tajik. (2017) reported that the usage of MG in Iranian fish farms is frequent. MG was detected in 108 fish samples, corresponding to 61.0% of the total examined samples, ranging between 0.35 and 7.12 µg/kg. 18 out of 177 samples contained residue of MG higher than 2 µg/kg [31].

Chloramphenicol is a broad-spectrum antibiotic with well-known bacteriostatic properties effective against both Gram-positive and Gram-negative bacteria, and other groups of microorganisms [32]. Chloramphenicol can be quickly eliminated from fish tissue. Bilandžić *et al.* (2011) suggested that trout muscle tissue could be compliant with health requirements for

consumption 10 days after withdrawal from chloramphenicol treatment [33]. In this study, the content of chloramphenicol was detected only one time at a concentration of 0.33 µg/kg, which may not harm consumers. Enrofloxacin is a fluoroquinolone antimicrobial agent. The antibiotic was used for rainbow trout against the main pathogenic bacteria *Aeromonas salmonicida*, *Yersinia ruckeri,* and *Flavobacterium psychrophilum* [34]. The content of enrofloxacin in our study was much lower than the level of MRL 100 µg/kg, which was set by the Commission of the European Union for the sum of the levels of enrofloxacin and ciprofloxacin in tissues from fishes in general [28]. With only one time detected during four years and low content, enrofloxacin is supposed not to be the chemical residue problem in rainbow trout culture in Lao Cai province.

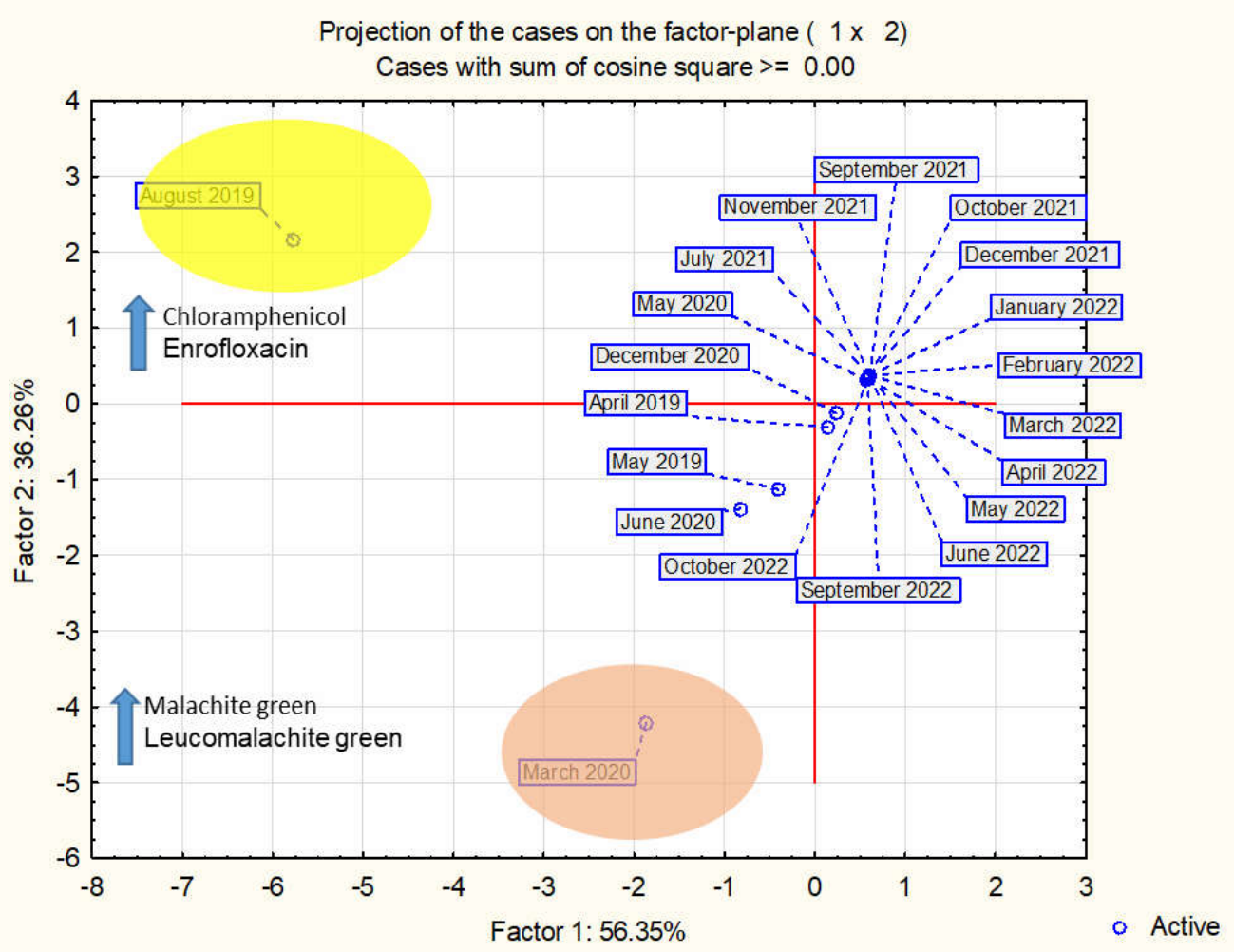

Figure 2. Principal component analysis of chemical residues in rainbow trout from 2019 - 2022.

Note: Principal component analysis (PCA) projection of cases (A) and variables (B) on factor 1 vs. 2. Variables are average concentrations of chemical residue in each investigation event.

We applied principal component analysis (PCA) to explore potential relationships between chemical residues and sampling time (Figure 2). The value of antimicrobial residue in each sampling event was labeled and presented in the form of month and year, for example, sampling on September 7th, 2022 was presented as September 2022. The projection of cases and variables on the principal component (PC) 1 and PC 2 plane in figure 2 shows a clear separation by time characteristics. Two PCs explain 92.6% of the total variance. Factor one was the negatively correlated

content of LMG, MG, chloramphenicol, and enrofloxacin residues. Which is representative of a better chemical residue control situation in 2021 and 2022. While high content of enrofloxacin and chloramphenicol was seen in August 2019. High levels of LMG and MG were seen in March 2020. These results demonstrate significant achievements in our efforts to minimize chemical residues in rainbow trout. These accomplishments have been made possible with the support of ICI Project Phase III (2019-2022), funded by Finland, which has implemented a range of awareness-raising and training solutions. However, it is important to continue implementing measures to ensure that the levels of these chemicals remain within safe limits.

4. CONCLUSION

Leuko malachite green and malachite green are the most common chemical residues present in rainbow trout.

The leukomalachite green and malachite green content in rainbow trout decreased significantly in 2021 and 2022.

It is necessary to continue implementing measurements to effectively control the levels of chemical residues in rainbow trout.

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ISOLATION AND CHARACTERIZATION OF LACTOBACILLUS FROM GRASS CARP (*Ctenopharyngodon idellus*) FOR USE AS PROBIOTIC IN AQUACULTURE

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ABSTRACT

In aquaculture, probiotics are given directly to animals to regulate their gut bacteria, enhance their immune systems, and aid digestion. Additionally, probiotics are introduced into the pond's water to enhance the water quality and increase the chances of survival for aquatic creatures. *Lactobacillus* belongs to the group of lactic acid bacteria (LAB), which are considered beneficial for aquaculture. Incorporating LAB into fish feed can enhance its nutritional quality. LAB produces various digestive enzymes that aid in food breakdown and digestion, promoting growth to the host. In this study, 95 isolates of lactic acid bacteria were recovered in the gut of grass carp in Thai Binh, Bac Giang and Nam Dinh province, Vietnam. Only 9 of 95 isolates had the inhibition effects on *Aeromonas hydrophila* and *Staphylococcus aureus*. The isolates L18 showed the strongest antimicrobial activity to *Aeromonas hydrophila* and *Staphylococcus aureus* with the diameter of antimicrobial zones were 18.22 ± 0.16 mm and 10.14 ± 0.23 mm, respectively. The strain L18 was identified as *Lactobacillus fermentum* based on its morphological, physiological, biochemical characteristics, and 16S rRNA sequences. The *L. fermentum* L18 is capable of withstanding concentrations of bile salts ranging between 0.0% and 0.5%. Moreover, it can tolerate stomach acid with a pH range of 1 to 4. Furthermore, the bacterial *L. fermentum* strain L18 can produce extracellular enzymes that include cellulase and amylase, which are responsible for breaking down starch and cellulose substrates. Thus, the *Lactobacillus* L18 strain could be considered a potential antimicrobial probiotic strain against fish pathogens and should be further studied for its probiotic benefits.

Keywords: *Aquaculture, probiotic, Lactobacillus fermentum.*

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1. INTRODUCTION

The fisheries and aquaculture sectors have been increasingly recognized for their essential contribution to global food security and nutrition in the twenty-first century. In 2020, farmed finfish reached 57.5 million metric tons (USD 146.1 billion), including 49.1 million metric tons (USD 109.8 billion) from inland aquaculture and 8.3 million metric tons (USD 36.2 billion) from mariculture in the sea and coastal aquaculture on the shore [1]. This shows that farmed aquatic animal species have grown steadily over the past decade. The industry is expected to reach over USD 120 billion in 2022. Efforts to intensify aquatic animal production have led to the excessive use of various antimicrobial agents, the adverse side effects of which have become uncomfortable and apparent to both producers and consumers. To fight those stressors and their negative impacts, probiotics have become recognized as effective substitutes, acting as immunity modulators and increasing resistance to various microbial pathogens [2, 3].

In aquaculture, probiotics confer several benefits and play significant roles in improving growth performance, disease resistance, immunity, health status, intestinal epithelial barrier integrity, gut microbiome, and water

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quality. In addition, probiotics in aquaculture diets could minimize antibiotic side effects. Probiotics are live beneficial bacteria introduced into the gastrointestinal tract through food or water, promoting overall health by enhancing internal microbial balance. Probiotic microbes produce bacteriocins, siderophores, lysozymes, proteases, and hydrogen peroxides, inhibiting harmful pathogens [4]. Probiotics stimulate the immune system, provide essential nutrients, and increase growth rates and feed efficiency. They can also improve water quality and reduce environmental pollution.

The genus *Lactobacillus* is the largest genus among lactic acid bacteria (LAB), consisting of more than 237 species [5], with a continuous diversity of species discoveries. *Lactobacillus* species are among the most widely used probiotics [2]. The mechanisms of action of *Lactobacillus* in aquaculture include the secretion of antagonistic compounds [6, 7], effects on quorum sensing mechanisms [8, 9], inhibition of adhesion and colonization by pathogenic bacteria via competitive exclusion [3], modulation of gut microbiota and immune reactions [10], antiviral effects [11], and the improvement of water quality and modulation of the aquatic microbiota [4, 12]. This research aimed to isolate, characterize, and identify the *Lactobacillus* isolates isolated from the intestine of grass carp to explore their probiotic properties and the potential of using them as probiotic products production.

2. MATERIAL AND METHOD

2.1. Isolation of microorganisms

A total of twenty-nine separate samples of fish guts from grass carp (*Ctenopharyngodon idellus*) were collected from local markets in Thai Binh, Bac Giang, and Nam Dinh, stored at 4°C by ice to avoid contamination and spoilage during transportation to the laboratory of Faculty of Biotechnology, Vietnam National University of Agriculture [13]. For the isolation of microorganisms, we followed the protocol described by Karami *et al.* (2017) [14] with some modifications. Fish gut samples were finely ground (one gram) and mixed with 99 ml of sterile physiological saline to release the microbiota in each sample. After homogenization, the samples were spread on a plate of MRS agar (g/l: Glucose 20.0, Meat extract 10.0, yeast extract 5.0, peptone 10.0, Tween 80 1 ml, triamonium citrate, 2.0, $K_2HPO_4.3H_2O$ 2.0, CH_3COONa 5.0, $MgSO_4.7H_2O$ 0.58, $MnSO_4.4H_2O$ 0.28, $CaCO_3$ 5.0, agar 15.0, pH = 6.5 ± 0.2) incubated at 30°C for 48 hours under aerobic conditions. From the isolated samples, strains of bacteria with typical shapes for *Lactobacillus* bacteria (small colonies, opaque white or colorless, and capable of decomposing $CaCO_3$) were selected, and primary biochemical characteristics such as Gram staining, oxidase and catalase test were performed according to Karami *et al.* (2017) [14].

2.2. Antimicrobial activity assay

The antibacterial activity was detected against different indicator strains (*Aeromonas hydrophila* and *Staphylococcus aureus*) by the agar-well diffusion method [15]. *Aeromonas hydrophila* and *Staphylococcus aureus,* obtained from diseased carp samples which were collected in Hung Yen province, were preserved, and stored at the laboratory of the Department of Microbial Technology, Faculty of Biotechnology, Vietnam National University of Agriculture. The overnight culture of 95 lactic acid bacteria isolates were centrifuged at 10,000 rpm for 10 minutes at 10°C. 100 µL of the cell-free supernatants (CFS) of culture broth were added to 5 mm wells on nutrient agar plates with were previously spread with a 100 µL suspension of *Aeromonas hydrophila* or *Staphylococcus aureus*, the plates were incubated at 30°C for 12 hours. The antimicrobial activity was expressed as the diameter of the inhibition zones (mm) around the wells. The presence of clear zones was an indication of the activity in the tested samples against the two pathogens.

2.3. Growth at different pH concentrations

Lactobacillus strains were grown at 30°C. The culture broth was then centrifuged at 10.000 rpm for 10 minutes to collect the cell pellet. The pellets

were then washed with distilled water to remove the residue and then added into MRS broth with a pH of 1 to 4. After 3 hours, 6 hours, and 9 hours. The OD values of cultures were determined at 620 nm every 3 hours by a UV-vis spectrophotometer [16]. Acid tolerance was determined based on ΔOD value > 0 (the difference between the OD value measured at 9 h and the OD value measured at 3 hours) at each pH concentration. The absorbance values obtained were plotted against the incubation time, and the acid tolerance of the strain was based on the time required for the absorbance value to increase by 0.3 units.

2.4. Growth at different bile concentrations

Lactobacillus strains were incubated in 3 ml of MRS broth supplemented with bile salts (Thermo Fisher Scientific, US) of 0.0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, and 0.8%. The bile salt tolerance of *Lactobacillus* strains was measured by OD at 600 nm after 0 hour, 12 hours, 24 hours, and 48 hours [17].

2.5. Genetic identification of bacteria

In this current study, the DNA of the *Lactobacillus* strain was extracted using the method of Han *et al.* (2018) [18]. PCR for the amplification of the 16S rRNA gene was carried out using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTGTGTGTGTTACGA CTT-3'). The PCR conditions were: initial denaturation of 5 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1.5 min at 53°C and 1 min at 72°C, and a final extension at 72°C for 10 min. PCR reactions were stored at 4°C. The PCR products underwent sequencing at the 1st BASE, Singapore. The sequences obtained were compared with the NCBI database through BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed using Mega X software based on the 16S rDNA sequences of the strain curated and the homologous sequences of other strains.

2.6. Determination of microbial extracellular enzyme activity

The extracellular enzyme production of selected bacteria was performed by plate assay according to Hmani *et al.* (2017) [19], with some modifications. Amylase and cellulase production were determined using Luria-Bertani (ThermoFisher Scientific, US) agar plates supplemented with 1% starch and 1% CMC, incubated for 24 hours at 30°C. The clear zone around the agar well revealed enzyme production.

2.7. Statistical analyses

Data were analyzed in Excel version 16.76.1 (Microsoft) using a statistical software package MIX2.0. All statistical analyses were performed using GraphPad Prism 9.0.2.

3. RESULTS AND DISCUSSION

3.1. Isolation of *Lactobacillus* bacteria

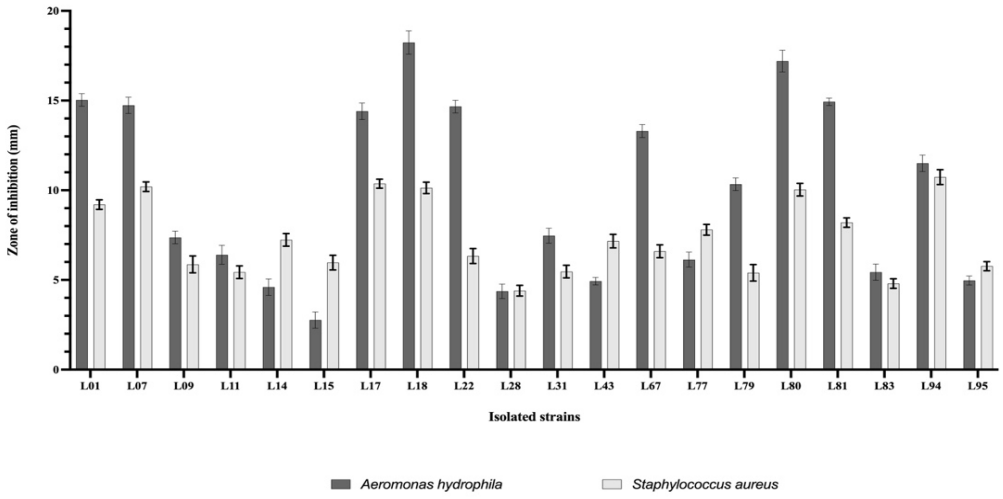

Figure 1. Diameter of inhibition zone (mm) of different *Lactobacillus* isolates against *Aeromonas hydrophila* and *Staphylococcus aureus*

From 29 different collected fish gut samples, 95 bacterial isolates with typical characteristics of lactic acid bacteria, such as milky white color and $CaCO_3$ resolving rings, were recovered. All isolates were Gram-positive, catalase-negative, and oxidase-negative. In general, all isolates were rod shaped. Those characteristics suggested the isolates corresponded to the LAB group. Thirty-six strains of bacteria with *Lactobacillus* characteristics are bacilli, while the remainder are cocci.

3.2. Screening of *Lactobacillus* strains for antimicrobial activity

Among the isolates that exhibited inhibition properties against targeted microorganisms (*Aeromonas hydrophila* and *Staphylococcus aureus*), 20 strains showed antimicrobial activity (Figure 1). The *Lactobacillus* strain L18 showed the strongest antibacterial activity with the largest antimicrobial zone diameter to *Aeromonas hydrophila* (18.22 ± 0.16 mm) and *Staphylococcus aureus* (10.14 ± 0.23) mm, respectively (Figure 3). This strain was identified as *Lactobacillus*, and it exhibited a broad spectrum of antibacterial activity. This study suggests that *Lactobacillus* L18 could be a promising natural antimicrobial agent. This finding indicates the potential of *Lactobacillus* L18 to be used in the development of novel and effective antibiotics to combat bacterial infections.

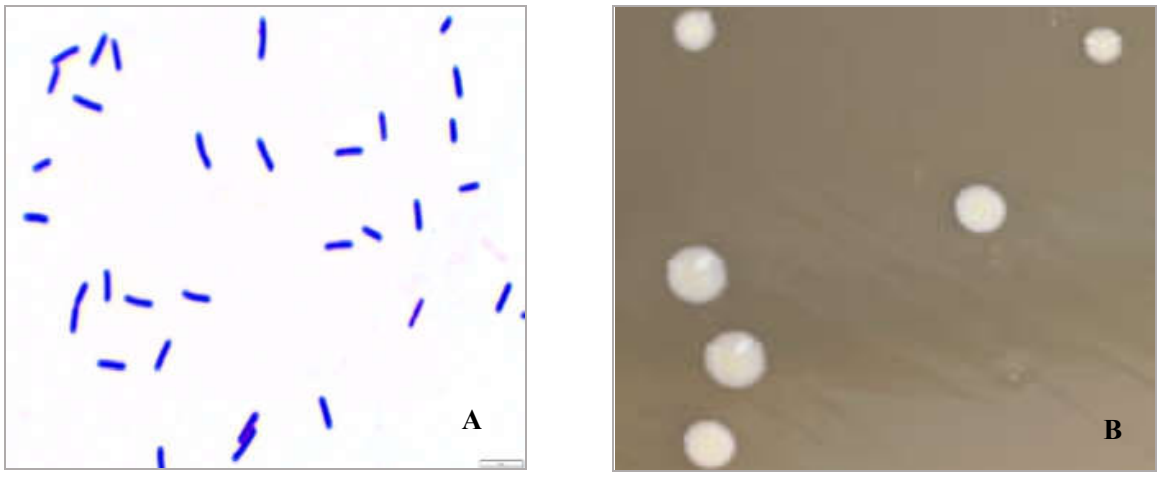

Figure 2. Bacilli shape, Gram positive isolate L18 with 1000-times magnification (A) and white and convex colonies of isolates L18 on MRS agar (B)

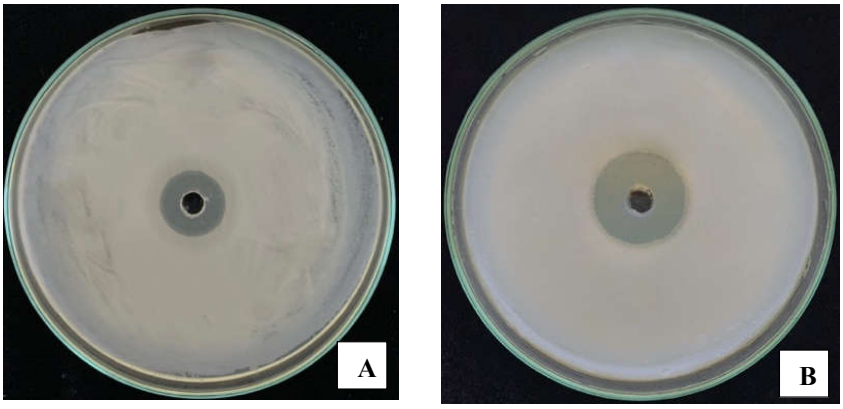

Figure 3. Antagonism of the *Lactobacillus* strain L18 to *Staphylococcus aureus* (3A) and *Aeromonas hydrophila* (3B)

This result is relatively consistent with Koohestani *et al.* (2018) [20] who investigated the resistance to *Staphylococcus aureus* of *Lactobacillus acidophilus* and *Lactobacillus casei*. They showed the ability to inhibit *Staphylococci* of *Lactobacillus acidophilus* with an antimicrobial zone that measures 16 mm, and *Lactobacillus casei* measures 13 mm. Their results indicate that *L. acidophilus* is more effective in inhibiting *Staphylococci* than *L. casei*. Furthermore, it was verified that these two strains of *Lactobacillus* can be used to reduce the presence of *Staphylococcus aureus* in food. In 2020, Ma *et al.* (2020) [21] investigated the resistance to *Staphylococcus aureus* ATCC 25923 of *Lactobacillus casei* strain KLDS 1.0338 with an antimicrobial zone diameter of 10.64 ± 0.15 mm. Additionally, Ma *et al.* (2020) also showed that *Lactobacillus casei* strain KLDS 1.0338 had superior inhibitory activity against *Escherichia coli* ATCC 25922, with an antimicrobial zone diameter of 11.64 ± 0.14 mm [21].

Our findings suggest that *Lactobacillus* strain L18 is a promising probiotic with the potential to improve gut health. However, further experiments need to be done to understand the long-term effects of this probiotic on gut health, how *Lactobacillus* L18 exhibits its antimicrobial activity, what is the dosage and delivery method for this probiotic for optimal gut health.

3.3. Effect of pH on the *Lactobacillus* bacteria

Gastric juice in the stomach is considered one of the first challenges faced by probiotic bacteria due to the low pH and antibacterial effects of pepsin [16, 22]. Probiotic bacteria are also exposed to bile salts and fatty acids that reduce their viability [23]. To survive these conditions, probiotic bacteria must be robust and highly resistant to harsh environmental conditions. Probiotic strains must survive in the gastrointestinal tract at an intestinal pH of 2.5 to 3.5, and they persist in the stomach for 2 - 4 hours or more before being transported to the intestinal tract.

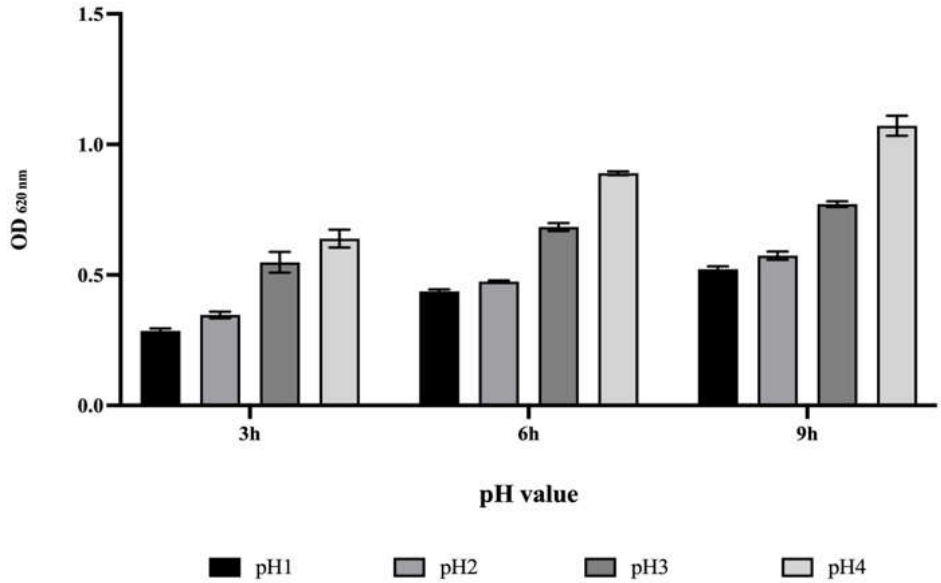

Figure 4. Acid tolerance of *Lactobacillus* strain L18

The bacterial *Lactobacillus* strain L18 was grown in MRS broth for 24 hours at 30°C and centrifuged at 5000 rpm for 5 min. Wash the cell residue with distilled water and then put it in a MRS broth with a pH range from 1 to 4. And monitor the results after 3 hours, 6 hours, and 9 hours.

The experimental results showed that the *Lactobacillus* strain L18 increased steadily in the pH range from 1 to 4 over 3 to 9 hours. At pH 4, the *Lactobacillus* strain L18 grew the fastest (Figure 4). This result is consistent with the author's study [24] that investigated the tolerance to stomach acid with a pH of 2 to 4 for 4 hours of

Lactobacillus plantarum LZ95 and *Lactobacillus plantarum* CY3. The results showed that the gastric acid tolerance of the two strains was not affected in the pH range of 2 to 4 for 2 hours. After 4 hours, 2 strains of *Lactobacillus plantarum* LZ95 and *Lactobacillus plantarum* CY3 showed a significant increase in their viability in the pH range of 2 - 4. The *Lactobacillus plantarum* strain CY2 viability was significantly impaired after 4 hours at pH 2 and pH 3 (60% loss of viability). The *Lactobacillus plantarum* strain CY2 viability was significantly affected after 4 hours at pH 4 (85% loss of viability). However, all three strains showed similar viability at pH 4 after 6 hours. These results suggest that *Lactobacillus plantarum* strains are moderately acid-tolerant. For example, the *Lactobacillus plantarum* strain CY2 showed 60% viability loss after 4 hours at pH 2, compared to 85% viability loss at pH 4.

According to the study of gastric acid tolerance, *Lactobacillus* strain L18 showed excellent stomach survival. Furthermore, *Lactobacillus* L18 displayed a higher ability to tolerate simulated gastric acidity than other *Lactobacillus* strains. It also has strong anti-inflammatory activity. This suggests that *Lactobacillus* strain L18 could be a promising probiotic for gastrointestinal diseases.

3.4. Effect of bile on the *Lactobacillus* bacteria

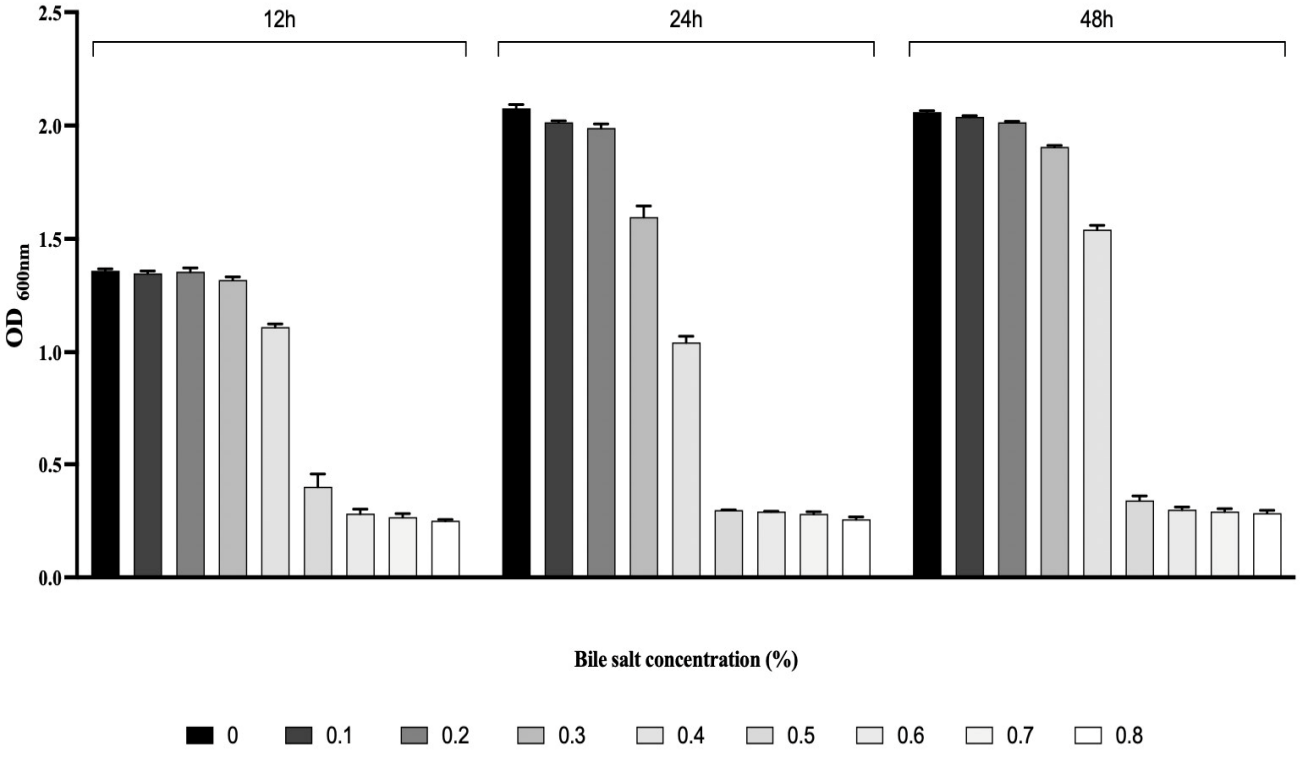

Figure 5. Bile tolerance of *Lactobacillus* strain L18

To exert beneficial effects in the gastrointestinal tract, probiotic bacteria must be able to pass through the stomach and tolerate concentrations of bile salts in the small intestine [25]. Bile salts play an essential role in lipid emulsification and also have antibacterial activity. Therefore, probiotic bacteria's ability to withstand bile salts is essential for survival, adhesion, and colonization in the small intestine. Some probiotic strains possess bile salt hydrolase activity, which allows them to break down bile salts and survive in the intestine.

The results showed that strain L18 grew well under bile salt conditions ranging from 0% to 0.2%. The OD values decreased with increasing bile salt concentrations between 0.5% and 0.8%. Thereby, it can be seen that high bile salt concentrations inhibit the growth and development of strain L18.Experimental results showed that *Lactobacillus* strain L18 can resist bile salts in the

range of 0% to 0.4%. The tolerance to bile salts gradually increased from 0.1% to 0.3% after 12 to 48 hours of culture. At 0.5%, there was growth from 12 to 24 hours but a decline at 48 hours. In addition, bacterial *Lactobacillus* strain L18 has poor tolerance to bile salts in the concentration range of 0.6 - 0.8%. At higher concentrations, strain L18 growth was inhibited (Figure 5).

The results showed that the optimal concentration of bile salts for strain L18 was 0.3 - 0.5%. The results provide insight into strain L18 bile salt tolerance. The results indicate that the *Lactobacillus* strain L18 is a promising probiotic candidate for use in bile-containing environments. Further studies should be conducted to understand the mechanisms underlying its bile resistance.

This result is consistent with Hassan *et al.* (2020) [6], which investigated *Lactobacillus* salt tolerance in the range of 0 - 1%. The results showed that the bile salt tolerance of the bacterial strains was highest in the range of 0.05–0.3%. However, bacterial strains are less tolerant at high concentrations of 0.6 to 1%. This suggests that the bacteria can better survive in saline environments with lower concentrations of bile salts. This could have implications for the use of *Lactobacillus* in food preservation and other industrial applications.

3.5. Genetic identification of bacteria

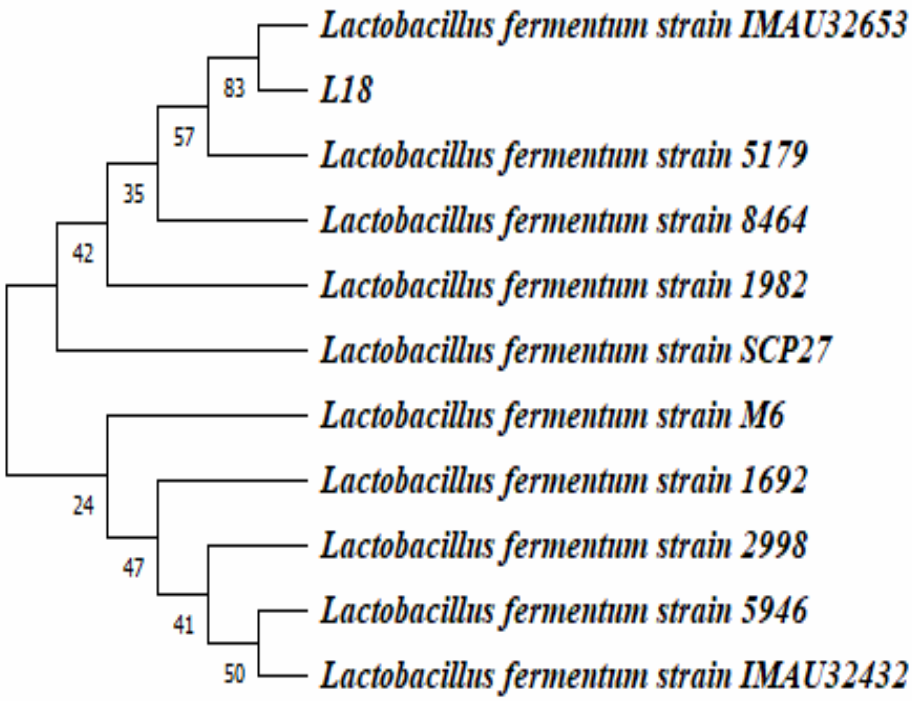

Figure 6. Phylogenetic tree based on 16S rRNA gene sequences representing *Lactobacillus* isolates. Numbers of nodes representing levels of bootstrap support (%) from a 1,000-record resample dataset

Based on the above characteristics and biochemical tests, L18 strain could be a species in *Lactobacillus* genus and therefore the information of 16S rRNA sequences could be contributed to the species identification of L18 strain. Here we used the primer pairs 27F and 1492R to amplify the 16S rRNA gene fragment and resulted in a single band of about 1500 bp. This is consistent with the theoretical size when multiplying the gene fragment with this primer pair. The results of sequence and phylogenetic tree comparison showed that strain L18 had 99.43% similarity to *Lactobacillus fermentum* strain IMAU32653 and was in the same branch as *Lactobacillus fermentum* strain IMAU32653 with the same value of Bootstrap 83% (Figure 6). In terms of reliability and similarity, strain L18 and *Lactobacillus fermentum* strain IMAU32653 are similar. The combination of biological and molecular characteristics shows that strain L18 is closely

related to *Lactobacillus fermentum* and is named *Lactobacillus fermentum* strain L18.

3.6. Extracellular enzyme production

Extracellular enzymes are biologically active substances that directly or indirectly inhibit and destroy pathogenic microorganisms in probiotics [26]. The results of this study showed that bacterial strain L18 can produce amylase and cellulase enzymes with a resolution ring diameter of 13.96± 0.35 mm and 10.07 ± 0.25 mm, respectively (Figure 7). These two enzymes degrade complex carbohydrates, which improves fish absorption. In addition, strain L18 enzyme can also inhibit pathogenic bacterial growth and help maintain intestinal micro-ecosystem balance. Therefore, L18 is a potential probiotic candidate for fish farming.

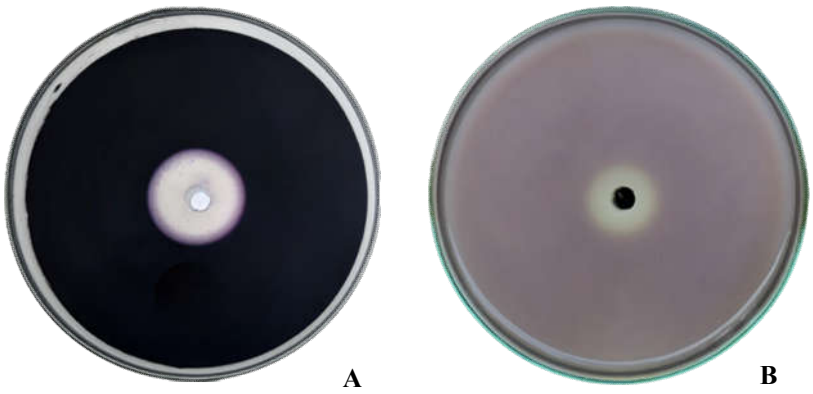

Figure 7. The amylase (A) and cellulase (B) enzyme activities of *Lactobacillus fermentum* strain L18

4. CONCLUSIONS

LAB strains isolated from tropical freshwater fish intestines have probiotic properties that make them potential candidates for probiotic applications. Among the isolates, *Lactobacillus fermentum* strain L18 exhibited significant probiotic activity along with significant antimicrobial activity against *Staphylococcus aureus* and *Aeromonas hydrophila*. Additionally, *L. fermentum* strain L18 was found to tolerate a wide range of pH and bile salt conditions. Further studies of *in vivo* assay and safety profiles are needed to determine their practical applications.

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CURRENT STATUS AND PROSPECTS OF SUSTAINABLE CLAM FARMING DEVELOPMENT IN VIETNAM

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ABSTRACT

This paper presents a comprehensive assessment of clam farming in Vietnam, focusing on *Meretrix lyrata* and *M. meretrix* species. The study assesses the current state of seed production technology, commercial farming, and its integration with processing and product commercialization. Clam farming plays a significant role in Vietnam's mollusc sector, with a vast farming area of 17,730 hectares and a total clam production of 236,463 tons. The country has successfully exported these clams to 42 countries, amounting to a total value of USD 107.121 million. The methods section describes the data collection process, including field surveys, interviews with stakeholders, and policy analysis. The results and discussion section presents key findings related to seed production, commercial farming, and cooperation among stakeholders. Challenges in the industry, such as insufficient seedling supply, unclear land use, limited access to technology and capital, market issues, and environmental concerns, are identified. The paper concludes by emphasizing the potential for sustainable clam farming and the need for research and cooperation to overcome challenges and foster the sector's future sustainable development.

Keywords: *Clam farming, Vietnam, sustainable development, challenges.*

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1. INTRODUCTION

Clam farming is a thriving marine aquaculture sector in Vietnam, thanks to its extensive coastline, numerous river mouths, vast tidal flats (660,000 hectares), and diverse species (2,200 identified). Notably, *Meretrix lyrata* (Ben Tre clam) and *M. meretrix* are key species. Vietnam has made significant strides in clam farming, advancing seed production, commercial farming, and integrating processing and product marketing. Coastal provinces like Hai Phong, Thai Binh, Nam Dinh, Ninh Binh, Thanh Hoa, Ben Tre, Tien Giang, Tra Vinh, Soc Trang, and Kien Giang have established productive farming areas. In 2021, Vietnam's clam farming accounted for 50% of mollusc aquaculture (17,730 hectares) and 50% of total aquaculture production (236,463 tons). Additionally, it contributed a remarkable 86% to mollusc aquaculture exports [1].

Clam farming's significance is prominently underscored in various strategic documents, notably Decision 339/QD-TTg [2], which is elaborated upon in Decision 1664/QD-TTg [3], focusing on advancing marine aquaculture through 2030 and projecting targets until 2045. These directives set forth clear objectives for mollusk clam cultivation. The primary goal is to attain a production volume of 460,000 tons by 2025, with an additional increase to 550,000 tons by 2030 in coastal areas.

Clam farming has made considerable progress in both seed and commercial farming technologies. For instance, Ben Tre and Tien Giang provinces primarily rely on natural clam seed sources with low densities, while the Northern provinces employ artificial clam seed sources with higher densities and productivity [1]. Mechanization has also been introduced in the production process, including breeding area transformation, harvesting, and sorting.

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The success of clam farming also hinges on the adoption of good practices in commercial clam farming, which enhance productivity and the quality of farmed clams. Community-based and collective production management models have been established in certain regions, such as cooperative models in Ben Tre and Nam Dinh provinces, alongside individual and corporate models. Many unregistered clam farming cooperatives have collaborated in production and business activities. Several enterprises have invested in modern processing facilities for clam exportation, such as the clam processing plant by HASUVIMEX in Thanh Hoa, FAQUIMEX in Ben Tre [4].

To ensure sustainable development in the clam farming sector, further research and assessment are needed on the success and coexistence of different production models in different regions. Sharing experiences, technology, and resources will enable the adoption of best practices and foster sustainable development within the industry.

Despite the potential for substantial growth, clam farming faces various challenges. Ensuring the quality of raw materials, processing, and product consumption, as well as environmental management, food safety, genetic deterioration, and land use management, are critical issues that need to be addressed to ensure the sustainable development of the sector.

Despite facing various obstacles, clam farming in Vietnam holds substantial potential for achieving sustainable development. To unlock this potential, it is essential to conduct a thorough assessment of the industry's current state, explore possibilities for technological advancements, optimize production organization, and consider relevant policies. By identifying and addressing challenges and proposing effective solutions, we can pave the way for the future growth of clam farming in the country and ensure its long-term sustainability.

2. METHODS

Research Duration and Locations: The investigation spanned a duration of seven months, commencing from January and culminating in July 2023.

Data Collection and Assessment: The study revolved around the compilation and meticulous analysis of an array of reports and documents pertinent to diverse dimensions of clam farming, processing, and consumption.

Field Surveys and Interview Methodology: The research was underpinned by a comprehensive approach that amalgamated both on-site surveys and insightful interviews. The geographical scope encompassed two distinctive regions: a concentration on Ben Tre in the southern region and an exploration of Thanh Hoa, Ninh Binh, Nam Dinh, and Thai Binh in the northern region. A total of 68 individuals were engaged in interviews, representing a diverse cross-section of stakeholders within the clam industry. The categorization of interviwees unveiled a composition of 79 people including 40 interviewees were farmers and traders representing cooperatives and associations, 9 interviewees affiliated with processing plants, 25 local officials, and 5 researchers.

- Ben Tre (22 interviewees): Department of Fisheries (2 interviewees); clam cooperatives including Rang Dong (5 interviewees), Dong Tam (4 interviewees), Tan Thuy (3 interviewees), An Thuy (4 interviewees), and Thanh Loi (6 interviewees).

- Thanh Hoa (28 interviewees): Department of Agriculture and Rural Development in Hau Loc District (4 interviewees); Association of Clam Production and Trade of Hau Loc (5 interviewees); Agriculture Research Institute of Thanh Hoa (5 interviewees); Thanh Hoa Seafood Import, Export Jsc - HASUVIMEX (9 interviewees) and Department of Fisheries (6 interviewees).

- Ninh Binh (5 interviewees): Kim Son Clam cooperative (5 interviewees); Department of Fisheries (2 interviewees).

- Nam Dinh (11 interviewees): Nghia Hung Clam cooperative (4 interviewees); Department of Fisheries (7 interviewees).

- Thai Binh (7 interviewees): Thuy Truong Clam cooperative (4 interviewees); Department of Fisheries (3 interviewees).

Expert interviews through semi-structured questionnaires were conducted, involving six experts from NGOs and associations: Center for Technology Transfer and Community Development in Agriculture and Fisheries - FACOD (2 experts), Ben Tre Fisheries Association (1 expert), International Cooperation for Sustainable Aquaculture and Fisheries Development - ICAFIS (1 expert), and Vietnam Association of Producers and Processors - VASEP (2 experts).

Focus Group Discussions were held with officers from the Ministry of Agriculture and Rural Development, totaling 17 participants. They represented the Department of Science, Technology and Environment (6 participants), Department of Fisheries (10 participants), and Vietnam Institute of Fisheries Economics and Planning - VIFEP (1 participant).

The profile of interviewees and participants in group discussion were meticulously crafted to encompass a varied spectrum of professionals extensively involved in the clam industry, spanning policy makers, local officers, clam farmers, traders, processors and members of clam cooperatives or associations. The primary aim was to gather viewpoints from various stakeholders deeply engaged in diverse aspects of clam cultivation, processing, and distribution.

3. RESULTS AND DISCUSSION

3.1. Seed production

3.1.1. Seed demand

Every year, the nationwide demand for clam seedlings is estimated at 70 billion. However, the current total production of clam seedlings, originating from artificial reproduction, is only around 15 - 20 billion per year, meeting approximately 30 - 40% of the seedling demand for commercial farming. The seedling production is concentrated in facilities located in provinces like Nam Dinh, Thai Binh, Ninh Binh, Tien Giang, and Ben Tre, while the remaining supply mainly relies on natural exploitation [5, 6].

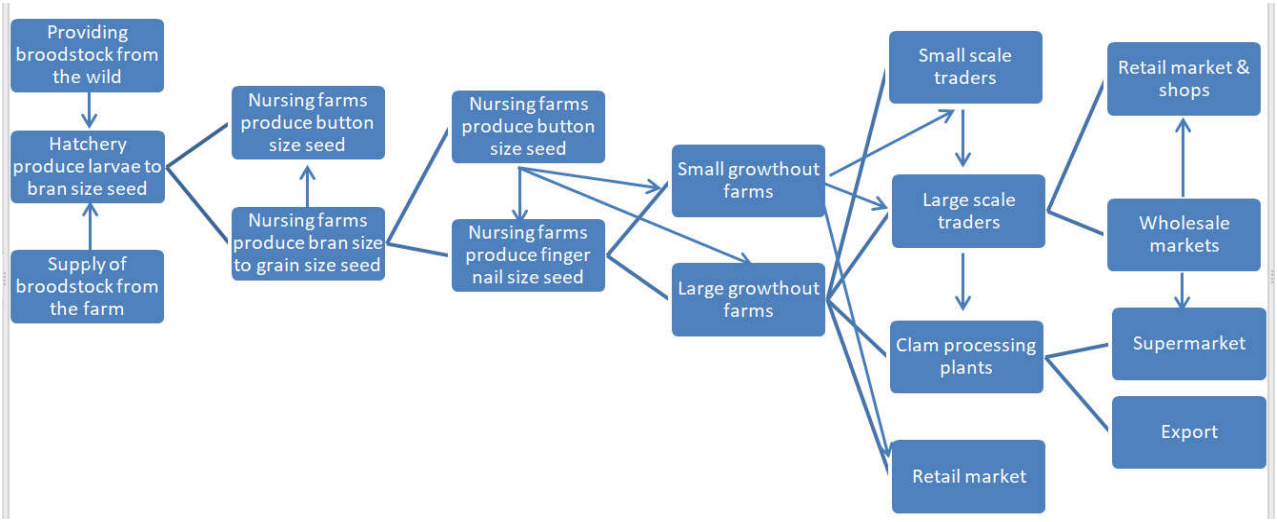

Figure 1. Value chain of clam in northern provinces of Vietnam.

Seed is nursed to different sizes for different growout practices

The increasing clam seedling demand has led to scarcity issues in most regions across the country, causing instability in clam farming. Currently, the primary source of clam seedlings is from natural extraction, which has limitations to meet the significant demand in provinces like Bạc Liêu, Tra Vinh, Ben Tre, and Tien Giang, where the demand reaches billions of clams. In the northern clam farming regions, spanning over 5,000 hectares from Quảng Ninh to Ha Tinh, the primary source of clam seedlings is local artificial reproduction facilities, but they cannot fully meet the local farmers' demand. Consequently, some traders from Thanh Hoa and Nam Dinh have to

travel to southern provinces like Tien Giang and Ben Tre to purchase clam seedlings at higher prices and sell them to farmers in their regions [5,6].

In response to the seedling scarcity, some regions in Nam Dinh, Thai Binh, and Ninh Binh are developing clam seedling nursing. They start with clam seeds and grow them to larger sizes in order to supply other regions. The formation of clam seedling farming could alleviate the seedling shortage and enhance the availability of quality clam seedlings for sustainable clam farming across the country [1, 6, 7].

3.1.2. Natural seed exploitation

In the southern region, the actual exploitation and natural distribution area of clams covers approximately 12,000 hectares along the coastal areas from Can Gio district (Ho Chi Minh city) to Ca Mau province, with the most concentrated areas being in Tien Giang, Ben Tre, and Tra Vinh provinces [8]. A total of 493 hectares were established by 7 clam harvesting cooperatives, including: Rang Dong and Dong Tam clam cooperatives in Binh Dai district; An Thuy, Tan Thuy, and Bao Thuan clam cooperatives in Ba Tri district; and Thanh Loi, Binh Minh clam cooperatives in Thanh Phu district [9]. The natural seed sources of clams appear irregularly every year, with unstable production and uneven sizes of seeds. Seed size smaller than 5000 pieces per kilogram is not allowed to be sold out of Ben Tre province without permisson of People Committee's of Ben Tre [10].

3.1.3. Seed production and research

Since 2003, the Research Institute for Aquaculture No.1 and No. 2 (RIA1 and RIA2) in Vietnam have been actively engaged in artificial reproduction technology for clams (*Meretrix* sp.). The successful implementation of this technology has resulted in notable technical achievements, including an impressive 80% participation rate of clams in the reproductive process, a remarkable 95% fertilization rate, and a promising 8 - 10% survival rate of first-stage seedlings, measuring 1mm in size [11]. The annual reproductive season of the Ben Tre clam is determined from mid-April to the end of August. The main reproductive season occurs from mid-May to the end of July [12]. These achievements demonstrate the significant progress made in advancing clam reproduction techniques, contributing to the sustainable development of the clam farming industry in various coastal regions. The production facilities for clam seedlings are also used to produce other mollusk species such as ark shells, limpets, Pacific oysters, clams, and blood cockles, with seasonal adjustments. There are over 400 production and business facilities for mollusk including clam seedlings nationwide, mainly concentrated in provinces such as Quang Ninh (8 facilities), Hai Phong (2 facilities), Thai Binh (14 facilities), Nam Dinh (120 facilities), Ninh Binh (276 facilities), Nghe An (2 facilities), and Ben Tre (9 facilities). The annual production capacity exceeds 13 billion seedlings, meeting over 60% of the demand. The remaining seedlings are collected from natural sources for commercial farming, mainly in provinces like Ben Tre, Tra Vinh, and Tien Giang [1].

However, the current scale of clam seedling production, as well as mollusk seedlings in general, in Vietnam mainly takes place in small-scale hatcheries, where the broodstocks used for seedling production are not subject to selection and screening processes. They are often chosen from commercially farmed clams for reproduction, resulting in inconsistent seedling quality, slow growth, low meat content, and susceptibility to diseases. Furthermore, the current seedling production technology is limited by its dependence on the amount of food (phytoplankton) cultured in bags or tanks with low and unstable densities, leading to frequent shortages. Moreover, the operational cost per batch of larval rearing is high due to the large volume of water required for the larval culture process. Another technological limitation is that most mollusk hatcheries, particularly clam hatcheries, do not pay enough attention to the larval rearing and adaptation of seedlings to the natural environment before being transferred to

commercial farming areas, resulting in low survival rates of seedlings after release. The high production cost while recent selling prices have decreased considerably has led to reduced profits for seedling producers, and the practice of releasing clams at high densities in commercial farms slows down clam growth.

In the 2025 - 2030 Seed program (initially the 2022 - 2025 period) of the Ministry of Agriculture and Rural Development, the priority is given to the seed development of mollusks, including bivalve, clams, oysters, and scallops, which are considered as the main mollusc target for export.

3.2. Commercial Farming

3.2.1. Area and production

Clam production in Vietnam has a rapid growth. In 1998, farmers in Nam Dinh province conducted an experiment of transferring clam seeds from Ben Tre to be farmed in intertidal areas, and achieved good results [5, 7]. In the past decade (2010 - 2021) clam farming area increased by 20%, clam production increased by 120%, export value from clam increased by 276%. The current status of clam production and consumption in Vietnam in the period 2010 - 2021 [1] is summarized in table 1.

Table 1. Status of mollusk and clam farming and exporting in Vietnam in the period 2010-2021

Targets	2010	2015	2019	2021
1. Cultivation area (ha)				
Total molluscs	25,560	40,685	41,500	35,570
Clam	14,760	18,720	19,200	17,729
Percentage (%)	58%	46%	46%	50%
2. Output (tons)				
Total molluscs	135,011	26,531	310,000	471,669
Clam	109,250	18,691	226,944	236,463
Percentatge	81%	70%	73%	50%
3. Export output (tons)				
Molluscs	23,705	32,500	41,100	87,700
Clam	17,755	23,150	30,300	78,000
Percentatge	75%	71%	74%	89%
4. Export value (x 1,000 USD)				
Molluscs	57,677	82,390	93,642	125,095
Clam	38,765	57,000	63,000	107,121
Percentatge	67%	69%	67%	86%

Source: DFISH (2022) [1]

In recent years, the mollusk farming industry in our country has experienced strong development, primarily focusing on two main species: Clams (*Meretrix* spp.) and *Crassostrea* sp. This industry is concentrated in coastal provinces, including Quang Ninh, Thai Binh, Nam Dinh, Ninh Binh, Thanh Hoa, Nghe An, Ha Tinh, Ben Tre, and Tra Vinh. In recent years, the production, exploitation, and processing of clams have gradually become an important livelihood for people in coastal areas. Commercial clam farming has played a significant role in economic development, contributing to poverty reduction and notably improving the lives of people in these coastal regions [1, 6, 7].

3.2.2. Farming technologies

There are significant differences in clam farming technology between the Northern and Southern regions, concerning seed sources, stocking densities, productivity, and harvesting practices.

In the Southern region, clam farming for commercial purposes mainly relies on natural seed sources, especially from natural clam populations in Ben Tre province. However, some large-scale farms have recently started using artificially reproduced seed sources from the Northern region. The stocking density in the Southern region is generally lower. On the contrary, in the Northern region, clams are mostly sourced from artificial reproduction, and only a few farms use natural clam seed of various sizes from provinces like Ben Tre and Tien Giang. The farming methods involve extensive farming, collecting natural seed, and low-density stocking, ranging from 5 to 94 clams/m^2, with an average of 25/m^2. However, in several provinces, some farms practice high-density stocking, with an average ranging from 493 to 600 piece/m^2, and in some cases, even up to 1,100 clams per square meter (with clam sizes around 2,000 clams/kg). Farming at high densities extends the farming period to about 24 to 28 months [1] to get sizes of 70 to 100 piece/kg. In the Northern provinces such as Thai Binh, Nam Dinh, Thanh Hoa, Ninh Binh, and Ha Tinh, the clam farming density is relatively high, ranging from 2,500 to 4,200 piece/m^2 (with clam sizes of 300-400 piece/kg), exceeding the recommended guidelines by 5 - 17 times [7].

The clam farming productivity varies between different ecological regions and localities. Thai Binh, for instance, reaches 25 tons/ha, and some exceptional areas achieve up to 50 tons/ha/crop. Nam Dinh records a productivity of 10-15 tons/ha/crop, with many models surpassing 20 tons/ha/crop, and certain production areas even exceed 40 tons/ha/crop. The Mekong Delta region attains an average productivity of 7.09 tons/ha/crop, with Tien Giang standing out at 11.33 tons/ha/crop [7]. Ben Tre has a slightly lower productivity of 7.5 tons/ha/crop, with 15-20% of the clams is left to maintain the spawning stock for subsequent years [6]. Tra Vinh and Kien Giang achieve productivities of 5 and 7 tons/ha, respectively. In regions with high production volumes (e.g., Thai Binh, Nam Dinh, Ninh Binh, Thanh Hoa), mechanical means are often employed for pond improvement and clam harvesting. In contrast, areas with conservation regulations, such as Ben Tre and Tien Giang, or regions with lower productivity, tend to opt for manual harvesting and extensive farming practices [6, 7]. It's important to acknowledge that the timeframe for selective harvest cycles in the South might be comparatively shorter than the simultaneous harvest approach employed in the North.

3.3. Cooperation among stakeholders in sustainable development

Currently, Ben Tre has seven clam cooperatives certified with MSC certificates, and information about 5 of them is presented with three other area of ASC certificate in Nam Dinh, Ninh Binh and Tra Vinh (Table 2). Currently, clam cooperatives mainly operate based on the 2012 Law on Cooperatives and are influenced by the unique historical formation and development as well as the specific conditions of each locality. The type and nature of user group participation in decision-making vary lightly based on the specific conditions of different cooperatives on the three coastal districts of Ben Tre, two districts of Tra

Vinh, Nghia Hung district in Nam Dinh and Kim Son district in Ninh Binh.

Ben Tre province in Mekong River Delta has successfully implemented a co-management system for clam farming since 1997 (Table 2), which brings together the government, the local community, and other relevant stakeholders. The provincial People's Committee plays a vital role by granting land use rights to cooperatives for clam cultivation, exploitation, and protection.

Table 2. Information about some cooperatives and linked clam (*M. lyrata*) farming areas that have achieved MSC or ASC certifications

Name	Year of Establishment	No. of households	Farming area (ha)	Certificate
Rang Dong (Binh Dai, Ben Tre)	1997	3,700	1,500	MSC
Dong Tam (Binh Dai, Ben Tre)	2002	2,600	242	MSC
An Thuy (Ba Tri, Ben Tre)	2004	4,500	352	MSC
Tan Thuy (Ba Tri, Ben Tre)	2004	2,964	325	MSC
Thanh Loi (Thanh Phu, Ben Tre)	2004	650	404	MSC
Lenger Farm linked Clam farming area (Nghia Hưng, Nam Dinh)	2020	n/a	500	ASC
Kim Son Clam Cooperative (Kim Son, Ninh Binh)	2022	110*	500	ASC
Clam farming area (Cau Ngang and Chau Thanh, Tra Vinh)	2023	n/a	433	ASC

*Note: * There are 95 households from Ninh Binh, 10 from Nam Dinh and 5 from Thanh Hoa; 10 are farmers cum traders.*

Source: Mai Van Tai and Pham Anh Tuan (2023)[6].

The coastal regions of Ben Tre were categorized into specific zones to serve diverse objectives encompassing conservation, cultivation, policy formulation, and regulatory measures associated with clam harvesting and farming. These zones also offer guidance for co-management entities and facilitate coordination across multiple endeavors, including the integration of scientific and technological advancements, partnerships with research institutions and NGOs, collaboration with coastal border guards and law enforcement, as well as conflict resolution and industry-related problem-solving [6, 9].

The local community, represented by cooperatives, plays an active role in decision-making regarding operational mechanisms, planning, and implementation of production and business activities. They follow laws and regulations specified in cooperative internal rules and have the autonomy to elect their representatives and participate in cooperative management. To support their initial operations and enhance the awareness and management capacity of cooperative members and staff, the cooperatives receive financial and technical assistance from local authorities.

NGOs like WWF, FACOD, ICAFISH, etc, along with research institutions, provide valuable expertise and support to the clam farming sector [1, 5, 6, 9]. The mandate of the National Department of Quality, Processing, and Market Development (NAFIQPM) encompasses overseeing food safety surveillance in designated regions [13], along with environmental and disease monitoring for aquaculture initiatives as

endorsed by Research Institute for Aquaculture No. 1, No. 2, No. 3 (RIA1, RIA2, RIA3).

Development of value chains

Support from both businesses and NGOs has significantly benefited clam farming areas, as exemplified by WWF and the Sustainable Fisheries Fund's assistance in the MSC certification process in Ben Tre, covering certification costs. Similarly, Lenger Vietnam and HASUVIMEX's support for ASC certification in Nam Dinh and Ninh Binh serves as valuable models for other regions to develop their standards. However, challenges persist for small-scale fisheries in achieving financial sustainability. In Ben Tre, clam farmers have gained access to domestic and international markets through Hung Truong Phat Company and Ben Tre Seafood Joint Stock Company, yet cooperation between cooperatives and processing companies remains limited, with delayed payments compared to traders. Export-oriented processors mainly purchase clams sized between 80 to 120 clams per kilogram, while larger clams (40-60 clams per kilogram) are primarily intended for the domestic market. Traders play a vital role in purchasing and marketing clams, meeting the requirements of processing companies for both export and domestic consumption. The MSC certification in Ben Tre and ASC in Nam Dinh, Ninh Binh and Tra Vinh have been pivotal in enabling clam farming to explore new markets in Europe, the United States, Japan, etc fostering a clam market with enhanced profitability. This certification proves to be a valuable support system, promoting sustainable practices and facilitating improved market access for clam farmers in the Vietnam.

There is a wide variety of processed products made from clams, including frozen IQF boiled clam meat, boiled whole clam in shell, fresh whole clams, and canned clam meat, among others. Overall, clam products are quite popular and well-consumed in both the domestic and export markets, with many items being available in major supermarkets within the country. The top 10 regions contributing to this industry are Thanh Hoa, Ben Tre, Nam Dinh, Binh Thuan, Thai Binh, Hai Phong, Soc Trang, Kien Giang, Quang Ninh, and Tien Giang, accounting for 97% of the total revenue. Among them, Thanh Hoa holds the largest share at 30%, followed by Ben Tre at 20%, and Nam Dinh at 10%. The three largest companies involved in this sector are Thanh Hoa Seafood Import-Export Joint Stock Company, Ben Tre Seafood Joint Stock Company, and Lenger Vietnam Seafood Company.

3.4. Main constraints and challenges in clam farming development

3.4.1. Insufficient clam seedling supply

The existing insufficiency in both quantity and quality of clam seedlings fails to effectively fulfill the substantial market demand. Present provisioning merely addresses an approximate range of 30 - 40% of the requisition, consequently engendering scarcities and a state of volatility within the industry. The dependence on naturally occurring sources for the acquisition of clam seedlings is environmentally unsustainable owing to their constrained availability and the attendant hazards of resource exhaustion.

3.4.2. Unclear land use and competition with other industries

The absence of comprehensive planning and the occurrence of impromptu expansion within coastal regions have contributed to the precarious and non-viable progression of bivalve aquaculture. The encroachment upon coastal zones and the ensuing expansion of aquaculture endeavors have engendered a contest for limited coastal spatial resources, thereby engendering detrimental repercussions upon local transportation networks and the tourism sector. Furthermore, the ramifications of climate change exert deleterious effects upon the productivity, quality, safety, and economic efficacy of clam farming, culminating in substantial deleterious financial outcomes.

3.4.3. Limited access to scientific and technological advancements, infrastructure, and capital

The predominant approach to clam aquaculture predominantly centers around small-scale familial operations, characterized by a dearth

of significant technological innovations and suboptimal infrastructural support. The industry's progress towards sustainability is hampered by the paucity of financial resources allocated for holistic strategizing and development. Additionally, the constrained access to refined methodologies in clam cultivation and the restricted avenues for acquiring knowledge and emulating prosperous paradigms further contribute to the challenges faced by the sector.

3.4.4. Market challenges

Fluctuations in both clam product consumption trends and pricing dynamics have engendered a state of instability that exerts notable impacts upon both the domestic and export - oriented markets. The presence of suboptimal hygiene and food safety protocols has yielded impediments in the realm of compliance with stipulated exportation prerequisites. Additionally, a deficiency in the commitment towards upholding desirable attributes such as product quality, dimensions, and the crucial meat-to-shell ratio has conspicuously contributed to a compromised reception within the market sphere. The clam aquaculture context within Vietnam is categorized as belonging to classification B, a classification that constrains the direct exportation of live clams, thereby mandating the precondition of processing anterior to their international consignment. This multifaceted scenario thus presents a formidable quandary in the concerted endeavor to amplify the export valuation of said clam products..

3.4.5. Climate change, environmental management, disease, and food safety

Coastal clam aquaculture is encountering heightened susceptibility to the ramifications of climate change, precipitating irregular productivity patterns and financial setbacks. Escalating turbidity of sediments in clam farming regions, coupled with scarcities in freshwater attributed to prolonged drought conditions and the diversion of water via reservoirs serving hydroelectric and irrigation purposes, has induced elevated salinity levels. This elevated salinity significantly compromises the growth trajectory and viability of clam populations. Concurrently, scholarly efforts and vigilant oversight pertaining to ecological governance and pathogenic countermeasures have lagged behind the imperatives of production demands. The absence of adequate disease mitigation protocols exacerbates episodic mass mortalities among clams, engendering economic adversity and environmental contamination. In order to surmount these multifaceted challenges and instate a trajectory of sustainable expansion within clam aquaculture, a comprehensive approach is imperative. This approach should encompass rectification of clam seedling supply inadequacies, refinement of spatial land management protocols, allocation of resources towards scientific and technological innovations, reinforcement of marketing strategies, and steadfast dedication to enhancing climate resilience and safeguarding ecological integrity.

4. CONCLUSION AND POLICY RECOMMENDATION

The clam farming industry in Vietnam has risen to prominence as a noteworthy marine aquaculture sector, holding substantial economic value and promising developmental prospects. With its expansive coastline, extensive tidal flats, and diverse range of clam species, Vietnam possesses the ideal conditions for successful clam farming, particularly centered around *Meretrix lyrata*, commonly known as the Ben Tre clam, and *M. meretrix*.

The evolution of clam farming in Vietnam has witnessed remarkable advancements in seed production techniques, commercial farming methodologies, and seamless integration with processing and product marketing strategies. Concentrated farming zones have been established in numerous coastal provinces, yielding significant outputs. This sector significantly contributes to the country's marine aquaculture domain and amplifies its export worth.

Prominent strategic directives, exemplified by Decision 339/QD-TTg [2] and Decision 1664/QD-TTg [3], underscore the pivotal role of clam farming while delineating development strategies

up to 2030 and envisioning targets until 2045. These directives underscore focused expansion within designated regions, investments in seed production technologies, and the augmentation of export-oriented processing to ensure the enduring growth of the clam farming sector.

However, despite the evident growth potential, clam farming confronts several challenges. Ensuring the quality of raw materials, processing, and end products, alongside effective environmental management, food safety measures, genetic preservation, and land utilization strategies, are pivotal concerns that warrant immediate attention to guarantee the sector's sustainable progression.

To actualize sustainable development within the clam farming domain, it is imperative to conduct comprehensive research and evaluations that assess the harmonious coexistence and success of varied production models across diverse regions. Collaborative efforts and the exchange of experiences, technologies, and resources among stakeholders will expedite the adoption of best practices and foster sustainable expansion within the industry.

A primary challenge lies in the inadequate availability of clam seedlings to meet the escalating demand. Dependency on natural sources for clam seeds is untenable and leads to shortages, particularly in specific provinces. Cultivating clam seedlings assumes paramount importance in alleviating this concern and securing a consistent supply of top-tier seedlings to support sustainable clam farming nationwide.

Prudent land use planning and management are indispensable to mitigate clashes with other sectors and ensure the constancy of clam farming. The ramifications of climate change and adept environmental stewardship hold pivotal significance in safeguarding the industry's resilience and productivity. The focal points should encompass disease prevention, meticulous environmental surveillance, and the enhancement of hygiene and food safety protocols to align with both domestic demands and international market prerequisites.

Investments in scientific breakthroughs and technological innovations, coupled with substantial infrastructural enhancements, stand as pivotal determinants in enhancing the productivity and efficiency of clam farming. Furthermore, the augmentation of market strategies is imperative to stabilize consumption patterns and pricing for clam products, catering to both local and global markets.

In summation, the clam farming industry in Vietnam harbors substantial potential for sustainable growth, contingent upon effectively addressing the prevailing challenges. By prioritizing the enhancement of clam seedling supply, the implementation of judicious land use strategies, investments in scientific and technological progress, bolstering market approaches, and fortifying climate resilience and environmental preservation endeavors, Vietnam can chart a course toward a thriving and enduring future for the clam farming sector.

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