Food Quality and Safety, 2022, **6**, 1–12
https://doi.org/10.1093/fqsafe/fyac036
Advance access publication 21 May 2022
Article

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Bacterial pathogens and factors associated with *Salmonella* contamination in hybrid red tilapia (*Oreochromis* spp.) cultivated in a cage culture system

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Abstract

Microbial food safety in cultured tilapia remains a challenge to public health worldwide, due in part to intensive aquaculture leading to poor water quality and high organic matter deposition. This study aimed to determine the prevalence of indicator and potential pathogenic bacteria in hybrid red tilapia (*Oreochromis* spp.) and their cultivation water and to identify environmental parameters and other bacterial contaminants associated with *Salmonella* contamination. A total of 120 fish were sampled, which were partitioned into fish carcasses (n=120), muscle (n=120), intestine (n=120), liver and kidney (n=120), and cultivation water (n=120) from three commercial farms in western Thailand from October 2019 to November 2020. The prevalence of fecal coliforms and *Escherichia coli* (*E. coli*) in these 600 samples was 74.8% and 56.7%, respectively. The prevalence of *Salmonella*, *Vibrio cholerae* (*V. cholerae*), *Aeromonas hydrophila*, and *Vibrio vulnificus* (*V. vulnificus*) was 23.0%, 17.5%, 2.5%, and 1.7%, respectively. None of the samples tested positive for *Streptococcus agalactiae*. Cultivation water exhibited a high prevalence for *Salmonella* (58.3%). Among fish samples, *Salmonella* had the highest prevalence at 14.1%, which was mainly from fish intestine. There was a significant association of *Salmonella* with the presence of fecal coliforms, *E. coli*, *V. cholerae*, and *V. vulnificus*. The predominant serovars of *Salmonella* included Saintpaul, Neukoelln, Escanaba, and Papuana. Grazing ducks that were raised in proximity to these cultured tilapia shared the same isolates of *Salmonella* based on the similarity of their rep-PCR DNA fingerprints, suggesting that ducks may function as either a biological reservoir for tilapia or at minimum participate in the environmental replication of this strain of *Salmonella*. Taken together, the results suggest that the environment used for tilapia aquaculture may be contaminated with pathogenic bacteria; therefore, food safety precautions are needed during processing, transportation, cooking, and consumption.

Keywords: *Aeromonas* spp.; grazing duck; *Salmonella* spp.; tilapia; *Vibrio* spp.

Introduction

To meet the high demands for global fish consumption, fish production has increased significantly from 76.5 million tons (MT) in 2016 to 82.1 MT in 2018 (FAO, 2020). *Oreochromis* spp., also known as tilapia, is one of the most important farmed freshwater fish. Tilapia production contributed to 8.3% of the total finfish products in 2018 (FAO, 2020), with Thailand and other Southeast Asian countries representing approximately 23.4% of total production. Due to the increase in global consumption of tilapia, microbial food safety has become an increasing concern for food safety and public health.

The major zoonotic bacteria found in cultured tilapia are *Salmonella*, *Aeromonas hydrophila* (*A. hydrophil*), *Vibrio cholerae* (*V. cholerae*), *Vibrio vulnificus* (*V. vulnificus*), *and Streptococcus agalactiae* (*S. agalactiae*) (Chen *et al.*, 2006; Suanyuk *et al.*, 2008). Although *Salmonella* infection in aquatic animals is generally nonpathogenic for the host animals, this bacterium can be transmitted as a foodborne pathogen and then function as a cause of severe gastroenteritis in humans. A previous study indicated that up to one-third of cultured tilapia can be contaminated with *Salmonella* (Awuor *et al.*, 2011). *S. agalactiae*, which belongs to group B *Streptococcus* spp. (GBS), has been a concern for human and animal health and contributes to severe losses in tilapia production worldwide (Jantrakajorn *et al.*, 2014; Barato *et al.*, 2015). For example, in Thailand, GBS serotypes Ia and III

Received 10 March 2022; Revised 16 April 2022; Editorial decision 10 May 2022

have been reported in both human patients and tilapia samples (Suanyuk *et al.*, 2008; Dangwetngam *et al.*, 2016). *A. hydrophila* is ubiquitous in freshwater environments and is a cause of epizootic ulcerative syndrome, leading to high mortality in fish. Moreover, *A. hydrophila* infection in humans induces gastroenteritis and extra-intestinal disease (i.e. meningitis and endocarditis; Zhang *et al.*, 2012). Last, *V. vulnificus* and *V. cholerae* are pathogenic bacteria commonly reported in tilapia from various countries, including Thailand, China, and Israel (Chen *et al.*, 2006; Senderovich *et al.*, 2010; Dong *et al.*, 2015). Copepods may function as a source of *V. cholerae* contamination in tilapia, but the source of *V. vulnificus* remains to be elucidated (Chen *et al.*, 2006). Previous studies have mainly focused on bacterial isolation from moribund fish or disease outbreaks; therefore, food safety data are needed on bacterial contamination in healthy fish intended for human consumption to protect public health.

In Thailand, aquaculture for hybrid red tilapia is primarily based on cage culture systems, which are dependent on natural surface water sources and therefore susceptible to contamination from urban discharges or municipal wastewater. The Kwae Noi River is one of the key locations for cage-based tilapia aquaculture due to its perennial flows and optimal environmental conditions for fish growth. High stocking density can trigger poor water circulation within the fish cages. The combination of fish fecal waste and appropriate environmental conditions can result in bacterial growth and accumulation. These cultivation factors can promote bacterial infection in tilapia, which then becomes an important vector for foodborne transmission to humans and a threat to public health. Therefore, the objectives of this study were to determine the prevalence of *Salmonella* spp., *A. hydrophila*, *S. agalactiae*, *V. cholerae*, *V. vulnificus*, fecal coliforms, and *Escherichia coli* (*E. coli*) in cultured hybrid red tilapia and to identify environmental parameters and other bacterial contaminants associated with *Salmonella* contamination in these cultured fish.

Materials and Methods

Sampling location and sample collection

Hybrid red tilapia is a hybrid of *Oreochromis mossambicus* and *Oreochromis niloticus*, which is raised in cages along the Kwae Noi River located in Muang district in Kanchanaburi Province, Thailand. It takes approximately 6–7 months to achieve a marketable body weight of at least 600 g for hybrid red tilapia. Tilapia are fed formulated pellet feeds three times per day, with temporary aeration provided as needed. Antimicrobial drugs, including enrofloxacin and oxytetracycline, are given in the feed when the fish show clinical signs such as swirling swimming, skin hemorrhage, or exophthalmia, or when there is a noticeable increase in morbidity and mortality.

A total of 120 tilapia were sampled, from which fish carcass rinses (n=120), fish muscle (n=120), intestine (n=120), liver and kidney (n=120) were obtained, and cultivation water (n=120) were collected from October 2019 to November 2020. Fresh fecal deposits from nearby grazing ducks (n=15), which were reared near the tilapia aquaculture site, were collected using a sterile plastic spoon and stored in a sterile plastic bag.

At each sampling event, the hybrid red tilapia (n=15) and cultivation water (n=15) were collected from three commercial tilapia farms, replicated eight times, with 1- to 2-month intervals between sampling events. All fish that were sampled appeared clinically healthy (no evident skin hemorrhage, ulcers, or abnormal swimming behavior). The fish were caught by hand net and individually collected in a double sterile plastic bag. Cultivation water (200–300 mL) from identical cages of harvested fish was collected at a depth of 45–60 cm below the surface. The water samples were kept in a sterile propylene bottle, with samples transported in refrigerated boxes kept at 4 °C and processed within 24 h after collection at the Department of Veterinary Public Health, Chulalongkorn University (Bangkok, Thailand).

Sample preparation

All hybrid red tilapia samples were weighed, and their width and length were recorded. The average weight±standard deviation (SD) of fish samples was (751.2±174.7) g, ranging from 503.0 to 1413.0 g per fish. The average±SD of width and length of fish were (14.0±1.5) cm and (29.1±3.0) cm, respectively.

An approximate 5 cm×5 cm area of the surface of each sampled fish was swabbed with sterile cotton for isolation of *A. hydrophila*. Next, for the fish carcass rinse, the entire external surface was rinsed with 50 mL of buffered peptone water (BPW; Difco, Becton Dickinson, Sparks, MD, USA) for the detection of fecal coliforms, *E. coli*, *Salmonella* spp., *V. cholerae*, *V. Vulnificus*, and *S. agalactiae*. The fish's external surface was then sprayed with 70% ethyl alcohol for decontamination, and the fish were aseptically dissected to collect 25 g of muscle, 1 g of kidney and liver, and 1 g of fish intestine. For cultivation water, a sterile cotton swab was immersed in the water for identification of *A. hydrophila*, and 25 mL of water was collected for detection or enumeration of fecal coliforms, *E. coli*, *Salmonella* spp., *V. cholerae, V. vulnificus*, and *S. agalactiae*. In addition, fecal materials from ducks were used for rep-PCR (repetitive extragenic palindromic polymerase chain reaction) characterization of *Salmonella* spp. The confirmation of *A. hydrophila*, *Salmonella* and *Vibrio* was performed by PCR. Genomic DNA from suspected colonies was extracted using the whole-cell boiling method (Levesque *et al.*, 1995). The PCR products were analyzed using electrophoresis in a 1.5% (mass concentration) agarose gel, stained with Redsafe™ Nucleic Acid Staining solution (Intron Biotechnology, Seongnam, Republic of Korea) and visualized by Omega Fluor™ gel documentation system (Aplegen, Pleasanton, CA, USA).

Enumeration of fecal coliforms and *E. coli*

The method of fecal coliforms and *E. coli* enumeration followed the procedure described in the United States Food and Drug Administration's Bacteriological Analytical Manual (U.S. FDA BAM; Feng *et al.*, 2002). To yield a 1:10 dilution, 25 g of muscle and 25 mL of sterile water were mixed with 225 mL of BPW (Difco). Fish carcass rinse, intestine, kidney and liver were mixed with 9 mL of BPW. One milliliter of the suspension was transferred to three replicate test tubes containing 9 mL of lactose broth (LB; Difco) with a Durham tube. Dilutions (10^{-1} to 10^{-3}) were used for cultivation water, muscle, and kidney and liver, while dilutions (10^{-1} to 10^{-5}) were used for intestine and fish carcass rinses.

All LB tubes were incubated at 37 °C overnight. Positive LB tubes with gas production were determined. A loopful of positive LB tubes was transferred to 9 mL of *E. coli* (EC) broth (Difco) and incubated in a water bath at 44.5 °C for 24–48 h with gas production in the EC tube indicative of positive fecal coliforms (most probable number (MPN)/g or MPN/mL).

For *E. coli* enumeration, a loopful of positive LB tubes was streaked on Levine–Eosin–Methylene Blue (L-EMB; Difco) agar and incubated at 37 °C overnight. The suspected colonies of *E. coli* on L-EMB agar are dark-centered, flat, and with or without green metallic sheen. The concentrations of *E. coli* were calculated as MPN/g (fish muscle, intestine, and kidney and liver) or MPN/mL (cultivation water and fish carcass rinse). Suspected colonies of *E. coli* were confirmed using biochemical tests, such as indole production and catalase tests.

Salmonella isolation and serotyping

Salmonella isolation followed the ISO 6579-1:2017 standard (ISO, 2017). Twenty-five grams of blended fish muscle and 25 mL of water were separately mixed with 225 mL of BPW. The intestine (1 g), kidney and liver (1 g), duck feces (1 g) and fish carcass rinse (1 mL) were individually mixed in a tube containing 9 mL of BPW. All mixture suspensions were incubated at 37 °C for 24 h. A 100-µL aliquot of solution was dropped on modified semi-solid Rappaport–Vassiliadis (Difco) medium and incubated at 42 °C. After overnight incubation, a loopful of incubated medium was streaked on xylose lysine deoxycholate (XLD; Difco) agar and incubated at 37 °C for 24 h. Typical colonies of *Salmonella* were red with or without black centers on XLD agar. For *Salmonella* confirmation, all isolates were screened for the *invA* gene using a pair of primers (*invA*-F/*invA*-R; 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′ and 5′-TCATCGCACCGTCAAAGGAACC-3′) with a product size of 284 bp (Kumar *et al.*, 2015).

Three to five suspected colonies of *Salmonella* were biochemically confirmed following the U.S. FDA BAM using triple sugar iron (TSI; Difco) slant agar (Andrews *et al.*, 2007). *Salmonella* colonies were purple to red in slant and yellow butt with H_2S production. Three *Salmonella* isolates per positive sample were selected, and serotyping was performed using slide agglutination test according to the Kauffmann–White scheme (Grimont and Weill, 2007) with available commercial antiserum (S&A Reagents Lab, Bangkok, Thailand).

A. hydrophila isolation

The detection of *A. hydrophila* was performed using standardized guidelines from the Public Health England (PHE) with slight modifications (PHE, 2015; Aboyadak *et al.*, 2017). Briefly, a sterile cotton swab of cultivation water, fish carcass rinse, muscle, intestine, and kidney and liver samples were streaked on Rimler–Shotts (RS) Medium Base (HiMedia Laboratories Ltd., Mumbai, India) supplemented with novobiocin 5 mg/L, and the plates were incubated at 35 °C overnight. Suspected colonies of *A. hydrophila* were round and yellow in color in the RS medium plate. The suspected colonies were further biochemically confirmed using TSI slant agar. Suspected colonies of *A. hydrophila* produced purple in slant and yellow in butt without H_2S production.

The confirmation of *A. hydrophila* was performed by PCR. Genomic DNA from suspected colonies was extracted by whole cell boiling method (Levesque *et al.*, 1995). Two 16S rRNA genes were amplified with genus-specific primers (Aer-F/Aer-R; 5′-CTACTTTTGCCGGCGAGCGG-3′ and 5′-TGATTCCCGAAGGCACTCCC-3′) and species-specific primers (AH-F/AH-R; 5′-GAAAGGTTGATGCCTAAT ACGTA-3′ and 5′-CGTGCTGGCAACAAAGGACAG-3′) with 35 cycles of the PCR conditions as follows: denaturation at 94 °C for 5 min, annealing at 50 °C for 40 s, and extension at 72 °C for 50 s (Ahmed *et al.*, 2018). The PCR products were analyzed using electrophoresis in a 1.5% (mass concentration) agarose gel, stained with Redsafe™ Nucleic Acid Staining solution (Intron Biotechnology) and visualized by Omega Fluor™ gel documentation system. (Aplegen).

V. cholerae isolation

Identification of *V. cholerae* was performed according to the U.S. FDA BAM (Kaysner and DePaola, 2004). In brief, 1 mL of the BPW mixture suspension from the sample preparation was added into 9 mL of alkaline peptone water (APW; Difco). The sample solution was incubated at 37 °C. After overnight incubation, a loopful of suspension was streaked on thiosulfate–citrate–bile salts–sucrose (TCBS; Difco) agar plate and incubated at 37 °C overnight. The suspected colonies of *V. cholerae* in TCBS agar are generally large, 2–4 mm in diameter, round, and yellow.

The suspected colonies of *V. cholerae* were confirmed on CHROMagar™ *Vibrio* (HiMedia Laboratories) and incubated at 37 °C for 24 h. The positive colonies of *V. cholerae* were observed to be green–blue to turquoise blue. The suspected colonies were also biochemically confirmed using TSI slant agar containing 2% NaCl. Positive colonies of *V. cholerae* were yellow in slant and butt without H_2S production. Molecular confirmation of *V. cholerae* was performed by *OmpW* gene (*OmpW*-F/*OmpW*-R; 5′-CACCAAGAAGGTGACTTTATTGTG-3′ and 5′-GAACTTATAACCACCCGCG-3′) with a product size of 588 bp (Sathiyamurthy *et al.*, 2013).

V. vulnificus isolation

V. vulnificus isolation was performed using the U.S. FDA BAM method (Kaysner and DePaola, 2004). The samples were enriched with APW and incubated at 37 °C. After overnight incubation, a loopful of suspension was streaked on TCBS agar, and the plate was incubated at 37 °C for 24 h. The positive colonies of *V. vulnificus* were green colonies. The suspected colonies were confirmed on CHROMagar™ *Vibrio* (HiMedia Laboratories). The positive *V. vulnificus* colonies are blue–green colonies. The presumptive colonies were biochemically confirmed by TSI (Difco) slant agar containing 2% NaCl. The positive *V. vulnificus* showed red slant and yellowish butt without H_2S production.

S. agalactiae isolation

Streptococcus isolation was performed according to the *Streptococcus* Laboratory, Centers for Disease Control and Prevention (USA; CDC, 2018), and the protocol from Laith *et al.* (2017), with a slight modification. In brief, the swab samples from internal organs were directly streaked onto brain heart infusion (Difco) agar supplemented with 6.5% NaCl and incubated at 30 °C overnight. The pinpoint colonies were

picked and confirmed on CHROMagar™ StrepB (HiMedia Laboratories) agar plates. The plates were incubated at 37 °C for 24 h. Positive colonies of *S. agalactiae* were mauve. The presumptive colonies of *S. agalactiae* were further biochemically confirmed by Gram-stain and catalase test.

Measurement of environmental parameters

Environmental parameters for water and weather were collected at 8 sampling time points. For water parameters, water temperature (°C), dissolved oxygen (DO; mg/L), pH, and salinity (‰) were recorded during sample collection. Portable water quality meters (SDL-100 and SDL-150, Extech Instruments, Nashua, NH, USA) were used for the measurement of water temperature, DO, and pH, while a refractometer (Master-S/MillM, Tokyo, Japan) was used to measure water salinity.

Weather data were collected both onsite and from online meteorological data. Ambient air temperature (°C), relative humidity (RH; %), average wind speed (m/s), maximum wind gust (m/s), dew point (°C), and heat index (°C) were recorded using a weather meter (Kestrel 3000, Nielsen-Kellerman, Boothwyn, PA, USA) at the sampling sites. The 7-d average for weather parameters, including rainfall (mm), wind speed (m/s), maximum wind gust (m/s), RH (%), and ambient air temperature (°C) data, were from the Thai meteorological department at the Kanchanaburi station (https://www.tmd.go.th/index.php).

The average±SD of water temperature ((30.8±2.2) °C), DO ((6.8±0.8) mg/L), pH (7.8±0.4), and salinity ((1.00±9.40)×10^{-7}) are presented in Table 1. Based on the online weather data, an average 7-d (±SD) rainfall of (1.1±1.0) mm, wind speed of (2.1±0.6) m/s, maximum wind gust of (9.4±1.5) m/s, RH of 65.6%±8.7%, and temperature of (29.0±2.6) °C are presented in Table 1.

Repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) fingerprinting

Thirty-one isolates of *Salmonella* serovars Saintpaul (*n*=18), Newport (*n*=4), Papuana (*n*=2), and Escanaba (*n*=7) were selected based on the availability of *Salmonella* serovars in tilapia and duck samples to perform rep-PCR DNA fingerprinting. The rep-PCR fingerprint of each bacterium was generated using two primer sets, i.e. Enterobacterial Repetitive Intergenic Consensus (ERIC1: 5′-ATGTAAGCTCCTGGGGATTCAC-3′, ERIC2: 5′-AAGT AAGTGACTGGGGTGAGCG-3′) and $(GTG)_5$: 5′-GTGGT GGTGGTGGTG-3′ (Prasertsee *et al.*, 2019; Santiyanont *et al.*, 2019). In brief, the 25-µL PCR mixture consisted of 100 ng bacterial DNA, 1× *Ex Taq* buffer, 0.2 mmol/L dNTPs, 0.8 µmol/L primer, and 0.625 U of *Ex Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan). The PCR conditions were as follows: one cycle of denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for ERIC and 40 °C for $(GTG)_5$ for 1 min, extension at 65 °C for 10 min and final extension at 65 °C for 20 min. Five microliters of PCR product were separated using 1% agarose gel electrophoresis. GelComparII version 5.10 (Applied Maths BVBA, Kortrijk, Belgium) was used for clustering of ERIC and $(GTG)_5$ fingerprint patterns. The fingerprint dendrogram was calculated using unweighted pair group method with arithmetic means based on composite data between ERIC and $(GTG)_5$.

Statistical analyses

One-way analysis of variance with multiple *post hoc* tests was used to test the association between the concentrations of fecal coliforms and *E. coli* and the type of sample. Similarly, Pearson's chi-square tests of independence were performed to test the association between the presence of *Salmonella*, *A. hydrophila*, *V. cholerae*, and *V. vulnificus* and the type of sample. The distribution of predominant *Salmonella* serovars for each sampling event was illustrated using a heatmap (Displayr, http://www.displayr.com). The association between the presence of *Salmonella* in the sample and various risk factors (e.g. presence of bacterial species evaluated in this study other than *Salmonella*, weather and other environmental parameters, water quality parameters) was performed using logistic regression, with a $P \leq 0.05$ based on the likelihood ratio test considered statistically significant and odds ratios calculated for the association between testing positive for *Salmonella* between the reference and comparison categories. All analyses were performed using Stata version 14.0 (StataCorp, College Station, TX, USA).

Results

Prevalence of fecal coliforms and *E. coli*

The overall prevalence of fecal coliforms and *E. coli* was 70.8% (340/480) and 50.0% (249/480), respectively, for the combined fish samples (carcass rinses, meat, intestines, liver and kidney) and 90.8% (109/120) and 75.8% (91/120) for the cultivation water (Table 2). Fish samples with the highest prevalence of fecal coliforms and *E. coli* were from fish carcass rinses (90.8%, 109/120) and intestines (84.2%, 101/120), respectively. The mean concentrations of fecal coliforms and *E. coli* were highest in fish intestines (2.4×10^4 MPN/g and 1.2×10^4 MPN/g, respectively). In contrast, fish muscle contained the lowest prevalence of fecal coliforms (45.0%, 54/120) and *E. coli* (14.2%, 17/120). For cultivation water, the prevalence for fecal coliforms and *E. coli* was 90.8% (109/120) and 75.8% (91/120), respectively, and the mean concentrations of these indicator bacteria were 1.4×10^2 MPN/mL and 1.9×10^1 MPN/mL, respectively. The mean concentration of fecal coliforms in fish intestine was significantly higher than that of cultivation water, carcass rinses, and muscle ($P<0.0001$); similarly, the mean concentration of *E. coli* in fish intestine was higher than other samples ($P<0.0001$).

Prevalence of pathogenic bacteria

Salmonella exhibited the highest overall sample prevalence of 23.0% compared to *V. cholerae* (17.5%), *A. hydrophila* (2.5%), and *V. vulnificus* (1.7%); no samples tested positive for *S. agalactiae* (Table 3). The prevalence for *A. hydrophila* in fish carcass rinses was 8.3% (10/120) and 4.3% (5/120) in cultivation water. *V. cholerae* was common in cultivation water (38.3%, 46/120) and fish intestine (20.8%, 25/120). Pearson's chi-square test indicated a significant association between sample type and the presence of pathogens, including *Salmonella*, *A. hydrophila*, *V. cholerae*, and *V. vulnificus*. Our results demonstrated that *Salmonella* was most abundant in cultivation water (58.3%, 70/120), followed by fish intestine

Table 1. Average and standard deviation (SD) for aquaculture cultivation water and weather parameters, stratified by sampling month from October 2019 to November 2020

Parameter	Monthly average (±SD)								Total average (±SD)
	10/19	12/19	1/20	3/20	5/20	7/20	9/20	11/20	
Water parameter									
Temperature (°C)	30.7 (±1.5)	27.5 (±0.4)	28.2 (±0.5)	31.7 (±0.5)	33.9 (±1.3)	33.0 (±0.6)	31.8 (±0.2)	29.7 (±0.2)	30.8 (±2.2)
Dissolved oxygen (mg/L)	6.8 (±0.7)	7.2 (±0.6)	7.7 (±0.1)	7.1 (±0.1)	5.3 (±0.3)	6.9 (±0.2)	6.2 (±0.06)	7.1 (±0.2)	6.8 (±0.8)
pH	7.5 (±0.1)	7.6 (±0.07)	7.6 (±0.1)	7.9 (±0.3)	7.5 (±0.4)	7.8 (±0.2)	8.6 (±0.2)	7.5 (±0.4)	7.8 (±0.4)
Salinity (‰)	1.0 ($\pm 6.0\times10^{-4}$)	1.0 ($\pm 2.5\times10^{-4}$)	1.0 (±0)	1.0 (±0)	1.0 ($\pm 6.2\times10^{-4}$)	1.0 (±0)	1.0 (±0)	1.0 (±0)	1.0 ($\pm 9.4\times10^{-4}$)
Weather parameter									
Ambient air temperature (°C)	34.1 (±1.1)	31.1 (±0.3)	34.0 (±0.6)	36.1 (±1.8)	36.2 (±1.7)	32.4 (±1.1)	32.4 (±1.8)	30.9 (±1.1)	33.4 (±2.3)
Relative humidity (%)	60.4 (±2.3)	58.9 (±2.3)	63.7 (±7.8)	57.6 (±4.70)	56.9 (±5.1)	64.9 (±4.9)	73.4 (±5.9)	82.6 (±7.4)	64.8 (±10.0)
Average wind speed (m/s)	1.2 (±0.3)	2.3 (±1.1)	0.7 (±0.5)	0.2 (±0.4)	0.3 (±0.5)	2.4 (±1.7)	1.0 (±0.1)	1.2 (±0.2)	1.2 (±1.2)
Maximum wind gust (m/s)	1.9 (±0.4)	2.9 (±1.1)	2.7 (±0.7)	2.8 (±0.7)	3.0 (±0.8)	10.4 (±3.0)	5.8 (±2.5)	4.9 (±2.7)	4.9 (±3.5)
Dew point (°C)	25.9 (±1.6)	21.8 (±0.4)	27.4 (±2.4)	27.5 (±2.2)	29.3 (±4.1)	25.0 (±1.6)	27.8 (±2.8)	27.2 (±1.5)	26.5 (±3.2)
Heat index (°C)	45.8 (±4.6)	35.6 (±1.8)	49.6 (±7.7)	48.7 (±4.4)	48.6 (±4.3)	40.3 (±3.9)	45.4 (±7.4)	42.5 (±5.1)	44.5 (±6.9)
Average 7-d rainfall (mm)	2.2 (±3.0)	0	0	0	0.9 (±1.6)	2.6 (±4.3)	1.0 (±2.2)	2.2 (±5.2)	1.1 (±1.0)
Average 7-d wind speed (m/s)	2.6 (±0.5)	2.0 (±0.5)	1.7 (±0.7)	2.7 (±0.5)	2.1 (±0.6)	2.3 (±0.9)	2.3 (±1.0)	0.7 (±0.5)	2.1 (±0.6)
Average 7-d maximum wind gust (m/s)	9.0 (±1.2)	10 (±1.0)	8.1 (±1.0)	10.6 (±1.18)	10.7 (±2.4)	10.0 (±1.4)	9.9 (±2.0)	6.3 (±1.0)	9.4 (±1.5)
Average 7-d relative humidity (%)	70.4 (±2.5)	59.3 (±4.3)	53.9 (±7.5)	55.3 (±10.2)	64.9 (±6.6)	69.6 (±4.8)	70.1 (±7.2)	81.7 (±6.8)	65.6 (±8.7)
Average 7-d ambient air temperature (°C)	29.9 (±0.8)	26.6 (±2.6)	27.4 (±0.7)	31.6 (±0.9)	31.9 (±1.4)	30.3 (±0.8)	30.1 (±0.8)	27.4 (±0.8)	29.0 (±2.6)

Table 2. Prevalence and concentration of fecal coliforms and *Escherichia coli* in red tilapia and aquaculture cultivation water (*n*=600)

Type of sample (*n*)	No. of positive (%)		Concentration (±SD) of positive sample (MPN/g or MPN/mL)	
	Fecal coliforms	*Escherichia coli*	Fecal coliforms	*Escherichia coli*
Carcass rinse (*n*=120)	109 (90.8%)	89 (74.2%)	4.1×10^{3} ($\pm1.8\times10^{4}$)	2.0×10^{3} ($\pm1.1\times10^{4}$)
Muscle (*n*=120)	54 (45.0%)	17 (14.2%)	2.6×10^{1} ($\pm2.2\times10^{2}$)	0.2×10^{1} ($\pm0.7\times10^{1}$)
Intestine (*n*=120)	108 (90.0%)	101 (84.2%)	2.4×10^{4} ($\pm4.0\times10^{4}$)	1.2×10^{4} ($\pm2.9\times10^{4}$)
Liver and kidney (*n*=120)	69 (57.5%)	42 (35.0%)	8.9×10^{1} ($\pm27\times10^{2}$)	1.0×10^{1} ($\pm4.9\times10^{1}$)
Total (*n*=480)	340 (70.8%)	249 (50.0%)	9.0×10^{3} ($\pm2.7\times10^{4}$)	1.1×10^{4} ($\pm2.3\times10^{4}$)
Cultivation water (*n*=120)	109 (90.8%)	91 (75.8%)	1.4×10^{2} ($\pm2.8\times10^{2}$)	1.9×10^{1} ($\pm4.8\times10^{1}$)
Grand total (*n*=600)	**449 (74.8%)**	**340 (56.7%)**	**5.6×10^{3} ($\pm1.6\times10^{4}$)**	**2.8×10^{3} ($\pm1.1\times10^{4}$)**

Table 3. Distribution of *Salmonella* spp., *Aeromonas hydrophila, Vibrio cholerae,* and *Vibrio vulnificus* in red tilapia (*n*=480) and aquaculture cultivation water (*n*=120)

Type of sample (*n*)	No. of positive (%)			
	Salmonella	*Aeromonas hydrophila*	*Vibrio vulnificus*	*Vibrio cholerae*
Carcass rinse (*n*=120)	21 (17.5%)	10 (8.3%)	2 (1.7%)	24 (20.0%)
Muscle (*n*=120)	0(0%)	0(0%)	0(0%)	2 (1.7%)
Intestine (*n*=120)	46 (38.3%)	0(0%)	6 (5.0%)	25 (20.8%)
Liver and kidney (*n*=120)	1 (0.8%)	0(0%)	1 (0.8%)	8 (6.7%)
Total (*n*=480)	68 (14.1%)	10 (2.0%)	9 (1.9%)	59 (12.3%)
Cultivation water (*n*=120)	138 (23.0%)	5 (4.2%)	1 (0.8%)	46 (38.3%)
Grand total (*n*=600)	**192 (32.0%)**	**15 (2.5%)**	**10 (1.7%)**	**105 (17.5%)**

(38.3%, 46/120) and carcass rinses (17.5%, 21/120). No muscle samples tested positive for the presence of *Salmonella* and *V. vulnificus*, and only approximately 2%–3% tested positive for *A. hydrophila* and *V. cholera*.

As shown in Table 4, the distribution of *Salmonella* serotypes was Saintpaul (18.9%, 74/394), Neukoelln (15.2%, 60/394), Escanaba (15.2%, 60/394), Papuana (15.0%, 59/394), and Virchow (8.6%, 34/394). In the hybrid red tilapia, the most common serotypes were Saintpaul (25.4%, 47/185), followed by Escanaba (23.8%, 44/185), Neukoelln (14.1%, 26/185), and Papuana (13.5%, 25/185). On the other hand, the predominant serotypes for cultivation water were Neukoelln (16.3%, 34/209), Papuana (16.3%, 34/209), and Saintpaul (12.9%, 27/209); for grazing duck feces, the *Salmonella* serotypes were Saintpaul (36.7%, 11/30), Escanaba (10.0%, 3/30), Fillmore (10.0%, 3/30), and Newport (10.0%, 3/30).

Logistic regression analyses for determination of risk factors associated with *Salmonella*

Logistic regression analyses indicated that the odds of detecting *Salmonella* in the fish and water samples were associated with the co-occurrence of fecal coliforms (OR 3.5, 95% confidence interval (CI): 1.1–11.2), *E. coli* (OR 2.9, 95% CI: 2.3–3.9), *V. cholera* (OR 2.3, 95% CI: 1.2–4.4), and *V. vulnificus* (OR 2.5, 95% CI: 1.7–3.8; Table 5). In addition, the odds of detecting *Salmonella* were positively associated with the mean 7-d maximum wind gusts (m/s) and negatively associated with the mean 7-d RH ($P<0.05$). Specifically, the odds of detecting *Salmonella* increased 1.08 times (OR 1.08) for each additional meter per second increase in the maximum wind gust; in contrast, the odds of detecting *Salmonella* decreased 0.97 (OR 0.97) times for each percentage increase in RH.

Fingerprinting of *Salmonella* serovars among tilapia, cultivation water, and duck fecal materials

The dendrogram of rep-PCR showed five major clades of *Salmonella*, designated as A, B, C, D, and E (Figure 1), all of which contained both fish and duck feces isolates. The cut-off value of the dendrogram was established at 80%, with the five clades highly segregated by serovar. Clade A, with the largest number of sequence-similar isolates, comprised only serovar *S.* Saintpaul, which was isolated from grazing duck feces, fish intestines, cultivation water, and fish carcass rinses. Clade B contained an identical genetic profile for four *S.* Newport isolates from duck feces and fish intestines. Clade C contained only two highly related isolates of *S.* Papuana, both from duck feces. Clades D and E were comprised of only *S.* Escanaba serovar with isolates from grazing duck feces, fish intestines, and cultivation water.

Table 4. *Salmonella* serovars isolated from red tilapia (*n*=480) and aquaculture cultivation water (*n*=120)

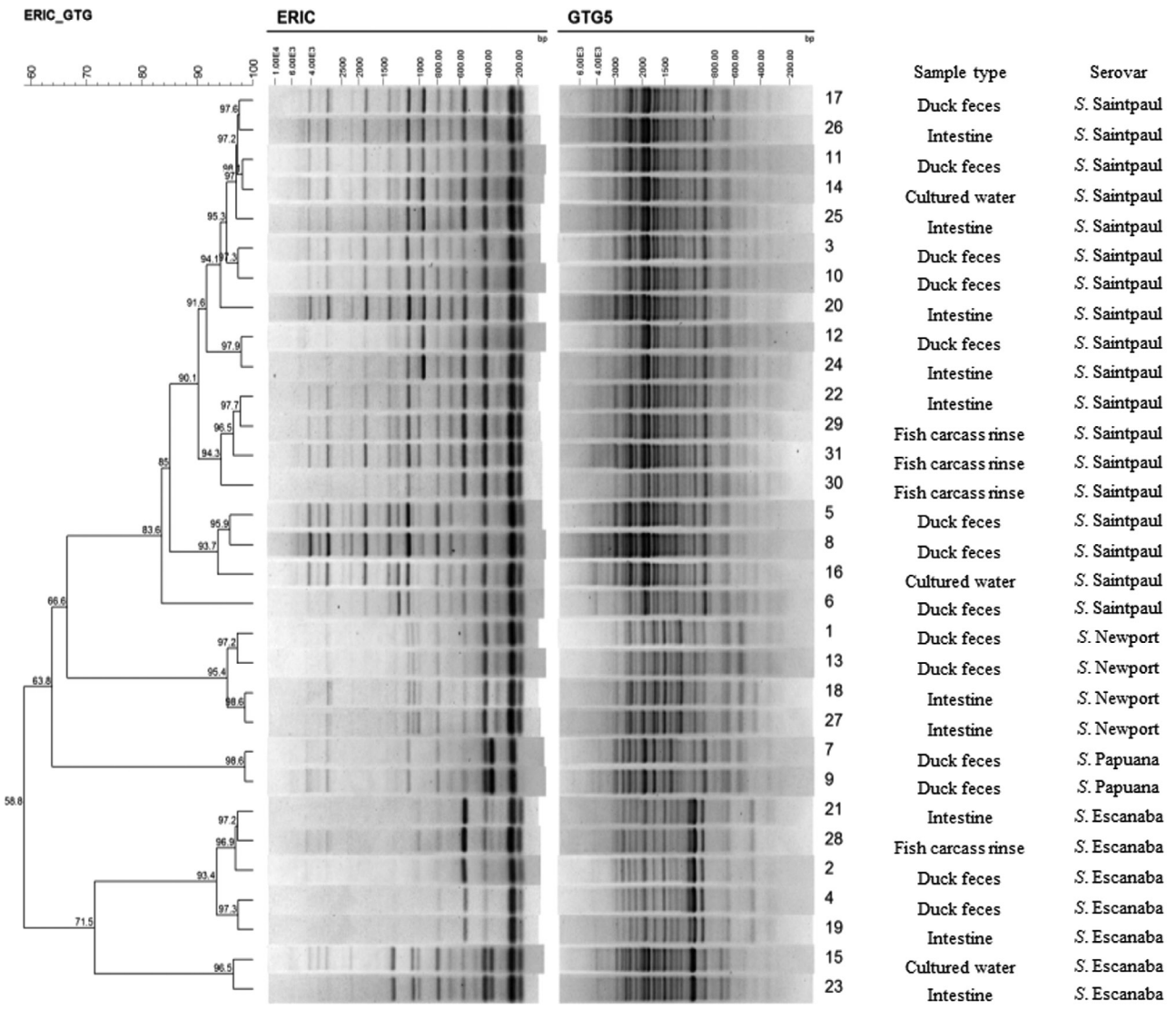
Salmonella serotype	Number of isolate[a] (%)				Total (%) (*n*=394)
	Fish carcass rinse (*n*=61)	Intestine (*n*=121)	Liver and kidney (*n*=3)	Water (*n*=209)	
Athinai	0 (0%)	0 (0%)	0 (0%)	2 (1.0%)	2 (0.5%)
Augustenborg	0 (0%)	0 (0%)	0 (0%)	2 (1.0%)	2 (0.5%)
Bradford	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Braenderup	0 (0%)	1 (0.8%)	0 (0%)	0 (0%)	1 (0.3%)
Brazzaville	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Breukelen	0 (0%)	0 (0%)	0 (0%)	3 (1.4%)	3 (0.8%)
Chartres	0 (0%)	3 (2.5%)	0 (0%)	3 (1.4%)	6 (1.5%)
Chester	0 (0%)	0 (0%)	0 (0%)	3 (1.4%)	3 (0.8%)
Derby	0 (0%)	2 (1.7%)	0 (0%)	0 (0%)	2 (0.5%)
Enteritidis	2 (3.3%)	2 (1.7%)	0 (0%)	0 (0%)	4 (1.0%)
Escanaba	21 (34.4%)	23 (19.0%)	0 (0%)	16 (7.7%)	60 (15.2%)
Galiema	0 (0%)	1 (0.8%)	0 (0%)	0 (0%)	1 (0.3%)
Hiduddify	0 (0%)	0 (0%)	0 (0%)	3 (1.4%)	3 (0.8%)
II	0 (0%)	2 (1.7%)	0 (0%)	4 (1.9%)	6 (1.5%)
Koessen	0 (0%)	0 (0%)	0 (0%)	2 (1.0%)	2 (0.5%)
Larochelle	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Menden	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Montevideo	0 (0%)	5 (4.1%)	0 (0%)	21 (10.1%)	26 (6.6%)
Neukoelln	15 (24.6%)	8 (6.6%)	3 (100%)	34 (16.3%)	60 (15.2%)
Newport	0 (0%)	2 (1.7%)	0 (0%)	0 (0%)	2 (0.5%)
Othmarschen	0 (0%)	3 (2.5%)	0 (0%)	16 (7.7%)	19 (4.8%)
Papuana	4 (6.6%)	21 (17.4%)	0 (0%)	34 (16.3%)	59 (15.0%)
Paratyphi B	0 (0%)	4 (3.3%)	0 (0%)	1 (0.5%)	5 (1.3%)
Rending	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Saintpaul	11 (18.0%)	36 (29.8%)	0 (0%)	27 (12.9%)	74 (18.9%)
Schwabach	0 (0%)	0 (0%)	0 (0%)	2 (1.0%)	2 (0.5%)
Singapore	0 (0%)	0 (0%)	0 (0%)	3 (1.4%)	3 (0.8%)
Stanley	0 (0%)	0 (0%)	0 (0%)	5 (2.4%)	5 (1.3%)
Strathcona	1 (1.6%)	1 (0.8%)	0 (0%)	2 (1.0%)	4 (1.0%)
Typhimurium	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Virchow	7 (11.5%)	7 (5.8%)	0 (0%)	20 (9.6%)	34 (8.6%)
Total	61 (100%)	121 (100%)	3 (100%)	209 (100%)	394 (100%)

[a]Samples from fish muscle were not positive for *Salmonella* spp.

Table 5. Logistic regression model for the association between the odds of detecting *Salmonella* in the red tilapia (*n*=480) and cultivation water (*n*=120) and the various microbiological and environmental parameters

Parameter	Odds ratio	SE	95% CI	*P*
The presence of fecal coliforms	3.51	2.08	1.10-11.24	0.034
The presence of *Escherichia coli*	2.94	0.41	2.25-3.86	<0.0001
The presence of *Vibrio vulnificus*	2.50	0.53	1.65-3.79	<0.0001
The presence of *Vibrio cholerae*	2.32	0.76	1.22-4.40	<0.0001
Average maximum wind gust (m/s)[a]	1.08	0.02	1.05-1.12	<0.0001
Average RH[a]	0.97	0.0092	0.95-0.97	0.001
Constant	0.23	0.22	0.032-1.55	0.131

AIC = 465.52.
[a]Average over 7 consecutive days.
SE, standard error; CI, confidence interval; AIC, Akaike Information Criterion; RH, relative humidity.



Sample type	Serovar
Duck feces	*S.* Saintpaul
Intestine	*S.* Saintpaul
Duck feces	*S.* Saintpaul
Cultured water	*S.* Saintpaul
Intestine	*S.* Saintpaul
Duck feces	*S.* Saintpaul
Duck feces	*S.* Saintpaul
Intestine	*S.* Saintpaul
Duck feces	*S.* Saintpaul
Intestine	*S.* Saintpaul
Intestine	*S.* Saintpaul
Fish carcass rinse	*S.* Saintpaul
Fish carcass rinse	*S.* Saintpaul
Fish carcass rinse	*S.* Saintpaul
Duck feces	*S.* Saintpaul
Duck feces	*S.* Saintpaul
Cultured water	*S.* Saintpaul
Duck feces	*S.* Saintpaul
Duck feces	*S.* Newport
Duck feces	*S.* Newport
Intestine	*S.* Newport
Intestine	*S.* Newport
Duck feces	*S.* Papuana
Duck feces	*S.* Papuana
Intestine	*S.* Escanaba
Fish carcass rinse	*S.* Escanaba
Duck feces	*S.* Escanaba
Duck feces	*S.* Escanaba
Intestine	*S.* Escanaba
Cultured water	*S.* Escanaba
Intestine	*S.* Escanaba

Figure 1. The dendrogram of rep-PCR profiles of *Salmonella* isolates from hybrid red tilapia and nearby duck feces.

Discussion

It is common practice to monitor indicator bacteria to evaluate water quality and aquaculture sanitation supporting fish production. The national standard of microbiological quality criteria for food and food contact containers in Thailand indicates that fish destined for human consumption should not have any detectable *Salmonella* spp. and *V. cholerae*, with acceptable levels of *Staphylococcus aureus* and *E. coli* being <100 colony-forming units/g and <10 MPN/g, respectively (BQSF, 2017). The International Commission on Microbiological Specifications for Foods (ICMSF) standard also recommends that the concentrations of fecal coliform and *E. coli* should not exceed 10 MPN/g (ICMSF, 1978). In this study, 62.3% (299/480) of the fish samples met the ICMSF and Bureau of Quality and Safety of Food (Thailand; BQSF) standards. Most of the fish muscle (94.2%, 113/120) contained no detectable *E. coli*, while 6% (7/120) of the muscle samples exceeded these *E. coli* standards.

Fish carcass rinses showed the highest prevalence of fecal coliforms. Our findings are consistent with a previous report suggesting that abundant indicator bacteria were found on tilapia skin surfaces due to possibly unhygienic aquaculture conditions (Rocha *et al.*, 2014). Long-term exposure to waterborne microbial contaminants is likely a major cause for the observed high prevalence of indicator bacteria in fish intestines and internal organs. Although fish muscle contains low concentrations of bacteria, it is possible that fish muscle can cross-contaminate tilapia skin or intestinal tissue during the fish preparation process. In this study, the levels of bacterial indicators in fish carcass rinses were similar to those in the cultivation water, which is consistent with the speculation that bacterial contamination in tilapia was largely the result of bacteria in cultivation water (Mandal *et al.*, 2009).

The most frequently identified *Salmonella* serovars were Saintpaul, Escanaba, Neukoelln, Papuana, Virchow, and Montevideo. The prevalence of those six serovars in eight sampling events was not uniformly distributed across time (Figure 2). For example, there was an increase in the occurrence of serovar Escanaba at time points IV, V, and VII. *S.* Neukoelln and *S.* Papuana were commonly detected at sampling time points II–IV, whereas the highest prevalence of *S.* Saintpaul was found at sampling time points VI–VIII (Figure 2).

Figure 2. The gradient distribution of the 6 major *Salmonella* serovars isolated from hybrid red tilapia during eight sampling events (*n*=328).

Interestingly, *Salmonella* serovars detected in this study were different from those in previous work. *Salmonella* serovars Brandenburg, Hadar, Heidelberg, and Saintpaul were reported in farmed fish in Brazil and Vietnam (Nguyen *et al.*, 2016; dos Santos *et al.*, 2019), whereas serovars Albany, Agona, Corvallis, Stanley, Typhimurium, Mikawasima, and Bovis-morbificans were observed in catfish and tilapia in Malaysia (Budiati *et al.*, 2016). Even though *Salmonella* serovar Saintpaul was abundantly found in tilapia in this study, this serovar has been commonly reported in pork in Thailand (Pungpian *et al.*, 2021). An outbreak of *Salmonella* Saintpaul, which was isolated from cucumbers, was responsible for 84 illnesses in the USA (CDC, 2013).

Identifying the source(s) of *Salmonella* contamination in tilapia aquaculture system would be challenging given that *Salmonella* is naturally found in the gastrointestinal tract of a wide variety of vertebrate animals. Grazing ducks raised near the tilapia production site were postulated to be a potential source of *Salmonella* contamination. Serovars Saintpaul (36.7%) and Escanaba (10.0%) were commonly found in duck feces and were also detected as the major serovars present in the tilapia farms. Previous studies also suggested that the presence of Saintpaul and Escanaba has been observed in livestock animals (Negi *et al.*, 2015; Eguale *et al.*, 2018; Awad *et al.*, 2020).

Although previous studies compared molecular typing methods for *Salmonella* (Nath *et al.*, 2010; Fendri *et al.*, 2013; Ferrari *et al.*, 2017), pulsed field gel electrophoresis (PFGE) was set as a standard method for many years for *Salmonella* typing. The discriminatory degree of various typing procedures was compared using 92 strains of *Salmonella* Typhimurium (*S.* Typhimurium). PFGE, multiple-locus variable number of tandem repeats analysis, and ERIC PCR differentiated 72, 53, and 63 types, respectively (Almeida *et al.*, 2015). These findings indicated that the discriminatory efficiency of PFGE was greater than others; however, PFGE can be a time-consuming and labor-intensive method (Winokur, 2003). Despite the moderate discriminatory power, ERIC PCR is rapid and reproducibly distinguishes epidemiological relationships among groups of *Salmonella*. The discriminatory index of ERIC PCR was as high as 0.9981 and 0.983 in typing of 113 and 74 *Salmonella enterica* isolates, respectively (Winokur, 2003; Nath *et al.*, 2010). Therefore, ERIC PCR was utilized in the present study to characterize the DNA sequence similarity of serovars *S.* Saintpaul, *S.* Newport, and *S.* Escanaba in fish and grazing ducks. Based on the DNA similarity of these isolates, the results suggested that nearby grazing duck feces may be a source of *Salmonella* that can contaminate the tilapia cultivation site, but such data cannot definitely prove the original source of contamination, either ducks to fish, fish to ducks, or some third *Salmonella* vertebrate reservoir contaminating both fish and ducks. For example, precipitation and subsequent overland flow could function to erode fecal materials from land-based sources and then run-off into tilapia cultivation sites.

V. vulnificus has been commonly found in estuarine water and shellfish, with the prevalence ranging from 13.6% to 15% (Cruz *et al.*, 2016; Baker-Austin and Oliver, 2018; King *et al.*, 2020). However, *V. vulnificus* could also be found in freshwater tilapia raised in Taiwan (China) and Egypt, and the prevalence ranged from 1.7% to 12.5% (Chen *et al.*, 2006; Younes *et al.*, 2016). In the USA, *V. vulnificus* infection in humans had a high fatality rate compared to other foodborne pathogens, and these bacteria were responsible for more than 95% of seafood-related deaths (Jones and Oliver, 2009). In aquatic animals, the clinical signs of *V. vulnificus* infection include dark coloration, lethargy, and hemorrhagic skin lesions.

In this study, only a small proportion of the fish intestines and internal organs tested positive for *V. vulnificus* (0.8%–5.0%), but none of the fish muscle was positive, indicating that the risk of *V. vulnificus* contamination might be small if wholesale processors and retail consumers of tilapia are careful during cleaning of fish and handling of filets.

In this study, the prevalence of *A. hydrophila* in fish was less than that in previous studies (Ahmed *et al.*, 2018; Zaher *et al.*, 2021). The prevalence of *A. hydrophila* found in fish carcass rinses (8.3%) and cultivation water (4.2%). This may be because healthy fish were included in our study, while high prevalence of *A. hydrophila* was commonly detected in clinically diseased fish (Salem *et al.*, 2020; Zaher *et al.*, 2021). Even though *S. agalactiae* was not detected during this study, it should be considered because *S. agalactiae* sequence type 283 has been associated with foodborne disease outbreaks due to raw tilapia consumption (Barkham *et al.*, 2019). *S. agalactiae* was also frequently reported in diseased tilapia in Thailand (Areechon *et al.*, 2016; Niu *et al.*, 2020).

These production sites were suitable for raising hybrid tilapia in cage-based system. On average, the quality of water, pH (6.8) and DO (7.8 mg/L) in this study were within the required ranges stated in the Thai Aquaculture Standard (TAS) for tilapia cultivation (pH: 6.5–8.0; DO>4 mg/L; TAS, 2010). However, NH_3-N and alkalinity, which are important parameters for cultivation quality, were not collected in the study. Further investigations should be performed to explore the quality of water for tilapia cultivation.

The presence of *Salmonella* in the fish and cultivation water samples was significantly associated with the presence of fecal coliforms, *E. coli*, *V. vulnificus*, and *V. cholerae* ($P<0.05$), with the odds of *Salmonella* detection being 2.3–2.5 times higher when these bacteria were also present. Seven-day mean maximum wind gusts and RH were positively and negatively associated with the odds of *Salmonella* in the samples, respectively. The highest wind gusts occurred during the sampling months of July and September; it is possible that high wind gusts could function to transfer *Salmonella* into the tilapia growing area from terrestrial sources, or this environmental parameter is collinear with some other unknown factor(s) causing the increase in bacterial levels. High RH was associated with a lower odds of *Salmonella* detection, which occurred especially during the sampling months of September and November, and the remainder of the year had lower RH values (Table 1). Interestingly, a previous study showed that high RH (85%) enhanced the survival of *S.* Typhimurium in a controlled chamber environment (López-Gálvez *et al.*, 2018). More research is needed to clarify the mechanism(s) causing these associations between weather parameters and the odds of *Salmonella* in tilapia.

Conclusions

High levels of fecal coliforms and *E. coli* and the presence of pathogenic bacteria were observed during this study, indicating that food safety precautions are needed regarding human consumption of tilapia. Based on the DNA similarity of bacterial isolates, nearby grazing ducks were identified as a potential source of *Salmonella* contamination for tilapia and the cultivation environment, but such speculations based on matching of DNA fingerprints do not prove causality, and more data are needed to confirm this speculation. Good animal husbandry, effective farm biosecurity, and, where possible, water treatment interventions for tilapia aquaculture may be helpful to reduce environmental levels of bacterial contamination. Furthermore, given the occurrence of bacterial pathogens in various tilapia tissues, it may be prudent to maintain hygienic processing of fish fillets, temperature control during transport, food preparation and adequate cooking temperature to reduce the risk of bacterial transmission from cultivated tilapia to humans.

Author Contributions

Study conception and design: Saharuetai Jeamsripong; Data analysis and interpretation: Varangkana Thaotumpitak and Saharuetai Jeamsripong; Study experiment: Varangkana Thaotumpitak, Jarukorn Sripradite, and Surapun Tepaamorndech; Drafting manuscript: Varangkana Thaotumpitak, Jarukorn Sripradite, Edward R. Atwill, Surapun Tepaamorndech, and Saharuetai Jeamsripong; Critical revision: Edward R. Atwill and Saharuetai Jeamsripong.

Acknowledgements

The authors would like to thank Mullika Kuldee, Jutanat Srisamran, Saran Anuntawirun, and Sutida Chalee for their field sampling and laboratory assistance.

Funding

This study was supported by the University of California, Davis, USA (A19-4577-S001), partially funded by the Faculty of Veterinary Science, Chulalongkorn University, Thailand (RG17/2559), and the 90th Anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund), Thailand. Varangkana Thaotumpitak is a recipient of the Royal Golden Jubilee PhD Program, and this study was supported by the National Research Council of Thailand (NRCT5-RGJ63001-017).

Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

Ethical Principles and Guidelines for the Use of Animals in this study were approved by the ethics committee of Chulalongkorn University Animal Care and Use Committee (IACUC; Approval No. 2031048). This study involving hazardous pathogens and nonpathogens used was reviewed and approved by the Institutional Biosafety Committee (IBC; Approval No. 2031027) of the Faculty of Veterinary Science, Chulalongkorn University, Thailand.

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