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Sireh extract (*Piper betle*) treatment in growth performance and disease prevention of catfish *Pangasius hypophthalmus* culture at Pahang River, Malaysia

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Abstract
The efficacy of *Piper betle* extract (*Sireh* leaves) as a functional diet in the growth performance and health status of catfish *Pangasius hypophthalmus*, which is also locally known as *Patin*, was determined via monthly sampling of catfish. The sampling was conducted to evaluate growth performance, health status and bacterial isolation from the internal organs of the fish. The feeding trials for the catfish culture were conducted at floating cage farms in Pahang River, starting from grow-out stages with an average body weight between 100 g and 200 g until harvest. During the two feeding trials, which were conducted from May to September 2017 (trial 1) and October 2017 to January 2018 (trial 2), the catfish were cultured in cages with dimensions of 10′ x 12’ x 5’. The cages had a stocking density of 1500 fish per cage and were located in Kampung Bintang, Temerloh and Bera, Pahang. Two regimes of *Sireh* extract treatment at 1 mL kg^{-1} of feed (100 mg kg^{-1}) for five times per week and three times per week were tested and compared with a control. A total of 270 catfish were sampled for body weight measurement, physical/necropsy examination of health status and bacterial isolations from the internal organs of the fish. The bacteria were cultured on tryptic soya agar and were identified using the API system (Biomérieux SA, France) after biochemical tests were conducted. In September 2017 (first trial), pathological changes such as redness on body and fins, congestion/enlargement of the spleen, kidneys and liver were observed along with the isolation of *A. hydophila/caviae/sobria* (2-10%). Similarly, in January 2018 (second trial), pathological changes were observed and bacterial isolation of *E. tarda* and *E. horshinae* (2.5-10%) were made. *E. aerogenes* and *E. cloacae* (2-25%) were commonly isolated from the internal organs. Overall, the treatment groups showed a statistically significant ($P \leq 0.05$) increase in appetite and growth performance compared to the control group. The SirehMax™ treatment significantly improved the growth performance of catfish culture at Pahang River.

Keywords: Bacterial, catfish, growth performance, *Sireh* extract

1. Introduction
Aquaculture is a major food sector in the global supply of animal protein. Fish and seafood account for 17% of all animal protein intake and this proportion has been increasing (FAO, 2012) [8]. The total fish supply was 154 million tonnes in 2011 and is expected to increase to 186 million tonnes by 2030. It is projected that 62% of the fish supply will come from aquaculture (Mimako *et al*. 2015) [11]. Tilapia and shrimp are expected to experience the fastest growth in aquaculture, followed by salmon, catfish (*Pangasius*) and carp (Mimako *et al*. 2015) [11]. In Malaysia, catfish farming and other freshwater aquaculture contributed 163,757 tonnes valued at RM992 millions of total aquaculture production in 2012 (Yusoff, 2015) [19]. The *Pangasius* sp., which is also known as *Patin* (*Pangasius hypophthalmus*), is a valuable catfish species cultured at Pahang River, Malaysia. Temerloh is known for its *Patin* culture. In 2017, Temerloh produced over 14,000 tonnes of fish, including *Patin* and Tilapia from its 2,018 cage culture units which were operated by 283 operators (Zaki, 2017) [20]. Others reported that rivers in Malaysia contributed up to 560 tonnes of freshwater fish landings worth RM2.2 million, with Temerloh alone accounting for 12.5 tonnes valued at over RM60,000 (The Star, 2017) [18].
Harsh climatic conditions such as high-water temperature during dry season, rainfall fluctuations and pollutions have caused various negative impacts on aquaculture (FAO, 2009) [7].

Pangasius sp. is susceptible to bacterial diseases, especially Motile Aeromonads Septicaemia (MAS) which is caused by *Aeromonas hydrophila* (Nur-Nazifah *et al*. 2016) [13]. Fish infected with *A. hydrophila* exhibit clinical symptoms such as lack of appetite, swimming abnormalities, pale gills, dropsy, generalised skin haemorrhage, inflammation/reddening of the mouth and anal region, and ulcers (Rimatulhana *et al*. 2016) [15]. Experimental infection of *A. hydrophila* in catfish *Ictalurus punctatus* showed diseased and moribund fish with reddened fins, external/internal septicaemia and iridial haemorrhage (Hanson *et al*. 2014; Zhang *et al*. 2016) [9, 21]. The symptoms vary depending on several factors, including the virulence of bacteria, resistance of fish to infection, presence or absence of septicaemia, and other stress factors related to the health status of fish and aquatic environment.

Betel leaves (*Piper betle*) contain various compounds such as carbohydrates, proteins, fat, minerals, vitamins, tannin, fibre, alkaloid, steroidal compounds and essential oil (Pradhan *et al*. 2013) [14]. However, the main active compounds responsible for the antibacterial effect are hydroxychavicol, sterol and tannin (Ali *et al*. 2010; Chakraborty and Shah, 2011) [3, 6]. Due to their high antimicrobial potential, betel leaves can be an excellent alternative to synthetic antimicrobial agents. The ethanolic crude extract of betel leaves at a concentration of 100 mg mL^{-1} demonstrated significant inhibition against *V. alginolyticus* (Ahmad-Baihaqi *et al*. 2018) [1]. Betel leaves have been found to contain phenol and chavicol that act as antiseptic to inhibit and kill bacteria (Syahidah *et al*. 2017) [17]. Using betel leaf extract as an antimicrobial agent in feed not only can reduce *A. hydrophila* infection in catfish (*Clarias gariepinus*) and gourami (*Ospronemus gouramy Lac*.), but can also improve their appetite and growth performance (Soeprapto & Sharif, 2018)[16]. Gourami fed with a higher concentration of betel leaf flour (6%) showed increased weight gain compared to those that were fed with lower concentration (2 and 4%). The gourami also had higher survival rates, with percentages of 93.0%, 88.8%, and 93.3% respectively (Soeprapto and Sharif, 2018)[16]. Hence, a field study was conducted to determine the efficacy of Sireh extract (SirehMax™) in feed as an antibacterial agent and growth promoter for catfish *Patin* culture in floating cages in Pahang River, Malaysia.

2. Material and Methods

2.1 Sireh extract (SirehMax™)

SirehMax™ is a herbal product made from *Piper betle* leaf extract. The process for the production of SirehMax™ is patented (Patent No: MY-176273-A). The recommended dosage of this product is 1 mL for 1 kg of formulated feed.

2.2 Sampling location

The field trial was carried out at two locations. The first location, which was Kampung Bintang (3.4944º N, 102.4345º E) in Temerloh, was selected for the grow-out *Patin* culture farms downstream of Pahang River and the trial was conducted from May to September 2017. The second trial was carried out at Kampung Kuala Teriang, Bera (3.2705º N, 102.4539º E) in Pahang. The field study for one cycle culture production was carried out from October 2017 until January 2018.

Fig 1: Patin culture in floating cages in Pahang River, Temerloh, Pahang.

Fig 2: Patin culture at Pahang River, Bera (3.2705º N, 102.4539º E), Pahang.

2.3 Sampling of fish

Sampling of *Patin* fish treated with SirehMax™ and a control group were carried out monthly from 12 selected cages of grow-out catfish. The catfish were treated with Sireh extract at 100 mg kg^{-1} of feed five times per weeks (SE 5x/week, n=50), SE 3x/weeks (n=50) and control (n=50). A total of 150 catfish were sampled in the first trial (May to September 2017) and 120 catfish were sampled in the second trial (October 2017 to January 2018) for measurements of body weight (growth performance), post-mortem examination, bacterial culture and isolation.

2.4 Observation, physical and post-mortem examinations

The body weight (g) of the sampled fish was measured. Any abnormalities and pathological changes observed on the body and in the internal organs were recorded, followed by bacterial isolation from the liver, kidney, spleen, brain and eye.

2.5 Bacterial isolation on culture media

Bacterial from the internal organs and skin lesions were isolated using Tryptone Soy Agar (TSA), followed by subculture and gram staining of pure isolate. A series of biochemical tests were performed for Gram negative bacteria, including oxidase, catalase, oxidative-fermentative glucose test, motility, growth on McConkey agar, Methyl red and Vogues Paskauer (MR/VP), followed by API 20NE or API 20E (Biomérieux SA, France) for Enterobacteriaceae.

Confirmation of *Aeromonas* spp. (*A. hydrophila*, *A. sobria* and *A. caviae*) was carried out using API 50 CH. Similarly, for Gram-positive bacteria, isolation and identification of genera and species were carried out after gram staining. This involved the identification of haemolytic characteristics growth on blood agar, oxidase, catalase and API STAPH or API 20 STREP.

2.6 SPSS analysis of growth performance

Statistical analysis (IBM SPSS v.25, Window, USA) was performed using ANOVA One-way to determine the significance ($P \leq 0.05$) of two regimes of SirehMax™ treatment on monthly body weight growth performance compared to the control group.

3. Results

3.1 Gross observation and necropsy examination

Pathological changes observed in fish varied from mild to moderate lesions such as inflammation/haemorrhages on skin, fin, tail, mouth or operculum (20-33%), congestions /enlargement of spleen, kidney and/or liver (40-53%) and observation of white/black nodule in spleen (30-38%) (Figure 3). Other fish showed abnormalities such as pale/yellow or green-coloured liver (11-16%). Pathological changes associated with inflammation and suggestive of bacterial infection was found in fish sampled in September 2017 and January 2018. Mild to moderate lesions associated with redness/haemorrhage on the body, fin and tail (Figure 4) as well as congestions of kidney, spleen or liver with white/black nodules in spleen (Figure 5) were found in 20% to 33% of the fish examined. However, no mortalities were reported by farmers. The bacteria *E. aerogenes* was isolated in 2% to 25% of fish sampled that showed signs of inflammation /congestions of the internal organs, with mixture of bacteria such as *A. hydrophila* (2-10%), *P. fluorescens* (2%), *P. shigelloides* (2-10%), *E. tarda* (2-10%), *S. haemolytica* (2%) and *P. pneumotropica* (2%) being isolated from the internal organs.

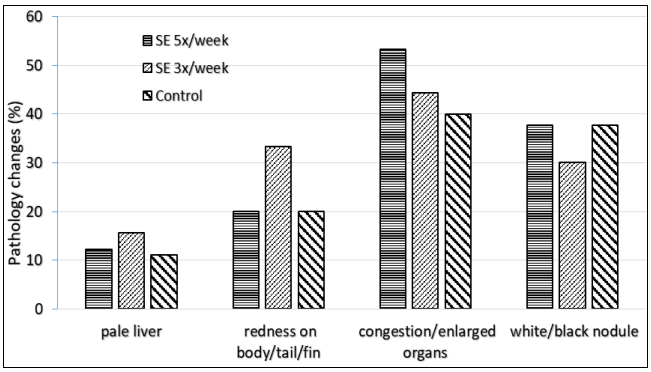

Fig 3: Physical observation and post-mortem findings in catfish

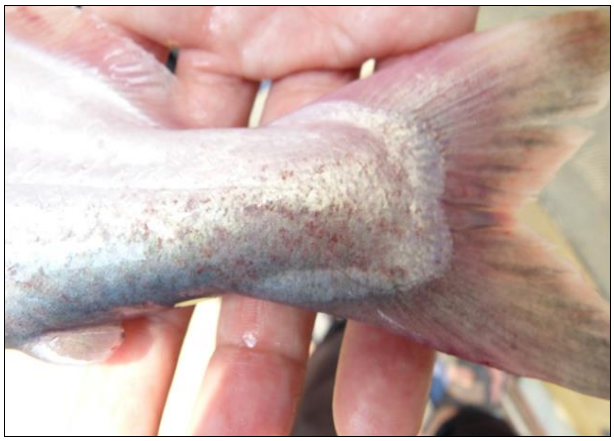

Fig 4: Pin-point haemorrhage at tail and caudal peduncle area

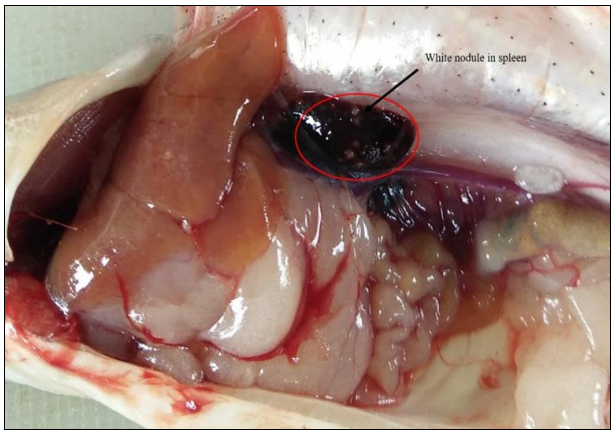

Fig 5: White and black nodule in spleen associated with bacterial infections

3.2 Bacterial isolation and prevalence (%)

Colonies of *Aeromonas* spp. were creamy white on TSA, with smooth and rounded colonies about 2 mm to 3 mm in diameter. They were Gram-negative motile rods that gave a positive reaction for cytochrome oxidase, gelatin hydrolysis, indole production, glucose, sucrose and mannitol fermentation, arginine dehydrolase and ß- galactosidase tests. They were also positive for Voges-Proskauer, methyl red, lysine decarboxylase and arabinose fermentation tests. Confirmation of *A. hydrophila* from *A. caviae* was carried out using API 50 CH (Table 1). Four isolates were identified as *A. sobria*, while only one isolate was identified as *A. caviae*. All the four isolates of *A. sobria* were ß–haemolytic on blood agar, whereas *A. caviae* showed γ-haemolytic characteristics. One isolate of *A. hydrophila* was ß–haemolytic on blood agar, whereas two isolates showed γ-haemolytic characteristics.

Table 1: Identification of *Aeromonas* spp. based on API 50 CH Kit

Test	API 50 CH	Code	AH-1	AH-2	AH-3
1.	CONTROL		-	-	-
2.	Glycerol	GLY	+	+	+
3.	Erythritol	ERY	-	-	-
4.	D-Arabinose	DARA	-	-	-
5.	L-Arbinose	LARA	-	-	+
6.	Ribose	RUB	+	+	+
7.	D-Xylose	DXYL	-	-	-
8.	L-Xylose	LXYL	-	-	-
9.	Adonitol	ADO	-	-	-
10.	β-Methyl - D-Xyloside	MDX	-	-	-

11.	Galactose	GAL	+	+	+
12.	Glucose	GLU	+	+	+
13.	Fructose	FRU	+	+	+
14.	Mannose	MNE	+	+	-
15.	Sorbose	SBE	-	-	-
16.	Rhamnose	RHA	-	-	-
17.	Dulcitol	DUL	-	-	-
18.	Inositol	INO	-	-	-
19.	Mannitol	MAN	+	+	+
20.	Sorbitol	SOR	-	-	-
21.	a-Methyl-D-Mannoside	MDM	-	-	-
22.	a-Methyl-D-Glucoside	MDG	+	+	-
23.	N-Acetyl Glucosamine	NAG	+	+	+
24.	Amygdalin	AMY	-	-	-
25.	**Arbutin**	ARB	-	+	+
26.	**Esculin**	ESC	-	+	+
27.	**Salicin**	SAL	-	+	+
28.	**Cellobiose**	CEL	-	-	+
29.	Maltose	MAL	+	+	+
30.	Lactose	LAC	-	-	-
31.	Melibiose	MEL	-	-	-
32.	**Sucrose**	SAC	-	+	+
33.	Trehalose	TRE	+	+	+
34.	Inulin	INU	-	-	-
35.	Melezitose	MLZ	-	-	-
36.	Raffinose	RAF	-	-	-
37.	Starch (AmiDon)	AMD	+	+	+
38.	Glycogen	GLYG	+	+	+
39.	Xylitol	XLT	-	-	-
40.	Gentiobiose	GEN	-	-	-
41.	Turanose	TUR	-	-	-
42.	D-Lyxose	LYX	-	-	-
43.	D-Tagatose	TAG	-	-	-
44.	D-Fucose	DFUC	-	-	-
45.	L-Fucose	LFUC	-	-	-
46.	D-Arabitol	DARL	-	-	-
47.	L-Arabitol	LARL	-	-	-
48.	Gluconate	GNT	+	+	+
49.	2-Keto Gluconate	2KG	-	-	-
50.	5-Keto Gluconate	5KG	-	-	-
51	Sensitivity 0129 (10/150)	129	R/S	R/R	R/R
52.	Triple sugar iron (slant/butt/H2S)	TSI	K/A,G	K/A,G	A/A
53.	Catalase test	CAT	+	+	+
	Genus/species	ID	*A. sobria*	*A. hydrophila*	*A. caviae*

Confirmation analysis using API 50 CH differentiated *Aeromonas* spp. based on several tests such as sucrose, cellobiose, arbutin, salicin and esculin. *A. sobria* showed negative results for these tests, whereas *A. hydrophila* and *A. caviae* showed positive results (Table 1). An additional test using Vibriostatic agent O129 (10/150) for *A. sobria* produced a resistant isolate at 10 µg concentration but was sensitive to 150 µg (O129 10/150 – R/S). *A. caviae* gave positive results for sucrose, cellobiose, arbutin, esculin and salicin. An additional test using TSI showed an acid slant/butt (A/A) with no gas and hydrogen sulphate formation for *A. caviae*. *A. hydrophila* was different from *A. caviae* in several tests such as L-Arabinose (-ve), mannose (+ve), α-Methyl-D-Glucoside (-ve) and cellobiose (-ve). The results for *Aeromonas* spp. identification are shown in Table 1. Confirmation for identification of isolates using API data software analysis was acceptable with results of similarity between 98% and 99.9%.

The main bacteria isolated from the internal organs of catfish were *Enterobacter* spp., specifically *E. aerogenes* and *E. cloacae*, with prevalence rates of 18-25%, 2-6%, and 4-20% in the SE5x/week, SE3x/week and control groups respectively (Figure 6 and 7). *Enterobacter* spp. and other *Enterobacteriaceae* (4-35%), which were commonly isolated from internal organs of catfish, were suspected to have originated from the water source (Pahang River). *Aeromonas* spp. (2-10%), *Edwardsiella* sp., *E. tarda* (2.5%) and *E. horshinae* (7.5%) were isolated at low prevalence rates in treated catfish.

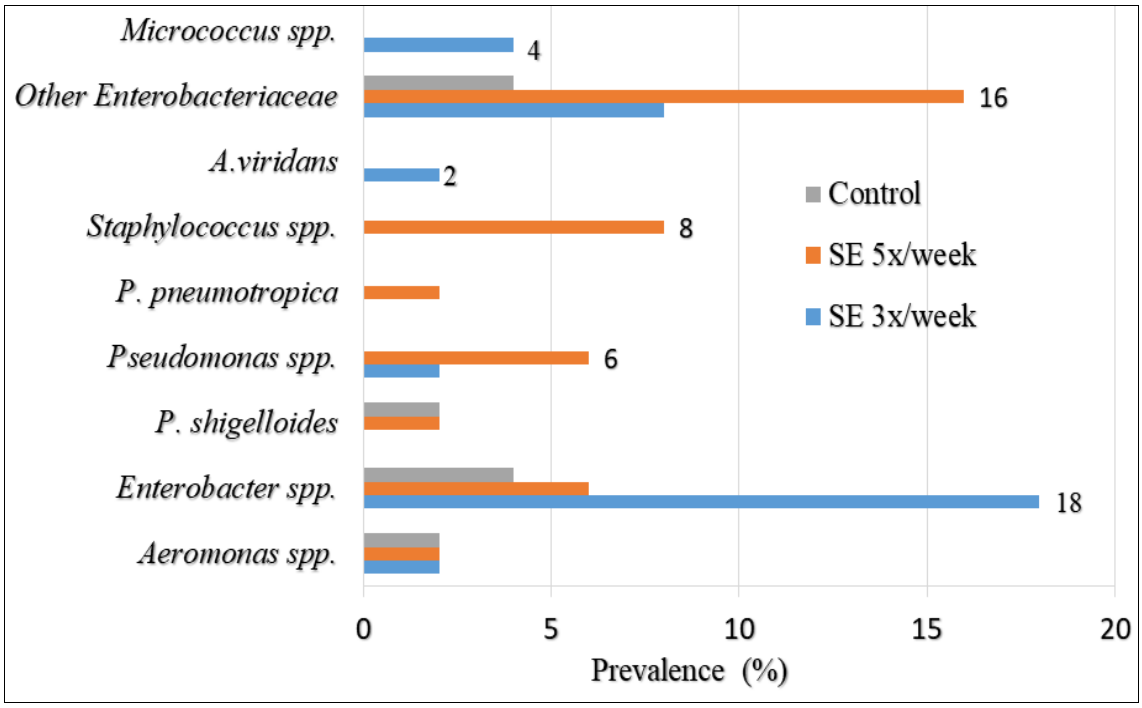

Fig 6: Bacterial prevalence (%) in catfish (May to September 2017)

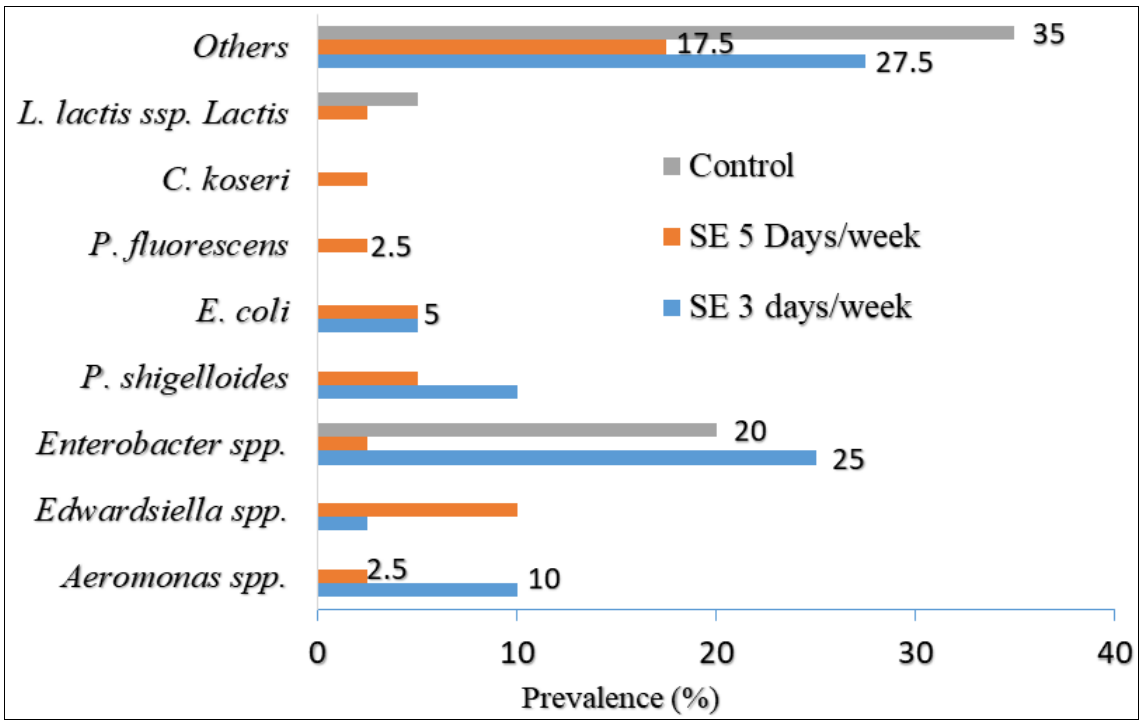

Fig 7: Bacterial prevalence (%) in catfish (October 2017 to January 2018)

3.3 Growth performance of fish culture

One-way analysis of variance (ANOVA) was used to determine the statistically significant difference between means of three independent groups (SE5x/weeks/SE3x/weeks/control). The results of the first trial, which were conducted from May to July, 2017, indicated that there was no significant difference in the body weight performance between catfish fed with sireh extract (SE5x/weeks and SE3x/weeks) and the control group ($P \geq 0.05$). However, their body weight increased significantly in August and September after undergoing three months of continuous treatment (Figure 8). The second trial, which were conducted from October 2017 to January 2018, showed that catfish treated with sireh extract had a significantly better weight gain compared to the control group ($P \leq 0.05$) (Figure 9). Sireh extract was also found to improve feed intake and appetite. Although catfish treated with sireh extract five times per week showed better monthly body weight performance, there was not statistically significant difference between the two regimes of treatment ($P \geq 0.05$).

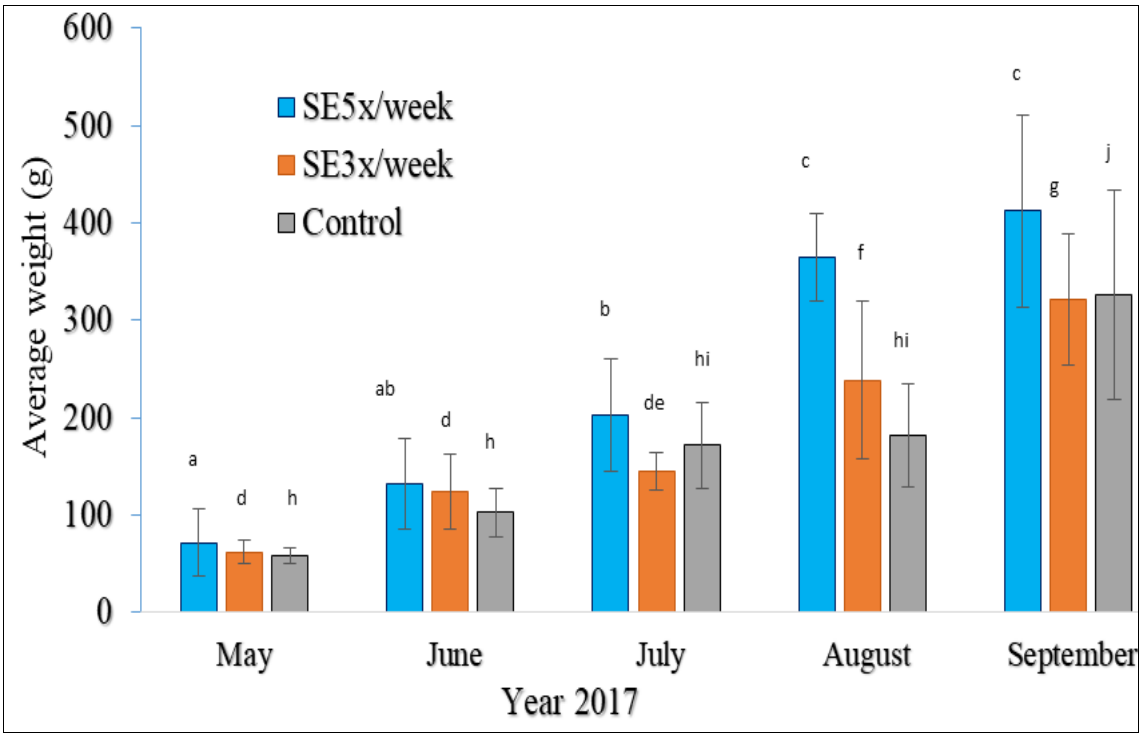

Fig 8: Statistical analysis showing significant growth performance of catfish culture ($p \leq 0.05$) in monthly average body weight gain (g) from August to September 2017

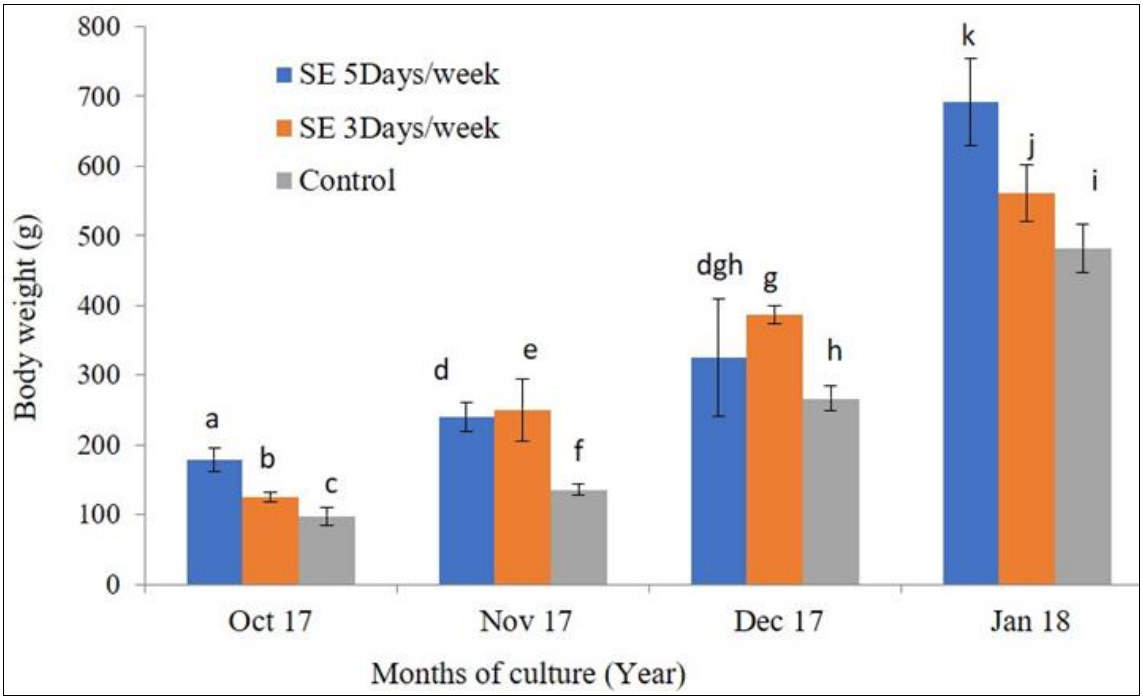

Fig 9: Growth performance of catfish culture in monthly average body weight gain (g) from October 2017 to January 2018

4. Discussion

It was anticipated that there would be a high prevalence of *A. hydrophila* in the SirehMax™ treatment groups since bacterial prevalence of 10% to 85% in fish cultivation is common (Austin and Austin, 2012; Molnar and Csaba, 2005) [5, 12]. *A. hydrophila* is typically a commensal or saprophytic bacterium found in the digestive system and skin, and its invasiveness as a secondary pathogen depends on previous infection or damage to the skin which is caused by other stress-related factors (Al-Harbi and Uddin, 2004; Austin and Austin, 2012; Harikrishnand, 2005) [2, 5, 10]. Thus, it is common to isolate this bacterium in the internal organs of fish under stressful conditions that are related to handling and minor injury. Other factors such as low dissolved oxygen (DO), high organic content and ammonium, and temperature fluctuations may also contribute to the colonisation of this bacterium in the internal organs associated with immune response mechanisms in the liver, spleen and kidney (Al-Harbi and Uddin, 2004; Harikrishnand and Balasundaram, 2005) [2, 10].

The presence of potential human pathogens such as *E. cloacae*, *Escherichia coli*, *Pseudomonas* sp. and *Klebsiella* sp. in the internal organs of fish indicates that the aquatic system is contaminated with high levels of human activities that are associated with food and animal waste. While *Klebsiella pneumoniae* is considered commensal in the fish environment,

it may pose a serious health threat to humans who consume fish with high contamination of this species (Ampofo and Clerk, 2010) [4]. Apart from posing a threat to fish health, the zoonotic nature of bacterial diseases and food safety is also a public health concern that needs to be considered.

Many factors are known to influence overall health performance and body weight gain in *Pangasius* culture. Environmental factors such as rainy season and rapid climatic change are known to affect water quality. The weather in Malaysia is characterised primarily by two monsoons, which are the Southwest Monsoon that starts from late May to September and the Northeast Monsoon that begins from November to March. Heavy rain associated with the monsoon season was reported from September to December 2017 until January 2018. The location of floating cages was shifted a few times during the rainy season due to heavy flow of current and the rise of water level at Pahang River, with a few incidences of flood in Temerloh and Bera, Pahang. Unusual mortalities and health problems, which were associated with skin injuries from an accumulation of debris within the cage area and muddy waters from upstream, were reported in September 2017 and January 2018. Bacterial *A. hydrophila* (2-10%) and *Edwardsiellosis* (2.5-10%) were isolated from the internal organs of the affected fish, suggesting bacterial infections at low prevalence. These studies showed that two regimes of sireh extract treatment (SE5x/weeks and SE3x/weeks) at 1 mL kg^{-1} feed in catfish culture throughout their grow-out stages would produce better growth performance and would be effective in the control of disease/mass mortalities of fish culture associated with bacterial diseases.

5. Conclusion

The therapeutic effects of sireh extract (SirehMax™) as a functional diet, which served as an anti-bacterial and appetite enhancer, were tested in two field trials of catfish culture in Pahang River, Pahang. The growth performance of the catfish culture was measured. Bacterial pathogen isolated from the fish was analysed and correlated with the history, pathological changes and laboratory findings to determine the infection status associated with bacterial diseases. Disease occurrences associated with *A. hydrophila* were reported in catfish in September 2017 (2%) and January 2018 (2.5-10%) but at low prevalence, including *Edwardsiella* spp. (2.5-10%). *Enterobacter* spp. (2-20%) and other Enterobacteriaceae (<35%) were the main bacteria isolated in fish samples throughout the study, which were associated with aquatic environment and water quality. In these studies, Sireh extract treatment, which served as a functional diet with suggested dose regimes of 1 mL Kg-1 feed for five times per week and three times per week, was found to significantly improve the growth performance of catfish culture in Pahang River. In conclusion, SirehMax™ treatment improved the overall growth performance of catfish culture and was effective in preventing common bacterial diseases in the cage culture system.

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