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ORIGINAL RESEARCH

Effect of intestinal autochthonous *Enterococcus faecalis* on the growth performance, gut morphology of Malaysian mahseer (*Tor tambroides*) and protection against *Aeromonas hydrophila*

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Abstract The study aimed to investigate the effects of dietary supplementation of two strains of potential intestinal autochthonous probiotics, *Enterococcus faecalis* on growth performances, body composition, gut morphology of Malaysian mahseer, *Tor tambroides,* and survival of the juveniles challenged with *Aeromonas hydrophila*. In this study, 270 fish were fed for 70 days with three different diets; a control (CON) and two diets supplemented with *E. faecalis* ($1x10^8$ $CFUg^{-1}$) as strains 2674 (T1) and FC11682 (T2). At the end of the study, growth performance, whole body fatty acid profiles, and gut morphology (villus length, width, and villus area) were significantly ($P < 0.05$) increased in probiotic treatment groups. The highest ($P < 0.05$) weight gain (%), SGR (% day^{-1}), and FCR were observed in T2. Additionally, the mortality after challenge test was 100% in the control group (CON) whereas values were the lowest (30%) ($P < 0.05$) in T2 followed by T1 (40%). Both the T1 and T2 showed significantly improved growth, gut morphology, and protection against *A. hydrophila,* with *E. faecalis* strain FC11682 (T2) being the most recommended of tested probiotics for Malaysian mahseer.

Keywords Autochthonous probiotic . Disease resistance . *Enterococcus faecalis* strain . Gut morphology . *Tor tambroides*

Introduction

The Malaysian mahseer, *Tor tambroides* (Bleeker 1854), is a cyprinid species with high commercial value, presenting good demand in aquaculture, and as a game and ornamental fish. However, its status in the wild is vulnerable due to anthropogenic activities (Esa et al. 2008; Asaduzzaman et al. 2018a; Lau et al. 2021). It is an omnivorous, feeding on molluscs, plants, small fish, and insects in its natural habitat (Ramezani-Fard et al. 2012), while being capable of utilizing plant-based ingredients in formulated diet (Ishak et al. 2021). Malaysian mahseer larvae fed formulated feed have shown higher levels of polyunsaturated fatty acid (PUFA) deposition in whole-body tissues than counterparts fed with live feed (Asaduzzaman et al. 2018b).

Diet and nutrition research studies on mahseer are growing rapidly (Lau et al. 2021). Another practical reason to find alternative dietary options for growth promotion is the slow growth of the fish which currently takes a longer period to reach marketable size in comparison to other cyprinid species (Chowdhury et al.

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2016). In these respects, probiotics have recently been proposed as dietary options for improving mahseer growth.

Asaduzzaman et al. (2018b) identified three host-derived probiotics (*Shewanella* sp. AFG21, *Bacillus* sp. AHG22, and *Alcaligenes* sp. AFG22) from the Malaysian mahseer. The study reported on mahseer juveniles fed with 10^8 CFU g^{-1} of each potential probiotic for 90 days which showed at conclusion that juveniles fed *Alcaligenes* sp. AFG22 had higher lipolytic, cellulolytic, and proteolytic counts, as well as larger villus area, villus length, and villus breadth.

Diseases and growth performance of aquaculture species are key problems limiting production in intensive commercial aquaculture (Stentiford et al. 2012, 2017; Cordero et al. 2016; Liu et al. 2020). Conventionally, antibiotics (oxytetracycline, erythromycin), and chemotherapeutics agents (formalin, Potassium permanganate) are being used to control disease outbreaks on farms, which leads to the both emergence of disease resistant bacteria and environmental pollution (accumulation of antibiotic residue) (Rattanavichai et al. 2015; Liu et al. 2020). As a result, probiotics have been developed as a novel functional product applied for the benefit of nutritional health of aquaculture species, such as in improvement of growth and disease resistance (Han et al. 2015; Asaduzzaman et al. 2018a; Cai et al. 2019), and are considered as potential alternatives for disease control which can also avoid the adverse results of antibiotics and antimicrobial chemicals (Gatesoupe 1999; Nayak 2010).

Probiotics used in aquaculture have mostly originated from terrestrial animals, but current research is increasingly using host gut probiotics isolated from the gastrointestinal (GI) tract of fish and/or culture water (Gatesoupe 1999; Lazado et al. 2015; Li et al. 2019). This shift has subsequently contributed to improvement of feed consumption and nutrient absorption, shown by positive influence on host growth parameters (Zhai et al. 2017; Asaduzzaman et al. 2018b; Nath et al. 2019; Dawood et al. 2019). Evidence has indicated that host associated probiotics (HAPs) would have better chance to maintain in the intestine even after probiotic withdrawal which would further enhance performance of the probiotics (Van Doan et al. 2018). To date, several bacterial species, such as *Bacillus* sp., *Lactobacilli* sp., *Lactococcus* sp., and *Enterococcus* sp. have been evaluated as wide-spectrum probiotics for aquaculture, while the search for more suitable species-specific probiotics ongoing (Lazado et al. 2015).

The dietary supplemental use of probiotics has been shown to influence both growth performance and body chemical composition (Zhou et al. 2009; Merrifield et al. 2010; Allameh et al. 2017; Asaduzzaman et al. 2018a). HAPs have been reported to improve gut morphology (villus height, width. and area) (Asaduzzaman et al. 2018a) and muscle morphometry (Asaduzzaman et al. 2018b; Shakur Aha et al. 2020), which in turn may assist in the disease resistance of the host fish (Merrifield et al. 2010). Fatty acids are important dietary nutrients needed by fish for optimum growth and good health management (Mejri et al. 2021). Several studies have also investigated the effect of HAPs on the fatty acid profile of cultured species such as *Cyprinus carpio*, and *Litopenaeus vannamei* (García de La Banda et al. 2010; Ramezani Fard et al. 2014; Baesi et al. 2017).

On the other hand, dietary supplementation of probiotics can be useful in control and improvement in handling stress, high stocking densities, reduced growth due to unbalanced physiology, limited reproductive capacity, and in suppression of pathogens thus allowing fish to survive against pathogens (Van Weerd and Komen 1998; Pulkkinen et al. 2010; Tachibana et al. 2020). Some studies have been carried out to investigate the effects of two closely related strains of one species on growth parameters, disease resistance and immune responses of aquaculture species and these studies observed significantly better performance compared to control (Díaz-Rosales et al. 2009; García de La Banda et al. 2010). Therefore, the current study identified the need to evaluate more potential HAPs, sourced specifically from Malaysian mahseer.

In a preliminary study, two strains of *Enterococcus faecalis* (strain 2674 and FC11682) were isolated from the gut of Malaysian mahseer (unpublished data). Subsequently, the current study was conducted to investigate the effectiveness of the dietary supplementation of these two closely related strains of *Enterococcus faecalis* on growth performances, body composition, and gut morphology of Malaysian mahseer. There is a scarcity of information about the impact of probiotics on disease resistance and fatty acid profiles of Malaysian mahseer. The development of suitable probiotics involves evaluation of their efficacy for disease resistance against common fish pathogens. Thus, the main aim of the present study was to evaluate the effects of dietary supplementation of two strains of probiotics on growth performance, body composition, and gut morphology, as well as disease protection of Malaysian mahseer juveniles against

pathogen *A. hydrophila.*

Materials and methods

Culture of microorganisms, preparation of experimental diets and feeding

Commercial feed (floating pellet code 9961, Thai Lux Enterprise Company Ltd, supplied by local supplier in Terengganu) containing 40% protein and 7% fat (Misieng et al. 2011; Ramezani-Fard et al. 2012; Kuebutornye et al. 2020a) were selected as basal feed in this study, with parameters confirmed by proximate composition analysis. One control diet, and two probiotic supplemented diets incorporated with different probiotics, were prepared according to method described by Asaduzzaman et al. (2018b) with some modification. In short, two closely related potential probiotic bacterial strains of *Enterococcus faecalis*. *(*strain 2674 and strain FC11682) were separately grown in 5 ml TSB broth tubes for 24 hours, and in 5 ml MRS broth tubes for 48-72 hours, at room temperature with incubator shaker set at 100 rpm. Cultures were centrifuged at 3000 rpm for 10 minutes and pellets were harvested and washed thrice in phosphate buffer solution (PBS, PH 7.4). Then, the pellets were resuspended in the same amount of PBS solution. The diluted probiotics in PBS solution were sprayed onto commercial floating pellet by bottle sprayer, which sterilized at each use using bleach (1ppm) and 70% ethanol (Kuebutornye et al. 2020). The number of culturable bacteria were determined by plate count method on TSA or MRS agar plate, to achieve $1x10^8$ CFUml^{-1}. After spraying, feed was dried under aseptic condition under laminar flow, and stored for maximum 7 days at 4°C before further use. Fresh experimental diets were prepared on a weekly basis to maintain the viability of bacteria ($1x10^8$ CFUg^{-1}) (Amstrong et al. 2016). The viability of the probiotic bacteria was tested in experimental diets by homogenizing 0.1 g of feed in 1 mL PBS. In triplicates, serial dilutions up to 10^8 were prepared and distributed on TSA/MRS agar plates, incubated for 24-48 hours at 30°C, with statistically viable plates (i.e., 30–300 colonies) subsequently counted (Asaduzzaman et al. 2018a; Abarike et al. 2018).

Design of experimental setup and sampling condition

Malaysian mahseer juveniles were collected from Selangor, Malaysia and acclimatized in a 5000-litre fiber glass circular tank with continuously aerated fresh water for two weeks, and maintained with commercial feed (Thai Lux Enterprise Company Ltd, supplied by local supplier in Terengganu) before commencement of the experiment. Fish were then measured (initial mean body weight 2.54±0.01g) and distributed randomly into three groups: one control (CON0) and two treatments (T1 and T2) in 9 different tanks (3 replicates for each group) measuring 150 litre each, and a 70 day (10 weeks) feeding trial conducted for T1, T2 and CON0 (Control). The Treatments T1, T2 and Control (CON0) groups were fed diets supplemented with probiotics *E. faecalis* strain 2674, strain FC11682 and control diet (CON0), respectively. The fish were fed at the rate of 2% of body weight twice daily (9 am and 4 pm) for 10 weeks. Uneaten feed was siphoned out, oven dried and weighed to calculate feed intake. Each tank received vigorous aeration via an air stone linked to a central air compressor, water was exchanged 100 percent weekly, and the tanks were cleaned weekly. Water quality parameters (DO, pH, water temperature, alkalinity, NO_2/NO_3 and alkalinity KH, NH_3) were measured daily using YSI probe (USA) and maintained at acceptable levels. Prior to start of the feeding trial, 10-12 fish were randomly stored at -80°C for proximate and fatty acid composition analysis. Triplicate groups of experimental diets were stored at -20°C for proximate composition analysis. At the end of trail fish were starved for 24 hours and 3 fish per tank (9 fish per treatment) were dissected to collect the gut for histological analysis. Fish were anaesthetized using clove oil (50uL/litre) prior to dissection (Mian et al. 2017). Another five fish from each replicant tank were collected and stored at -80°C for fatty acid analysis. A total 12 fish/tank, close to the average final weight, were transported to a challenge test laboratory for challenge with pathogenic bacteria *A. hydrophila*. All trials were carried out in accordance with Universiti Malaysia Terengganu and European legislation on laboratory animal welfare, as well as the guiding principles for the use and care of laboratory animals.

Challenge study against *Aeromonas hydrophila*

At the final period of the feeding study, 12 healthy fish per tank, close to average weight, from triplicate groups fed control, T1, and T2 diets, were challenged with *A. hydrophila*. All the above-mentioned treatments were subjected to experimental infection in triplicate. Prior to injection, fish were anaesthetized using clove oil (50uL/litre). Then, 0.1ml bacterial suspension with $1x10^8$ CFUml^{-1} (LD_{50}; determined in a prior experiment) of *A. hydrophila* was injected intra-peritoneally into fish fed probiotics diet, while control fish were injected with the same amount of PBS (Amend 1981). Clinical signs (such as, isolation of fish, hemorrhage) and mortality patterns were observed and recorded for 7 days. Cumulative mortality percent and relative percent of survival (RPS%) were computed to compare disease resistance capability demonstrated by each treatment for comparison with the control group. The following formula was used to compute cumulative mortality (%) and relative percent survival (RPS%) (Liu et al. 2017).

$$\text{Cumulative mortality (\%)} = \left(\frac{\textit{Total mortality in each treatment number after challenge}}{\textit{Total number of fish challenged for same treatment}}\right) x100 .$$

To assess the possible protection provided by probiotics, the relative percentage of survival (RPS) (Amend 1981) was computed as follows:

$$\text{RPS (\%)} = \left(1 - \frac{\textit{Percent mortality in each treatment number after challenge}}{\textit{Percent mortality in control group}}\right) x100 .$$

Growth performance

To determining feed conversion ratio (FCR), the total amount of feed given to each experimental group was recorded. Other growth-related parameters were determined by following formula such as, weight gain (g)= final weight (g) initial weight (g) and specific growth rate (SGR % day^{-1}) = 100 (ln W2-lnW1)/T (W1 and W2 are the initial and final weight (g), respectively, and T is the number of days in the feeding period) and apparent FCR=feed fed (g)/weight gain (g) (Amend 1981; Tukmechi et al. 2011; Abarike et al. 2018).

Proximate composition and fatty acid composition of whole-body Malaysian mahseer juveniles fish, and diets

To analyse the proximate compositions of harvested samples, whole body samples of Malaysian mahseer were used in triplicate using traditional AOAC methods (AOAC 1997). Moisture content of the sample was determined by drying it at 105°C to a constant weight. Ash was measured after oven treatment at 550°C for 12 hours. Crude protein content was determined using the Kjeldahl technique (KBL 40 S Digestion System, Gerhardt GmbH & Co., and Kjeltec 2100 Distilling Unit, FOSS Tecator AB, Högendäs). Crude lipids were determined using the Soxhlet method (FOSS Labtec ST310, Högendäs). Following the one-step approach developed by Abdulkadir and Tsuchiya (2008), the fatty acid (FA) composition of freeze-dried whole-body samples of Malaysian mahseer was analysed. Freeze-dried 200-300 mg of whole-body mahseer samples were extracted and esterified in a single tube. The fatty acid methyl esters (FAMEs) were extracted and quantified using gas chromatography with flame ionisation detection (GC-FID-QP2010 Ultra). By comparing the peak area of each FA to the overall peak area of all FAs in the samples, composition in terms of individual FAs was calculated quantitatively (as a percentage).

Histological analysis for gut morphology

At the final period, fish were starved for 24 hours, and 3 fish from each replicate, were selected close to the mean treatment weight, and anaesthetized using clove oil (50 mgL^{-1}) and dissected aseptically following method described by (Liu et al. 2021) with some modifications. Briefly, mid-gut samples were immersed in Bouin's solution (10:1) for 24 hours. Following that, the samples were transferred to 70% ethanol solution, and subsequently processed with a series graded level alcohol in an Leica™ automatic tissue processor, followed by paraffin embedding. The implanted tissue blocks were sectioned at 5-7 mm intervals, stained with haematoxylin-eosin (H&E), and examined under a light microscope (Nikon Eclipse Ni-U). At 40X

Table 1 Growth performances of Malaysian mahseer juveniles fish fed with two closely related strains of intestinal autochthonous probiotics

	Experimental diets		
	Control (CON0)	T1 (*E. faecalis* strain 2674)	T2 (*E. faecalis* strain 11682)
Initial weight	2.54±0.01[a]	2.54±0.01[a]	2.54±0.01[a]
Final weight	4.66±0.02[c]	5.01±0.01[b]	5.64±0.07[a]
Weight gain	2.12±0.01[c]	2.470±0.01[b]	3.100±0.06[a]
Weight gain%	83.08±0.23[c]	97.33±0.47[b]	122.21±5.68[a]
SGR%	1.01±0.01[c]	1.13±0.01[b]	1.33±0.19[a]
Survival %	97.33±0.66[a]	98.00±0.81[a]	100.00±0.00[a]
FCR	2.87±0.03[a]	2.08±0.03[b]	1.87±0.33[c]

Values=Mean ± Standard error of triplicate measurements (n-3). Different superscript letter for each parameter in the same row expressed significant differences at P ≤0.05. ANOVA was followed by Tukey test.

Table 2 Proximate composition (%) of whole body of Malaysian mahseer juveniles fed diets with Enterococcus faecalis (strain 2674 and strain FC11682), and control diets for 70 days.

Parameters	Experimental diets with two probiotic strains and control			
	Initial	Control (Con0)	T1 (*E. faecalis* strain 2674)	T2 (*E. faecalis* strain 11682)
Moisture	74.16±1.44	71.37±5.09[a]	71.13±1.30[a]	70.98±1.03[a]
Crude protein	11.61±1.25	11.07±1.53[a]	12.23±0.73[a]	12.76±1.30[a]
Crude lipid	7.10±0.50	8.60±0.41[a]	9.52±0.17[a]	9.26±0.12[a]
Ash	3.83±0.51	3.65±0.06[a]	3.44±0.40[a]	3.64±0.29[a]

Values=Mean ± Standard error of triplicate measurements (n-3). Different superscript letter for each within the same row expressed significant differences (P <0.05). ANOVA was followed by Tukey test.

magnification, the number of intestinal villi, and average villus height, villus width, villus area, crypt depth, and intestinal wall thickness were measured randomly, observing 5 to 10 villi per slide (minimum 5 to maximum 10 villi), using a computer-operated image picture analysis system. Where higher than 10 villi could be studied, the villi were selected to be spread evenly throughout the intestinal samples.

Statistical analysis

In all trials, triplicate samples were utilized. Before the analysis, the assumptions of normal distribution and variance homogeneity were double-checked. SPSS (version 20) was used to perform one-way analysis of variance (ANOVA) on data from each treatment. When significant differences (P <0.05) were identified, data from each treatment was subjected to a one-way analysis of variance (ANOVA) using SPSS (version 20; IBM, Chicago, IL, USA), followed by a Tukey multiple range test to examine differences between treatments.

Results

Growth parameters

Malaysian mahseer juveniles fed with the two autochthonous strains of probiotic, *E. faecalis* strain 2674 (T1) and *E. faecalis* strain FC11682 (T2) over 70 days feeding experiment showed significantly higher (P <0.05) final mean body weight (g), weight gain (g), weight gain percent (%), specific growth rate (SGR%), and FCR compared to control fish. Statistical results showed that the best growth performance was observed for the fish in for T2 (Table 1), the second highest growth performance was observed for the fish in T1, while the lowest values were recorded for the control group. However, survival rate of mahseer were not influenced by any of the strain and control diets.

Biochemical composition: proximate and fatty acid analysis of whole-body

Proximate analysis is shown in Table 2 and whole-body fatty acid composition of Malaysian mahseer

Table 3 Fatty acid composition (%) of whole body of Malaysian mahseer juveniles fed diets with two strains of *E. faecalis* (strain 2674 and strain FC11682), and control diets for 70 days.

Fatty acid	Experimental diets with two probiotic strains, and control			
	Initial	Control (CON0)	T1 (*E. faecalis strain* 2674)	T2 (*E. faecalis* strain 11682)
Total SFA	37.03±1.12	38.10±1.08^{a}	35.27±0.54^{b}	31.72±0.58^{c}
Total MUFA	20.03±1.02	21.22±0.86^{c}	28.94±0.55^{b}	32.44±0.46^{a}
Total n3	25.45±0.50	24.91±0.57^{a}	19.87±0.18^{c}	20.31±0.20^{b}
Total n6	17.47±0.78	14.75±0.09^{a}	15.90±0.12^{a}	15.51±0.81^{a}
Total PUFA	42.93±0.31	39.67±0.48^{b}	35.78±0.07^{b}	35.83±0.65 ^{b}
n3	25.45±0.50	24.91±0.57^{a}	19.87±0.18^{b}	20.31±0.20^{b}
n6	17.47±0.78	14.75±0.09^{a}	15.90±0.12^{a}	15.51±0.81^{a}
PUFA/SFA	1.16±0.04	1.01±0.03^{a}	1.01±0.03^{a}	1.13±0.03^{a}
n3/n6	1.46±0.09	1.68±0.05 ^{a}	1.25±0.02 ^{a}	1.31±0.08 ^{a}
DHA/EPA	3.41±0.40	3.06±0.07^{a}	1.81±0.09^{b}	1.78±0.03^{b}
C18:3n-3	2.92±0.49	2.33±0.27^{b}	4.66±0.31^{a}	4.44±0.22 ^{a}
C18:2n-6	7.77±0.75	6.51±0.71^{c}	9.38±0.08^{a}	8.95±0.07^{b}

Values=Mean ± Standard error of triplicate measurements (n-3). Different superscript letter for each within the same row expressed significant differences (P <0.05). ANOVA was followed by Tukey test.

Table 4 Gut histo-morphology of Malaysian mahseer juveniles fish fed *E. faecalis* 2674, FC11682, and control diets for 70 days

Parameters	Control (CON0)	T1 (*E. faecalis* strain 2674)	T2 (*E. faecalis* strain 11682)
Villus height (VL)	82.96±0.47^{c}	95.68±0.46^{b}	105.49±0.84^{a}
Villus width (VW)	46.43±0.66^{c}	59.41±1.54^{b}	65.32±1.67^{a}
Villus area (VA)	3516.03±58.19^{c}	4260.16±81.56^{b}	5557.84±152.13^{a}
Intestinal wall thickness (IWT)	22.25±1.12^{a}	26.24±1.29^{a}	26.18±1.16^{a}
Crypt depth (CD)	16.93±1.24^{a}	19.22±2.81^{a}	17.09±1.02^{a}

Values=Mean ± Standard error of triplicate measurements (n-3). Different superscript letter for each parameter in the same row expressed significant differences at P ≤0.05. ANOVA was done followed by Tukey test.

juveniles fed probiotics diets, and control diet, for 70 days are shown in Table 3. The highest (%) saturated fatty acids (SFA) (38.10±1.08%) were found in control fish, followed by T1, and the lowest SFA value was recorded for T2 fish fed diets with *E. faecalis* strain FC11682. The total MUFA was recorded significantly higher (P <0.05) for treatments groups, with the highest value recorded for T2 group. Conversely, the total PUFA was higher (P <0.05) in control group compared to probiotic groups. Higher amount of alpha linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6) were found in both probiotic treatment groups (T1 and T2) compared to control. DHA/EPA ratio was the highest in the control (Table 3).

Gut histomorphology

The evaluation of intestinal morphometric measurements of Malaysian mahseer juveniles at the end of a 70 days feeding trail with host associated and commercial probiotic are displayed in Table 4, and microscopic photographs are shown in Fig. 1. The parameters of gut morphology such as, crypt width, and breadth of intestinal wall were not significantly different within autochthonous probiotic strains. Three major intestinal morphological features, height, width and area of the villi area were significantly (P <0.05) increased by the effect of different strains compared to control. The highest villus height and width were found in T2 fed diets with *E. faecalis* FC11682, followed by T1 fed diets with strain 2674, and the lowest was recorded for control.

Challenge study: protection against pathogenic infection

Fig. 2 shows the cumulative mortality percentage of Malaysian mahseer juveniles challenged with *A. hydrpohila* with the LD_{50} (Lethal dose) after 70 days feeding with diets supplemented with two probiotic strains *E. faecalis,* 2674 and *E. faecalis* FC11682. The cumulative mortality recorded was significantly (P <0.05) higher in treatments compared to control, with mortalities of 100% in control, 40% in T1, and 30% in T2. The RPS (%) was found the highest in (T2) group (70%), followed by T1 group (60%), and the

a) T1 b) T2 c) C

Fig. 1 a)-c). Light microscopic measurements of gut morphology of *Tor tambroides* juvenile fed diets supplemented with two strains of *E. faecalis* for 70 days, 40X magnification, scale bar 20µm i= villus height, ii=intestinal wall thickness, iii=villus width, iv=crypt depth, and v=villus area.

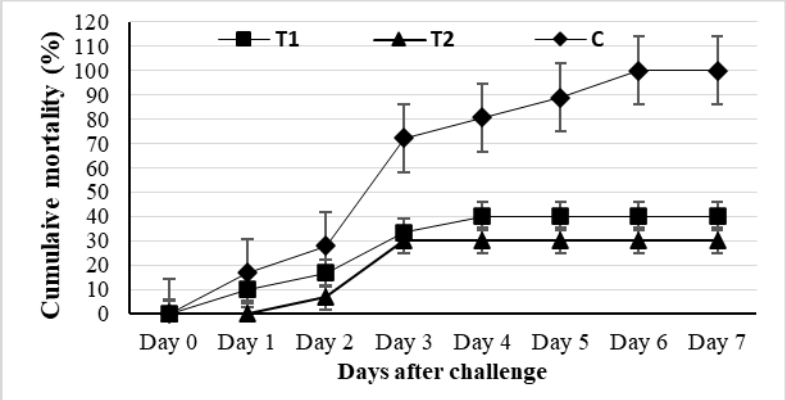

Fig. 2 Effect of dietary application of *Enterococcus faecalis* (strain 2674 and FC11682) on the cumulative mortality (%) of Malaysian mahseer juveniles after 7 days challenged against *A. hydrophila* (mortality=mean±SD).

lowest (0%) was in control group. There were no significant ($P < 0.05$) differences observed within the two probiotic strain treatments.

Discussion

The Genus *Enterococcus* is one of the major probiotics within *lactobacillus* group that are used in aquaculture (Van Doan et al. 2020). *E. faecalis* species have been screened from the gastrointestinal tract of freshwater carps, *Labeo rohita* and *Cirrhinus mrigala* (Shahid et al. 2017), *Channa argus* (Kong et al. 2021), and giant freshwater prawn, *Macrobrachium rosenbergii* (Azahar et al. 2018; Khushi et al. 2020). The other important member of this genus is *Enterococcus faecium,* which was isolated from the intestine of flat head grey mullet and common carp (Gopalakannan and Arul 2011). Another species, *E. hirae*, was evaluated on growth and immunity of hybrid catfish (*Clarias gariepinus X Clarias macrocephalus*) but was isolated from fermented vegetable waste, not isolated from fish origin (Hamid et al. 2021). An increase in fish growth by consuming probiotic enriched diet has been observed by numerous authors (Ghosh et al. 2008; Asaduzzaman et al. 2018a, b; Khushi et al. 2020; Silva et al. 2021). Similarly, results from this study showed that fish provided *E. faecalis* strain 2674 (T1), and FC11682 (T2) probiotics-supplemented diets improved growth performance in Malaysian mahseer (Table 1). In addition, other beneficial effects of probiotics *E. faecalis* on the Malaysian mahseer, such as biochemical composition and prevention of disease, have been evaluated. The effects of *Enterococcus* spp. have similarly been evaluated for other aquaculture fish species, including *Labeo rohita, Cirrhinus mrigala* (Shahid et al. 2017)*, Channa argus* (Kong et al. 2021) crayfish, and *Astacus leptodactylus* (Safari and Paolucci 2018), where probiotic bacteria such as *E. faecalis* were applied as dietary supplementation. Even though both bacteria tested in this study were from the same genus, *Enterococcus*, and had the same origin, their influence on growth performances was distinct after 70 days of feeding experiments. This growth may be due to the digestive enzyme activities

of dietary probiotics as reported by Allameh et al (2017), who found a significant increase in protease and lipase activity when the probiotic *E. faecalis* was applied to a culture of Javanese carp, *Puntius gonionotus,* (Bleeker 1850). This present study also evaluated the effect of two closely related bacterial strains of *E. faecalis* on whole body proximate analysis, fatty acid composition, gut morphology, and protection against pathogenic bacteria *A hydropila*. Despite higher growth performances by *E. faecalis* supplementation, there were not observed any effects on proximate contents within treatments (Table 2). This result is inconsistent with the earlier studies (García de La Banda et al. 2010; Rodriguez-Estrada et al. 2013; Asaduzzaman et al. 2018a).

Whole body fatty acid composition (Table 3) was significantly influenced by both probiotic strains diets as compared to control, where fish fed probiotics strain FC11682 (T2) was more influential than fish fed by *E. faecalis* strain (T1). In this connection total PUFA, total MUFA, linoleic acid (C18:3n-3), and linolenic acid (C18:2n-6) values were recorded significantly higher in T2, followed by T1, with the lowest value recorded for control group. Conversely, the total saturated fatty acids (SFA) were recorded significantly lower in the treatment groups compared to the control. This is consistent with previous research (García de La Banda et al. 2010) in which two closely related strains of *Shewanella* sp. were used as probiotics diets in Senegalese sole (*Solea senegalensis*). It was reported that MUFA values were significantly higher in fish fed probiotic diet supplement with higher amount (5% of total fatty acids) for linoleic acid (C18:2n-6), linolenic acid (C18:3n-3), total n6 PUFA in liver, and lower DHA level. Another study, Baesi et al. (2017) showed that when common carp (*Cyprinus carpio*) was fed commercial *lactobacillus*, the fatty acid profile of fish was affected, resulting in a substantial reduction in saturated fatty acids in fish muscle fed probiotic diets, and MUFA values that were greater than in the control. Diets containing probiotics had a greater n-3 fatty acid content and lower n-6 fatty acids compared to control. Some previous studies have explained that the function of SFAs is to increase the risk of cardiovascular disease, where the three main fatty acids, myristic acid, lauric acid, and palmitic acid, play this role (Mahan and Escott-Stump 2008). In this present study, fish fed with two closely related probiotic strains reduced the total SFAs compared to the control. It was concluded that this could improve the flesh quality of fish. Both probiotic strains could be used as feed additives to provide essential nutrients with outcome to improve the beneficial fatty acids in fish through production of enzymes and vitamins.

The thickness of the intestinal muscular layer, the height (VL), width (VW) and area (VA) of the villi, crypt depth (CD), and goblet cells can be used as biological indicators to determine health of intestine (Khojasteh 2012; Elsabagh et al. 2018). Given the importance of the gut as a digestive, absorptive, and defensive organ, prior research has looked at the influences of probiotics on the morphology of the intestine in fish (Daniels et al. 2010; Merrifield et al. 2010; Kristiansen et al. 2011; Asaduzzaman et al. 2018a). The intestinal absorption surface increases as VL and VW levels rise. Goblet cells, for example, produce mucus that has antibacterial characteristics, and speeds up transfer through the testicular epithelium, preventing introduction of pathogenic bacteria (Elsabagh et al. 2018). Probiotic *Alcaligens sp., Bacillus sp., Shewanella sp. and B. amyloliquefaciens* have influenced gut morphology and showed similar results (Reda and Selim 2015; Asaduzzaman et al. 2018a; Kuebutornye et al. 2020; Liu et al. 2020). In this regard, in our present study, fish fed with two closely related intestinal autochthonous probiotic strains of *E. faecalis* were investigated for gut morphology characteristics (Table 4). The findings showed that strain FC11682 performed better in terms of resultant gut morphological parameters than strain 2674 (Table 4). These two probiotic strains did not affect crypt depth or width of intestinal wall. Similar to our results, dietary application of autochthonous probiotics *(Alcaligens sp., Bacillus sp., and Shewanella sp.)* have increased gut morphology of Malaysian mahseer (*Tor tambroides*) (Asaduzzaman et al. 2018a), and single or conjoint application *E faecalis* has increased muscular thickness and villus width of *Channa argus* fish (Khushi et al. 2020). Although *Alcaligenes* sp. supplementation in the diet has been shown to be beneficial, and to have significantly improved villus height, villus width and villus area and no effects were observed on intestinal wall thickness and crypt depth. Nile tilapia *(Oreochromis niloticus)* fish fed with probiotic supplemented diet showed a significant greater villus length compared to control fish (Pirarat et al. 2008; Ramos et al. 2017). Merrifield et al. (2010) established that probiotics bacteria (*Pediococcus acidilactici*) increase enterocyte microvilli in the intestine of rainbow trout (*Onchorhyncus mykiss*).

Host-associated probiotics, which were previously part of the host's microbiome, were able to safeguard gut health and increase villus height. Some previous researches (Cerezuela et al. 2013; Ridha and Azad 2016)

have been demonstrated probiotics to aid not only in enhancing the host's development and immunology, but also in changing a disrupted host microbiome, and in modifying intestinal morphology for improved absorption. Bermudez-Brito et al. (2012) reported that probiotics combat infections in the stomach of fish in different ways, such as where probiotic bacteria compete with pathogens for the same nutrients, which causes pathogens to suffer from nutritional insufficiency through greater success in consumption of nutrients by probiotics (Sornsenee et al. 2021). Probiotics also reduce the surface area for pathogen colonization thus preventing adhesion to the intestinal wall, and also transmit signals to immune cells to create cytokines which promote the leukocytes to eliminate infections. Infections are attacked through the release of bacteriocins by probiotics. In this study, we showed that autochthonous probiotic boosted the resistance of Malaysian mahseer against *A. hydrophila*, displaying significantly higher survival rate (Fig. 2). The greater RPS (%) observed in probiotic fed mahseer fish might be caused by the host-associated probiotic stronger colonisation and persistence in the fish gut, resulting in increased competitiveness between pathogenic bacteria and probiotics for nutrients and space, thus affecting pathogen exclusion and subsequently increasing fish immunological response. Our results are supported by previous studies where the antibacterial activity of *E. faecalis* against common fish infections were investigated. Safari and Paolucci (2018) showed that *E. faecalis* in combination with synbiotics could improve survival of crayfish, *Astacus leptodactylus* against *A. hydrophila*. In another study, *E. faecalis* isolated from Indian major carps, *Labeo rohita* and *Cirrhinus mrigala* had potential antipathogenic activity against *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Escherichia coli* (Shahid et al. 2017). Furthermore, *E. faecalis* had inhibitory effect against pathogenic *Vibrio anguillarum* and *Photobacterium damsel*ae (Touraki et al. 2012), establishing that the intestinal autochthonous probiotics strains of *E. faecalis* (2674 and FC11682) can increase the protection of Malaysian mahseer juveniles against the common fish pathogens *A. hydrophila*.

Conclusion

In this present study, dietary administration of *Enterococcus faecalis* (strain 2674, and FC11682) demonstrated significantly higher growth indices, improved gut morphological parameters of Malaysian mahseer juveniles, and exhibited significantly ($P < 0.05$) better protection against *A. hydrophila* infection, including higher RPS (%) and lower cumulative mortality (%), in fish provided diets with potential probiotics at the concentrations of $1x10^8$ $CFUg^{-1}$. In proximate composition analysis, there were no significant ($P < 0.05$) variations observed within two strains, except that *Enterococcus faecalis* (strain FC11682) displayed significantly ($P < 0.05$) higher values for growth indices, for some important fatty acids (%) concentration, for improved gut morphology, and as well as for improved RPS (%) compared to strain 2674. The findings showed that dietary application of both strains may speed up growth and provide protection from *A. hydrophila* infection in juvenile Malaysian mahseer. However, the *Enterococcus faecalis* strain FC11682 performance was better compared to strain 2674. Further studies, however, are needed to evaluate the influences of the potential probiotics on various size group of Malaysian mahseer fish, with observation of some nonspecific immune parameters such as albumin, globulin, lysozyme, and myeloperoxidase activities.

Competing interests The authors declare that they have no competing interests.

Authors' contribution Shumpei Iehata and Ambok Bolong Abol-Munafi designed the study. Mohammod Kamruzzaman Hossain conducted experiments, data analysis, and wrote draft manuscript. All authors reviewed and approved the manuscript.

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