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Host gut-derived probiotic bacteria promote hypertrophic muscle progression and upregulate growth-related gene expression of slow-growing Malaysian Mahseer *Tor tambroides*

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ABSTRACT

In modern aquaculture, dietary supplementation of probiotics is a novel approach for enhancing growth performance of slow-growing fish. However, the actual role of probiotics in regulating muscle growth at cellular and molecular levels in fish still needs to be clarified. In this study, we hypothesized that host gut derived probiotic bacteria would enhance cellular muscle growth, and upregulate growth-related gene expression in slow-growing Malaysian mahseer *Tor tambroides*. Therefore, three host-associated probiotics (*Bacillus* sp. AHG22, *Alcaligenes* sp. AFG22, and *Shewanella* sp. AFG21) were isolated from the gastro-intestinal tract of *T. tambroides* and screened based on their digestive enzyme activity. A fishmeal and casein based control diet (40% crude protein and 10% lipid) was formulated, and three different probiotic supplemented diets were prepared by immersing the control diet in each isolated host-derived bacteria, suspended in sterile phosphate buffered saline (PBS), to achieve a final concentration of approximately 1.0×10^8 CFU g^{-1} feed. Triplicate groups of *T. tambroides* juveniles (initial weight 1.39 ± 0.06 g) were stocked in twelve glass aquaria (100 L capacity) with stocking density of 20 individuals per aquarium. The feed was applied twice daily at 3.0% of the fish body weight per day for 90 days. Growth performance (weight gain and specific growth rate) of *T. tambroides* juveniles were significantly higher in *Alcaligenes* sp. AFG22 and *Bacillus* sp. AHG22 supplemented diet treatments. Muscle morphometric analysis revealed that dietary supplementation of host-associated probiotic bacteria did not influence the frequency distribution of hyperplastic (class 10) small diameter fibers (≤10 μm). However, hypertrophic (Class 50, Class 60 and Class 70) large diameter fibers (> 50 μm) were significantly higher in *Alcaligenes* sp. AFG22 and *Bacillus* sp. AHG22 supplemented treatments, indicating that increased growth rate of *T. tambroides* in these treatments was mostly governed by increased muscle fibers hypertrophy, rather than by hyperplasia. Real-time PCR data demonstrated that the relative mRNA expression of *GH* and *IGF1* was upregulated in juvenile *T. tambroides* fed the diets supplemented with *Alcaligenes* sp. AFG22 and *Bacillus* sp. AHG22. These results indicate that host-derived probiotics, especially *Alcaligenes* sp. AFG22 and *Bacillus* sp. AHG22, have a significant potential as autochthonous probiotics for the stimulation of growth performance of slow-growing *T. tambroides* in aquaculture.

1. Introduction

Malaysian mahseer (*Tor tambroides* Bleeker), a species of an important group of freshwater cyprinids collectively known as mahseers (*Tor* spp.), inhabits fast flowing mountain rivers throughout the Southeast Asian and *trans*-Himalayan regions (Ambak et al., 2007). This species, locally known as *kelah* or *empurau*, is an important and highly valued game, food and ornamental fish (Ng, 2004). Due to the drastic decline in natural populations of *T. tambroides*, it has become one of the most expensive freshwater food fish in Malaysia. Recently, there has been an increasing awareness and interest among the relevant authorities of Malaysia for its artificial propagation for aquaculture production and conservation purposes. The successful hatchery production of *T. tambroides* has created an opportunity for the mass-production of

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larvae for commercial aquaculture (Ingram et al., 2007). In contrast to other freshwater cyprinids, which grow faster to a large final size, the growth rate of *T. tambroides* is much slower, although they reach a similar final body size as other commonly cultured freshwater cyprinid species. It takes a year to reach 500–600 g in the wild (Ng, 2004) and 33 months to reach a mean weight of 800 g (range 380–1250 g) in pond culture (Ingram et al., 2007). Therefore, in recent years a number of studies were conducted on feed development to improve growth performance of *T. tambroides* larvae and juveniles (Ng et al., 2008; Ng and Andin, 2011; Ismail et al., 2012; Kamarudin et al., 2012; Ramezani-Fard et al., 2012a, 2012b; Asaduzzaman et al., 2016, 2017).

The central goal of finfish aquaculture is the production of myotomal muscle. Postnatal muscle growth in teleost fish involves the activation and proliferation of a set of quiescent myogenic precursor cells termed satellite cells, located at the periphery of the muscle fibers (Johnston et al., 2009). Their nuclei are absorbed by existing fibers as they expand in size (hypertrophy) or fuse together to form multinucleated myotubes and new fibers (hyperplasia). In fish, muscle grows continuously via hyperplastic and hypertrophic mechanisms throughout life. In a variety of aquaculture species, two distinct phases of hyperplasia have been observed, namely stratified hyperplasia involving formation of new muscle fibers restricted to the germinal zones only and mosaic hyperplasia involving formation of new muscle fibers throughout the myotome (Asaduzzaman et al., 2013). The majority of muscle fibers are risen by mosaic hyperplasia in most of the aquaculture species which show indeterminate growth and reach a large body size (Johnston, 1999; Asaduzzaman et al., 2013). Mosaic hyperplastic muscle growth is of great interest in commercial aquaculture because it contributes to the market size of the fish. In general, hypertrophy occurs when protein synthesis rate is greater than protein degradation rate, resulting in an increased muscle fiber size, which ultimately leads to an increase in myotomal muscle growth.

Hyperplastic and hypertrophic muscle growth in fish are controlled by several genetic factors such as growth hormone (*GH*), insulin-like growth factors (*IGFs*), and *myostatin* (*MSTN*) (Johnston et al., 2009). Signaling by these factors affects the balance between anabolic and catabolic processes within muscle fibers and growth occurs when protein synthesis outweighs degradation. *GH* stimulates muscle growth in fish through inducing proliferation of myogenic cells and muscle hyperplasia and/or hypertrophy. IGFs are peptides structurally related to insulin that include *IGF-1* and *IGF-2* (Duan et al., 2010). The main target tissue of these factors is skeletal muscle, although they can also affect other tissues (LeRoith et al., 2001). In muscle, *IGFs* directly stimulate muscle cell proliferation, differentiation, and hypertrophy and inhibit muscle atrophy. Therefore, all of these genes act as positive regulators for muscle growth that promote hyperplastic and/or hypertrophic muscular growth. *MSTN*, a member of the transforming growth factor-β superfamily, is expressed predominantly in skeletal muscle of fish and the downstream function is to prevent the progression of myogenic cells into the cell division cycle (Thomas et al., 2000). This suggests that *MSTN* produced by the myocytes is a negative regulator that inhibits differentiation, and thus limits hyperplastic muscle growth.

Dietary supplementation of probiotics, also known as 'bio-friendly agents', is a novel approach to increase the myotomal muscle growth and wellbeing of farmed aquatic animals (Andani et al., 2012; Talpur et al., 2012; Han et al., 2015). Until now, several bacterial candidates have been evaluated as probiotics to improve growth parameters, nutrition, immune response, and disease resistance in several cyprinids (Kumar et al., 2008; Bandyopadhyay and Mohapatra, 2009; Wang 2011; Giri et al., 2012; Das et al., 2013; Chi et al., 2014; Wu et al., 2015). At the same time, the search for new host-derived microorganisms that could be used as probiotics continues. In recent years, there has been an increasing attention for the use of host-derived microbiota as a source of probiotics (Lazado et al., 2015). This is because the physiological peculiarities and characteristics of every host and the significant influence of environmental factors make it difficult to develop a probiotic microorganism that has a universal application (Lazado et al., 2015). Therefore, in this study, three potential probiotics (*Bacillus* sp. AHG22, *Alcaligenes* sp. AFG22, and *Shewanella* sp. AFG21) from the gastrointestinal tract of adult *T. tambroides* were isolated and screened based on their digestive enzyme activity to evaluate their effects on growth performances of the same fish species. A number of studies previously isolated *Bacillus* spp. from the gastrointestinal tract of rainbow trout (Newaj-Fyzul et al., 2007), yellow fin bream *Acanthopagrus latus* (Ridha and Azad, 2012), groupers *Epinephelus coioides* (Sun et al., 2012) and Indian major carps (Nayak et al., 2007) for using them as host-derived probiotics. The genus *Shewanella* has also been identified as one of the most frequent cultivable bacterial group isolated from intestines of fish (Martín-Antonio et al., 2007; Tapia-Paniagua et al., 2014), and some strains of this genus have been evaluated as host-associated probiotic microorganisms for the culture of farmed fish (De la Banda et al., 2012; Guzmán-Villanueva et al., 2014; Jiang et al., 2013; Tapia-Paniagua et al., 2014). Unlike two other host-associated probiotics, there is paucity of information on the probiotic ability of *Alcaligenes* spp. in aquaculture, although it has been found in fish intestine (Austin, 2002; Al-Hisnawi et al., 2014). *Alcaligenes* sp. has been used for different purposes such as production of polyhydroxybutyrate (PHB) (Wang et al., 2013) or ammonium removal treatment from wastewater (Joo et al., 2006) and sewage sludge (Shoda and Ishikawa, 2014), and as a growth promoting substance in Okra plant (*Abelmoschus esculentus*) (Ray et al., 2016).

Although a number of studies reported the positive effect of different probiotics on the growth performance of various cyprinid fish species, very few studies have evaluated host-derived microorganisms as a potential probiotic for improving the growth performances of cyprinids. Despite the importance of improving growth and nutritional performances of *T. tambroides*, there are limited published reports about the effects of probiotics on growth performances of this species. In addition, the underlying cellular and molecular mechanisms of enhanced growth under the influence of probiotics is poorly documented in fish. In this study, we hypothesized that dietary administration of host-associated bacteria would enhance growth performance of slow-growing *T. tambroides* by promoting cellular muscle growth and upregulating growth related gene expression. Growth performance of *T. tambroides* is evaluated at the cellular level by morphometric analysis of muscle fibers, and at the molecular level by analyzing relative mRNA expression of major growth-related gene.

2. Materials and methods

2.1. Fish and husbandry conditions

About 300 *T. tambroides* juveniles were collected from the Mahseer Hatchery, School of Fisheries and Aquaculture Sciences, University Malaysia Terengganu, Malaysia (UMT), and acclimated to the laboratory conditions for one week. During this period, *T. tambroides* larvae were fed with a commercial feed containing 40% crude protein (Cargill Feed Sdn. Bhd, Malaysia). From the 300 fish, 240 *T. tambroides* juveniles (initial body mass: 1.39 ± 0.05 g) were selected and evenly distributed into 12 rectangular shape glass aquaria (100 L capacity) with stocking density at 20 fish per aquarium based on our previous experience of aquarium and laboratory-based culture of this fish. Each aquarium was maintained individually in a closed system to avoid cross-contamination among different probiotics at temperatures of 26 ± 0.5 °C. Continuous aeration was provided in each tank through an air stone, which was connected to a central air compressor. Left over feed and faeces were siphoned out as required. The tanks were cleaned weekly to reduce the risk of bacterial growth and ammonia saturation.