



Effects of host gut-derived probiotic bacteria on gut morphology, microbiota composition and volatile short chain fatty acids production of Malaysian Mahseer *Tor tambroides*



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ABSTRACT

Three host-associated probiotics (*Bacillus* sp. AHG22, *Alcaligenes* sp. AFG22, and *Shewanella* sp. AFG21) were isolated from the gastrointestinal tract of *Tor tambroides*, and their effects were evaluated on gut morphology, microbiota composition and volatile short chain fatty acids (VSCFAs) production of the same species. A 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 host-derived isolated probiotic, suspended in sterile phosphate buffered saline (PBS), to achieve concentration at 1.0×10^8 CFU g⁻¹ feed. Triplicate groups of *T. tambroides* juveniles (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 body weight per day for 90 days. The intake of probiotics drastically modified the gut microbiota composition. The average number of OTUs, Shannon index and Margalef species richness were significantly higher in host-associated probiotic treatments compared to the control. A significant increase of lipolytic, proteolytic and cellulolytic bacterial number were observed in the gastrointestinal tracts of *T. tambroides* fed the diets supplemented with *Alcaligenes* sp. AFG22 compared to the control. Villus length, villus width and villus area were significantly higher in *T. tambroides* juveniles fed the diet supplemented with *Alcaligenes* sp. AFG22. Acetate and butyrate were detected as main VSCFA production in the gastrointestinal tract of *T. tambroides*. Acetate and total VSCFAs production in *Alcaligenes* sp. AFG22 supplemented treatment was significantly higher than control. These results indicate that host-derived probiotics, especially *Alcaligenes* sp. has a significant potential as an important probiotic to enhance the nutrients utilization and metabolism through increasing gut surface area and VSCFAs production, and adjusting gut microbiota balance of *T. tambroides* juveniles.

1. Introduction

Manipulation of intestinal microflora through the dietary supplementation of probiotics, also known as 'bio-friendly agents', is a novel approach to improve the gut health, growth performances and well-being of farmed aquatic animals (Andani et al., 2012; Talpur et al., 2012; Han et al., 2015). In the last decade, there are increasing trends to utilize probiotics in aquaculture due to their increasing demand and substitution of chemotherapeutics (Andani et al., 2012; Talpur et al., 2012; Han et al., 2015, Dawood and Koshio, 2016). However, selection

of probiotics is very critical because inappropriate microorganisms causes an imbalance in the ratio of good-to-bad bacteria that negatively influence metabolism of nutrients, contribution of the colonization resistance, antagonistic activity against pathogens, immunomodulation and etc. (reviewed in Lazado et al., 2015). An ideal probiotic, irrespective of its source should able to colonize, establish and multiply in the host gut. Most of the commercial probiotics used for terrestrial animals are now being used in aquaculture practices. However, sometimes commercially available probiotics are relatively ineffective because they cannot survive and/or remain viable at optimum

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concentration in fish gut due to their non-fish origin (reviewed in Lazado et al., 2015). Until now, several bacterial candidates such as lactic acid bacteria, bacilli and pseudomonads, and yeast (*Saccharomyces cerevisiae*) have been evaluated as probiotics in aquaculture to improve digestive enzyme activity (Suzer et al., 2008), and manipulation of gut microbial community (Tapia-Paniagua et al., 2010; Lobo et al., 2014) of several fish species. At the same time, search for new microorganisms that could be used as probiotics is continuously being undertaken (Lazado et al., 2015). This is because a particular probiotic candidate may be effective in one species of fish, but may be ineffective in another species. A single probiotic candidate may never could be used in all host species, because the physiological and physico-chemical conditions of the host and/or environment could influence these properties, which eventually affect the efficiency of a particular probiotics (Lazado et al., 2015).

In recent years, there is an increasing consideration to the use of the host-associated microbiota as an alternative source of probiotics to the aquaculture industry that has been too inclined to the use of terrestrial probiotics (Lazado et al., 2015). Host-derived probiotics could be best approach for better efficacy in host. There is a general consensus that probiotics from autochthonous source have a greater chance of competing with resident microbes and of becoming predominant within a short period of intake and to persist in the colonic environment for some time after the withdrawal of probiotics (reviewed in Lazado et al., 2015). The mucosal surfaces of gastrointestinal tract of aquatic animal harbor microorganisms of varied diversity and function, and can be used to isolate candidate microorganisms for probiotic development (Balcázar et al., 2007; Newaj-Fyzul et al., 2007; Caipang et al., 2010; Lazado et al., 2010; Maeda et al., 2014). Indeed, several studies have provided that candidate probiotics are naturally thriving as commensals of fish mucosal surfaces such as in Atlantic salmon (*Salmo salar*) (Jöborn et al., 1999), rainbow trout (*Oncorhynchus mykiss*) (Spanggaard et al., 2000), Atlantic cod (Fjellheim et al., 2007; Dhanasiri et al., 2011), channel catfish (*Ictalurus punctatus*) (Larsen et al., 2014), and many others.

The dietary administration of probiotics can exert some effect on the intestinal microbiota, digestive enzyme activities, morphology and volatile short chain fatty acids (VSCFAs) production in various aquatic animal species (Liu et al., 2009; Zhou et al., 2009; Daniels et al., 2010; Merrifield et al., 2010a; Cerezuela et al., 2012; Dagá et al., 2013; Allameh et al., 2017). Different authors have reported that the intake of probiotics modified intestinal microbiota composition (Gómez and Balcázar, 2008; Merrifield et al., 2010b). These gastro-intestinal bacterial flora of fishes in general, represents a very important and diversified enzymatic potential with capable of producing proteolytic, amylolytic, cellulolytic, lipolytic, and chitinolytic enzymes, which is important for digestion of proteins, carbohydrates, cellulose, lipids and chitin (Bairagi et al., 2002; Gutowska et al., 2004). Besides the diversified enzymatic potential, previous studies have reported that dietary supplementation of probiotics modulate the activity of the intestinal microbiota, which in turn influences the production of VSCFAs such as acetate, propionate and butyrate in the fish intestine (Smith et al., 1996; Kihara and Sakata, 1997; Burr et al., 2005; Allameh et al., 2017), provide energy for epithelial cells and modulate gut microbiota (Tropping and Clifton, 2001; Liu et al., 2014). VSCFAs also have an important influence in altering gut integrity, and are related to various health conditions such as phagocytic, anti-inflammatory and anti-microbial effects in fish and mammals (Tropping and Clifton, 2001; Liu et al., 2014; Allameh et al., 2017). Thus, the microbial modulation and improved morphology of the gastrointestinal tract exerted by host-associated probiotics may help to enhance the nutrients absorption and metabolism, and disease resistance of the host (Merrifield et al., 2010a).

Malaysian mahseer (*Tor tambroides* Bleeker) is a potential species for aquaculture because they are the highest valuable freshwater cyprinid and having a great demand as game and ornamental fish in Malaysia (Esa et al., 2008). There is a great interest in *T. tambroides* aquaculture

production in Malaysia both for consumer demand and conservation purposes. It has been reported that probiotics positively influence gut microbiota, gut histo-morphology, immune response, and disease resistance in several cyprinids such as common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), rohu (*Labeo rohita*), and catla (*Catla catla*) (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). Unlike other cyprinids, to the best of our knowledge, there are no published report about the effects of any probiotics on gut microbiota composition, gut histo-morphology and VSCFAs production of *T. tambroides*; although it is more important to improve the nutrition of this species due to their slow growth. In this study, we hypothesized that dietary administration of host-associated bacteria would enhance gut environment of *T. tambroides*. Therefore, we isolated three potential probiotics (*Bacillus* sp. AHG22, *Alcaligenes* sp. AFG22, and *Shewanella* sp. AFG21) from the gastrointestinal tract of adult *T. tambroides* and evaluated their effects on the gut environment of the same fish species. Gut environment of *T. tambroides* is assessed in terms of gut microbiota profile using denature gradient gel electrophoresis (DGGE), gut bacterial nutritional enzyme activity, volatile short chain fatty acid (SCFA), and histological analysis of gut morphology.

2. Materials and methods

2.1. Fish and husbandry conditions

About three hundred *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 1 week. During this period, *T. tambroides* larvae were fed with a commercial feed containing 40% crude protein (Cargill Feed Sdn. Bhd, Malaysia). From the three hundred fish, two hundred forty *T. tambroides* juvenile (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. Each aquarium was maintained individually in 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.

2.2. Isolation and screening criteria of candidate probiotic strains

Probiotic bacteria candidates were isolated from the gastrointestinal tract of adult *T. tambroides* by plating method onto Tryptone Soya agar (TSB; Himedia, India) and thereafter incubated for 24–48 h at 25 °C. These isolates were spotted on starch agar (SA) for amylolytic activity, Carboxymethyl cellulose agar (CMCA) with 1% congo red for cellulolytic activity, TSA medium containing 1% Tween 80 and 0.001% CaCl₂ 2H₂O for lipolytic activity, and TSA medium containing 1% skim milk for proteolytic activity, and thereafter incubated at 25 °C for 72–90 h. After incubation, colonies with wide clear zone were selected as candidate probiotic strain. Then, the isolates were identified by sequencing of 16S rRNA gene using universal primer set; 8F (5'-AGAGTTTGATCA TGGCTC-3') and 1492R (5'-GGCTACCTTGTACGACTT-3') (Ishino et al., 2012). After isolation and screening, three different probiotics (*Bacillus* sp. AHG22, *Alcaligenes* sp. AFG22, and *Shewanella* sp. AFG21) were selected according to their *in vitro* digestive enzyme activity. *Bacillus* sp. AHG22 (isolated from hindgut region) showed only proteolytic activity, *Shewanella* sp. AFG21 (isolated from foregut region) showed both lipolytic and proteolytic activity and *Alcaligenes* sp. AFG22 (isolated from foregut region) showed amylolytic, lipolytic and proteolytic activity (Table 1).

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