Aquaculture Reports 17 (2020) 100378

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Isolation and screening of lactic acid bacteria associated with the gastrointestinal tracts of abalone at various life stages for probiotic candidates

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ARTICLE INFO

Keywords:
Abalone
Antimicrobial compounds
Gastrointestinal tract
Lactic acid bacteria

ABSTRACT

Members of lactic acid bacteria (LAB) have been well-known for probiotic agents owing to their ability to produce diverse beneficial compounds for cultured species, as well as for having a status of generally recognized as safe microorganisms. Considering these facts, the current study investigated the presence of cultivable LAB associated with the gastrointestinal tracts (GIT) of hybrid abalone (*Haliotis laevigata* x *H. rubra* Brown 1992) at various life stages (larvae, juvenile, and adult), and screened them for their probiotic properties. GITs of 90 hybrid abalone (30 larvae, 30 juveniles, and 30 adult stages) were dissected out aseptically and then inoculated into de Man Rogosa Sharp (MRS) broth and agar. The result revealed that no LAB was isolated from the GITs of abalone at the larval stage. LAB were firstly recorded at the juvenile stage (44 isolates) and the adult stage (49 isolates). Based on partial sequences of the 16S rRNA gene, the 44 LAB in the juvenile stage were identified as five genera and belonged to 10 ribotypes. While the 49 LAB isolates from the adult stage were identified as three genera and belonged to eight ribotypes. Of the 93 LAB, five strains which were *Enterococcus faecium* MA002, *Enterococcus lactis* MA056, *Leuconostoc mesenteroides* MA064, *Enterococcus lactis* MA068, and *Enterococcus lactis* MA084, exhibited antagonistic activities against bacterial pathogens (*Vibrio alginolyticus* and *Listeria monocytogenes*). The five LAB strains did not show any hemolytic activity, possibly indicating that they were non-pathogen. These results confirmed that LAB colonized the intestinal tract of abalone at juvenile and adult stages and were capable of producing antimicrobial compounds. Acknowledging the presence of indigenous LAB in GITs of abalone, a prebiotic application can be considered for another strategy to modify the healthy microbiome of abalone.

1. Introduction

Aquatic organisms host diverse microorganisms in their gastrointestinal tracts, generally known as the microbiome of which have been frequently reported to strongly determine their host performances (Castañeda-Monsalve et al., 2019; Llewellyn et al., 2014). It is notable that the growth or disease resistance of the host organism becomes better when beneficial microbiomes dominate in the hosts' intestinal tract (Chauhan and Singh, 2019). The microbiome, which can improve performances of their hosts, are generally known as probiotics. Therefore, various strategies have been developed to stir microbial communities in the gastrointestinal tract of aquaculture species including fish, shrimp, and abalone. Currently, the most common method in aquaculture is by the administration of beneficial bacteria (probiotics) through feed (Amin et al., 2019) or rearing water or by the supplementation of materials that can support the growth of beneficial bacteria in the intestinal tract of hosts (prebiotics). Both approaches serve the main purposes of stirring or manipulating the intestinal tract with those beneficial bacteria in order to enhance the performances of cultured animals. In terms of probiotics, beneficial bacteria, a bacterial group of lactic acid bacteria (LAB), has elicited most interest due to their ability to release various beneficial compounds such as free fatty acids (FFA), digestive enzymes, and antimicrobial compounds (Reda et al., 2018). These developments have led many scientists to explore diverse members of LAB for probiotic purposes.

Many studies have reported that probiotics were bacteriostatic or bactericidal to various microbial pathogens through several mechanisms, including the releasing of antimicrobial compounds (Amin et al.,

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https://doi.org/10.1016/j.aqrep.2020.100378
Received 25 February 2020; Received in revised form 15 May 2020; Accepted 17 May 2020

2016), outcompeting for nutrients or adhesion sites in gut mucosa (Nikoskelainen et al., 2001), and through stimulating hosts' immune systems (Chiu et al., 2007; Panigrahi et al., 2005). With reference to antimicrobial compounds, probiotics have been documented to release variety compounds, such as bacteriocins (Lin et al., 2013; Verschuere et al., 2000), bacteriolytic enzymes, hydrogen peroxide (Verschuere et al., 2000) and various organic acids (Amin et al., 2016; Vazquez et al., 2005). Among the released antimicrobial compounds, bacteriocin has gained more interest because of its visceral characteristics, such as being generally recognised as safe (GRAS) substances, pH tolerance, heat tolerance, inactive and nontoxic to eukaryote cells, as well as showing a relatively broad antimicrobial spectrum towards pathogens (Hwanhlem et al., 2014). Due to these characteristics, LAB have been commonly used as a probiotic agent in many aquaculture species, including abalone. Several strains of LAB have been reported to synthesis essential free amino acids, short-chain fatty acids (SCFA), and water-soluble vitamins, which are essential for animal growth (LeBlanc et al., 2011; Masuda et al., 2012). Iehata et al. (2009) reported that supplementation of two LAB strains (*Lactobacillus* sp. strain a3 and *Enterococcus* sp. strain s6), could successfully inhibit the growth of three abalone pathogens, *Vibrio anguillarum*, *Vibrio harveyi* and *Virio carachaeriae* in *Haliotis midae*. In addition, *Lactobacillus* sp. strain a3, *Enterococcus* sp. strain s6, and *Pediococcus* sp. strains Ab1 were confirmed to produce alginate *lyase* and volatile short-chain fatty acids (VSCFAs), given a significant increase in growth of two abalone species; *Haliotis gigantae* and *Haliotis midae* (Iehata et al., 2009, 2010).

However, most of these LAB were isolated from terrestrial organisms such as horses or humans (Iehata et al., 2009). As a consequence, viability becomes a major challenge when these LAB were applied to aquatic species, which live under different environmental conditions (Iehata et al., 2010). In addition, Rawls et al. (2004) stated that the colonization of LAB in the intestinal tract involved specific microbe-host interactions, which made certain LAB very host-specific. Additionally, the intestinal microflora of aquatic species has been previously reported to be different among different life-cycle stages. These factors together determine the capacity of LAB to colonize the intestinal tract of aquatic species. Regarding these issues, LAB strains originated from aquatic animals with specific life-cycle stages might become better approach for selecting probiotic candidates.

Therefore, this research was aimed at isolation and identification of cultivable LAB associated with the gastrointestinal tract of hybrid abalone at three different life-cycle stages, as well as screening the indigenous LAB for antimicrobial activity against eight bacterial pathogens. These research findings were expected to give additional information about the presence of LAB in the gastrointestinal tracts of hybrid abalone, and how the LAB strains might develop at various life stages of hybrid abalone.

2. Material and method

2.1. Experimental animal

A total of 90 hybrid abalone (*H. laevigata* x *H. rubra*) at three different life stages (30 larvae, 30 juvenile, and 30 adults) were collected for samples from the Abalone Tasmania (ABTAS) farm, Clarence point, Tasmania, Australia. The 30 abalone larvae (3.94 ± 0.44 mm in length) were reared in a rectangular concrete tank and fed by grazing on a natural diet such as microalgae growing on the surface of the rearing tank. While other 30 abalone at the juvenile stage (17.51 ± 1.22 mm of length) reared in a circular concrete tank and fed with commercial pellets were taken randomly. The other 30 abalone at the adult stage (82.79 ± 3.60 mm) reared at a circular concrete tank and fed with commercial pellets were also collected for the later life stage samples. All abalone were transported alive in an icebox in 1 h to the Laboratory of Aquatic Microbiology, Launceston, University of Tasmania. The abalone were sacrificed by immersion in Aquatic Anaesthetic (AQUI-S®) solution at a concentration of 30 mg/L for 15 min. Afterward, the abalone were dissected out aseptically for gastrointestinal tract samplings and the intestines were placed individually in 1.5 mL sterile plastic tubes.

2.2. Isolation of LAB

The gastrointestinal tract (GIT) of abalone was prepared according to a protocol of Amin (2018) with a slight modification. In brief, the GIT was removed aseptically and homogenized in phosphate-buffered saline (10 mM PBS, pH 7.2) by a Stomacher. One ml of the homogenate was inoculated into De Man Rogosa and Sharpe (MRS) broth and incubated anaerobically for 6 h at room temperature (~22 °C). Afterward, the broth culture was serially diluted in 0.85 % natural saline solution (NSS). 100 µL of each dilution was spread onto the surface of triplicate agar plates containing Bromo-Creso purple (BCP) as an indicator and incubated aerobically for seven days. Thereafter, colonies with yellow zones were purified by subculturing them into MRS broth for 48 h at room temperature. Pure isolates were subjected to standard phenotypical characterizations. Only those isolates which were Gram-positive, catalase, and oxidase negative and able to perform glucose fermentation were considered as LAB, and proceed to genotypic identification

2.3. LAB amplification by PCRs

Representative LAB isolates were genotypically characterized by a PCR according to Amin (2010), with a slight modification. The pure bacterial isolates were subcultured in MRS agar individually, and a single separated colony was picked up with a loop and diluted into 50 µL sterile MillQ waters. A universal set of primers was used in the PCR reaction and resulted in ~900 bp product: 27 F (5′-AGA GTT TGA TCC TGC CTC AG-3′) and 907R (5′−CCG TCA ATT CCT TTG AGT TT-3′) (Lane, 1991). The following amplification conditions were used: a 7 min denaturing step at 94 °C, followed by 30 denaturation cycles (94 °C for 1 min), annealing (55 °C for 1 min), and extension (72 °C for 1 min), and a final extension (72 °C for 15 min). All of the amplification assays contained 2 µL of template DNA, 28 µL of master mix (0.67 µM dNTP, 0.67 µM Forward primer, 0.67 µM Reverse, 0.04 U/µl *Taq* polymerase, 4.0 µL of 5x buffer HF buffer, 2 µL $MgCl_2$, 17 µL of H_2O), to achieve a final volume of 30 µL. All of the PCR assays were carried out in a MyCycler Thermal Cycler (BioRad, California, USA). PCR products were analysed on 2.5 % horizontal agarose gels. Before sequencing, PCR products were purified using a PCR Purification Kit (QIAGEN) and sent for sequencing. The sequenced isolates were compared to the published sequences using the BLAST search algorithm (GenBank National Centre for Biotechnology Information, NCBI) to retrieve the closest-known alignment identities.

2.4. LAB identification

The 16S rRNA gene sequences of the representative LAB and the closest-known strains derived from GeneBank were aligned to construct a phylogenetic tree according to Amin et al. (2016). In brief, the LAB sequences were examined for sequence homology with the archived 16S rDNA sequences from the GenBank at https://blast.ncbi.nlm. Subsequently, multiple alignments of sequences were performed with the ClustalW2 program, followed by a phylogenetic construction using the neighbor-joining DNA distance using Geneious software version 5.3.6. Thereafter, a resultant topology tree was evaluated by bootstrap analysis of neighbor-joining data sets based on 100 bootstrap replicates to construct distance-based trees.

2.5. Screening for antimicrobial activity

Each LAB isolate was screened for antimicrobial activity against eight bacterial pathogens, according to Amin et al. (2016) using an agar

well diffusion assay with the following steps:

Preparation of cell-free supernatant (CFS): Each LAB isolate was subcultured into 10 mL MRS broth. The inoculated broth was then placed inside an anaerobic jar with an anaerobic sachet (AN0035, Oxoid) and incubated for 24 h at room temperature. Afterward, the bacterial cells were harvested by centrifugation at 13,000 x *g* for 10 min at 4 °C, and the supernatant was collected in a sterile tube. Afterward, pH of the supernatant was adjusted to 6.5–6.8 by adding 1 M sodium hydroxide (NaOH) to diminish the antimicrobial influence of organic acids. Thereafter, the supernatants were sterilized by filtering through a 0.22 μm Millex Syringe Filter (Millipore) and stored at 4° C until further use.

Preparation of indicator pathogens: eight bacterial pathogens were used as indicator pathogens, which were: Aeromonas hydrophila, Vibrio alginolyticus, Vibrio anguillarum, Vibrio harveyi, Vibrio parahaemolyticus, Vibrio proteolyticus, *Yersinia ruckeri* strain UTYR 001A, and *Listeria monocytogenes*. The bacterial pathogens were obtained from culture collections in the Aquatic Microbiology Laboratory of the IMAS Launceston, and Microbiology Laboratory, Human Life Science, University of Tasmania. Each bacterial pathogen was cultured in 10 mL glass tube containing 5 mL Muller Hinton (MH) broth and incubated aerobically at ~22 °C for 24 h. Bacterial cells harvested by centrifugation at 13,000g for 60 s were diluted in 0.85 % normal saline solution (NSS) to have cell concentration of ~ 1.0×10^6 CFU/mL.

Agar-well diffusion assay: This assay was conducted according to Amin et al. (2016). In brief, one mL aliquot of each bacterial pathogen (~ 1.0×10^6 CFU/mL) was cultured by spreading technique onto MH agar plates and air-dried for 5 min. Thereafter, wells (~6 mm diameter) were aseptically punctured using the base tip of a 200 μL sterilized pipette. Subsequently, 80 μL CFS of each LAB was added to the duplicate wells and the same amount of sterilized MRS as the control, followed by incubation aerobically ~22 °C. After 24 h incubation, formation of a holo zone which indicated the presence of antimicrobial activity was measured.

2.6. Hemolytic activity

The toxicity of the LAB was determined, according to Nayak and Mukherjee (2011). In brief, each bacterial colony was spotted on Columbia Blood agar plate supplemented with 5% defibrinated sheep blood and incubated at 37 °C aerobically for 48 h. The hemolytic activity was considered positive when there was a formation of clearance zones around the LAB colony

3. Results

3.1. LAB associated with the GIT of abalone

A total of 93 LAB ribotypes were isolated from the gastrointestinal tracts of the 90-hybrid abalone; 0 from 30 larvae, 44 isolates from 30 juvenile and 49 isolates from 30 adult stages. Phenotypically, all ribotypes were Gram-positive, lack of catalase and oxidase, and were able to ferment glucose without CO_2 production. Based on their partial sequences of 16S rRNA genes (~ 900 bp), these 44 LAB isolated from the GITs of the juvenile stage showed high similarity to 10 LAB ribotypes, which belonged to five genera, Table 1. While the other 49 LAB associated with GITs of adult abalone were identified as eight ribotypes, which belonged to 3 genera, Table 2. The present study confirmed that LAB were also part of healthy microbiota in GITs of hybrid abalone.

3.2. Diversity of LAB isolated from abalone GITs in different life stages

LAB which were isolated from the GITs of hybrid abalone seemed to be quite diverse in terms of genus and ribotypes. A total of 15 LAB ribotypes were isolated, including *Carnobacterium divergens, Eenterococcus casselliflavus, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Enterococcus lactis, Enterococcus malodoratus, Enterococcus mundtii, Lactobacillus curvatus, Lactobacillus sakei, Lactococcus garviae, Leuconostoc mesenteroides, Pediococcus pentosaceus, Pediococcus lolii,* and *Pediococcus acidilactici*.

Furthermore, the present study also indicated that hybrid abalone at the juvenile stage hosted more LAB ribotypes compared to the adult stage; 10 ribotypes from juvenile and eight ribotypes from the adult, respectively (Table 3). In terms of genera, *Enterococcus* appeared to be the most dominant group of LAB in GITs of both juveniles and adults.

The LAB composition between the two life stages appeared to

Table 1
LAB associated with the gastrointestinal tract of abalone at the juvenile stage.

No	Strains	Species Identity	Cell shape	DNA similarity (%)
1	MA089	*Carnobacterium divergens*	rod	99.20
2	MA010	*Enterococcus caselliflavus*	rod	97.88
3	MA006	*Enterococcus caselliflavus*	rod	97.28
4	MA001	*Enterococcus durans*	coccus	99.89
5	MA036	*Enterococcus durans*	coccus/rod	99.66
6	MA038	*Enterococcus durans*	coccus/rod	99.12
7	MA041	*Enterococcus durans*	coccus/rod	99.54
8	MA050	*Enterococcus durans*	coccus	99.45
9	MA053	*Enterococcus durans*	coccus/rod	98.76
10	MA004	*Enterococcus faecium*	coccus	99.66
11	MA002	*Enterococcus faecium*	coccus/rod	98.97
12	MA003	*Enterococcus faecium*	coccus	98.79
13	MA005	*Enterococcus faecium*	coccus	96.60
14	MA007	*Enterococcus faecium*	coccus/rod	97.27
15	MA008	*Enterococcus faecium*	coccus/rod	97.91
16	MA037	*Enterococcus faecium*	coccus	99.66
17	MA055	*Enterococcus lactis*	coccus/rod	95.57
18	MA056	*Enterococcus lactis*	coccus	98.87
19	MA087	*Lactococcus garviae*	coccus	99.77
20	MA032	*Lactobacillus curvatus*	rod	98.10
21	MA034	*Lactobacillus curvatus*	rod	98.16
22	MA035	*Pediococcus lolii*	coccus	97.60
23	MA199	*Pediococcus acidilactici*	coccus	99.01
24	MA043	*Pediococcus pentosaceus*	coccus	99.23
25	MA044	*Pediococcus pentosaceus*	coccus	99.44
26	MA048	*Pediococcus pentosaceus*	coccus	99.55
27	MA049	*Pediococcus pentosaceus*	coccus	99.55

Table 2
LAB associated with the gastrointestinal tract of abalone at the adult stage.

No	Strains	Species Identity	Cell shape	DNA similarity (%)
1	MA061	*Enterococcus faecalis*	rod/coccus	96.51
2	MA065	*Enterococcus faecalis*	rod/coccus	98.76
3	MA198	*Enterococcus faecalis*	coccus	99.10
4	MA071	*Enterococcus faecalis*	coccus	98.28
5	MA057	*Enterococcus faecium*	coccus	99.66
6	MA069	*Enterococcus faecium*	coccus	98.01
7	MA068	*Enterococcus lactis*	coccus	98.41
8	MA084	*Enterococcus lactis*	coccus	99.00
9	MA015	*Enterococcus lactis*	coccus	98.97
10	MA060	*Enterococcus lactis*	coccus	99.21
11	MA074	*Enterococcus malodoratus*	coccus/rod	99.43
12	MA075	*Enterococcus malodoratus*	coccus	99.77
13	MA022	*Enterococcus mundtii*	coccus/rod	98.15
14	MA028	*Enterococcus mundtii*	coccus	97.89
15	MA030	*Enterococcus mundtii*	coccus/rod	97.90
16	MA031	*Enterococcus mundtii*	coccus/rod	98.26
17	MA066	*Lactobacillus sakei*	rod/coccus	99.89
18	MA058	*Lactobacillus sakei*	rod	99.33
19	MA059	*Lactobacillus sakei*	Rod	98.65
20	MA077	*Lactobacillus sakei*	rod	99.67
21	MA078	*Lactobacillus sakei*	rod	99.45
22	MA080	*Lactobacillus sakei*	rod	99.89
23	MA081	*Lactobacillus sakei*	rod	99.78
24	MA082	*Lactobacillus sakei*	rod	99.89
25	MA019	*Lactobacillus curvatus*	rod	98.60
26	MA070	*Leconostoc mesenteroides*	coccus	99.43

Table 3
LAB species associated with the GITs of abalone at juvenile and adult stages.

Larval abalone (3.94 ± 0.44 mm)	Juvenile abalone (17.51 ± 1.22 mm)	Adult abalone (82.79 ± 3.60 mm)
No LAB		

change as the abalone were growing. For instance, seven LAB ribotypes which were detected in GITs of the juvenile were not found in GITs of the adult stage, including *C. divergens, E. durans, E. caselliflavus, Lact. garviae, Pd. lolii, Pd. pentosaceus and Pd. acidilactici*. Meanwhile, 5 LAB ribotypes in the adult stage were not found in the juvenile. However, the result indicated that at least 3 LAB ribotypes were a presence at both life-cycle stages, which were *E. malodoratus, E. faecalis, E. mundtii, Leu. mesenteroides*, and *Lb. sakei*. While there were three LAB ribotypes which existed in GITs of both juvenile and adult stages which were *E. lactis, E. faecium* and *Lb. curvatus,* Table 3.

3.3. Phylogenetic analysis of cultivable LAB

Phylogenetic trees were constructed from sequences of 16S rRNA genes from representative isolates and the closest species obtained by the BLAST function from gene bank (Figs. 1 and 2) with Genious software version 5.3.6. The phylogenetic reconstruction was carried out to assign a systematic position for each generated DNA sequence.

3.4. Antimicrobial compound – producing bacteria

The pH values of the CFS extracted from the LAB-inoculated MRS broth in general decreased from 6.2 to 4–5 after 24 h incubation at room temperature, Table 4. Then, in order to nullify the antagonistic activity of lactic and acetic acids in the antimicrobial assay, pH of the CFS was neutralized by adjusting its pH values to 6.5–6.8 for further antimicrobial assay.

Five out of the 93 LAB ribotypes showed antimicrobial activity against one or more indicator pathogens using the agar well diffusion assay, indicated by a diameter of the clearance zone > 10 mm (Fig. 3 & Table 5). The antagonistic LAB belonged to *E. faecium* MA002, *E. lactis* MA056, *Leuc. mesenteroides* MA064, *E. lactis* MA068, and *E. lactis* MA084.

3.5. Hemolytic activity

All LAB ribotypes produce no holo zone on the blood agar, which may indicate that the LAB were producing no hemolytic activity or harmless to cultured animals.

4. Discussions

4.1. Viability of LAB throughout the life stages of abalone

LAB associated with GITs of aquaculture species have been extensively studied for probiotic purposes (Amin et al., 2016; Cui et al., 2015; Dash et al., 2016). Several studies have documented that the use of LAB enhanced the growth performances or disease resistance of several abalone species including *Haliotis gigantae* (Iehata et al., 2014), *Haliotis iris* (Hadi et al., 2014), and *Haliotis asinina* (Amin et al., 2019). However, even though LAB have been frequently applied in abalone, only a few authors have studied the presence of LAB in GITs of abalone. Consequently, the administered LAB species has faced several issues, including low viability and stability in the targeted organs (Amin et al., 2019; Iehata et al., 2009). Considering these issues, some offered solutions are indigenous probiotics (Fjellheim et al., 2010), or prebiotics which are non-digestible oligosaccharide to stimulate the growth and/or activity of a limited number of beneficial bacteria in the intestine (Kondepudi et al., 2012; Soukoulis et al., 2014; Wu et al., 2014). The indigenous probionts are presumed to be more adaptable to the ecological niche of GITs and may consequently have a higher chance of colonizing guts and conferring health benefits to their hosts (Amin et al., 2019), while prebiotics are considered simpler since they do not need to culture. However, before the prebiotic application, we need to

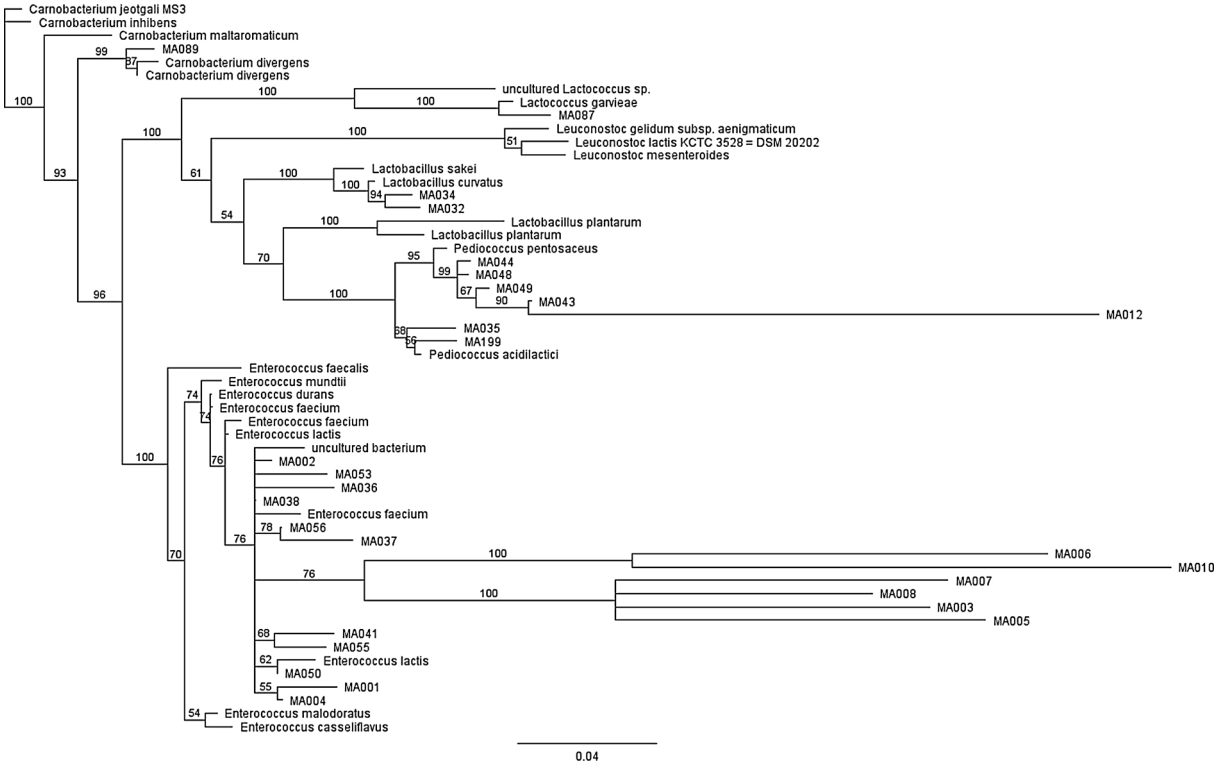

Fig. 1. Phylogenetic tree of LAB isolated from GITs of juvenile hybrid abalone. Sequences were alignment using ClustalW Alignment.

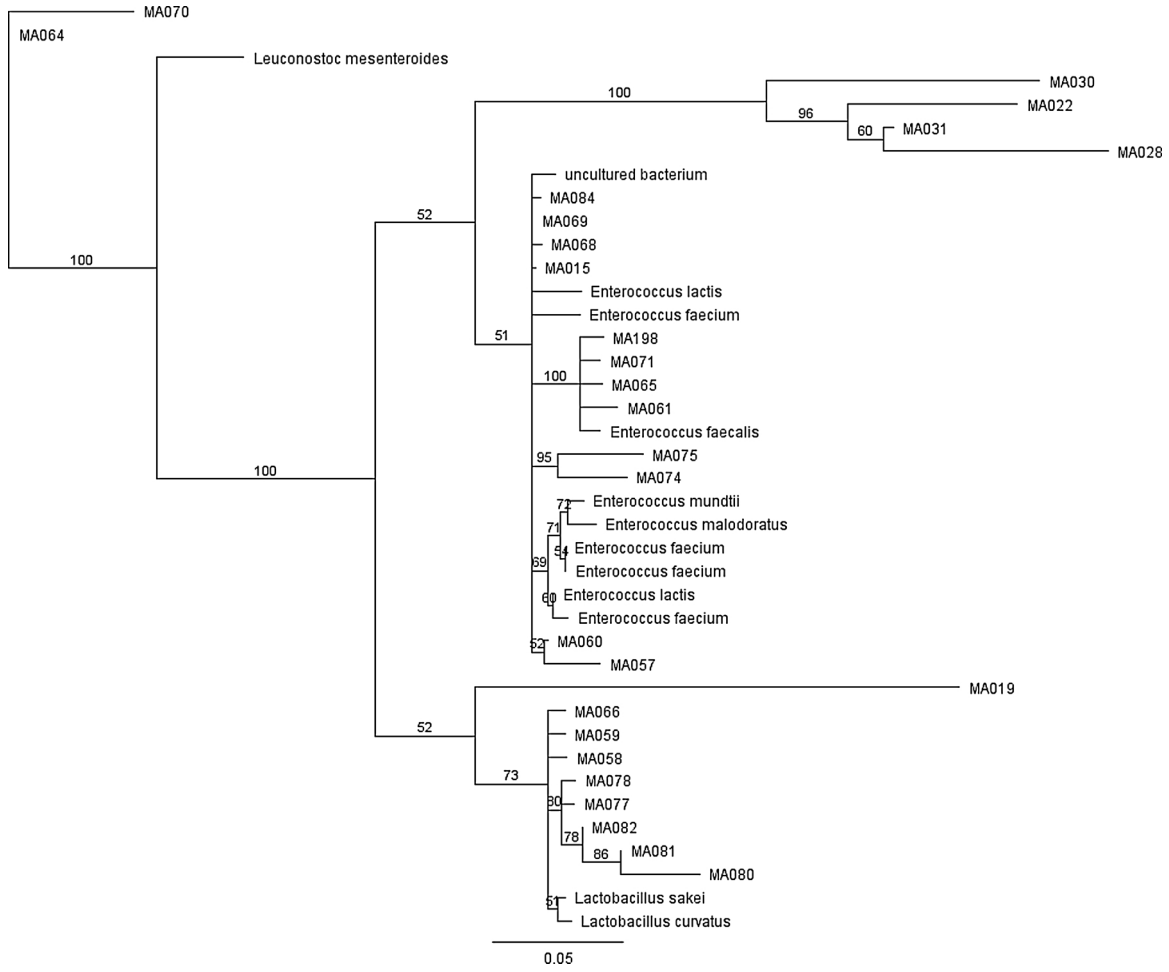

Fig. 2. Phylogenetic tree of LAB isolated from the gastrointestinal tract of adult hybrid abalone. Sequences were alignment using ClustalW Alignment.

Table 4
Values of pH of CFS and CFSn used in the screening of antimicrobial activity.

No	LAB Identity	pH	
		CFS	CFSn
1	*Enterococcus faecium* MA002	4.5 ± 0.2	6.7 ± 0.2
2	*Enterococcus lactis* MA056	4.6 ± 0.0	6.7 ± 0.1
3	*Leuconostoc mesenteroides* MA064	4.5 ± 0.2	6.6 ± 0.1
4	*Lactococcus lactis* MA068	4.7 ± 0.1	6.7 ± 0.0
5	*Enterococcus lactis* MA084	4.5 ± 0.0	6.8 ± 0.0

CFS: Cell-free supernatant, CFSn: neutralized cell-free supernatant.

first confirm that the targeted beneficial bacteria (LAB) are present in the cultured animals. Thus, this present study aimed to investigate the presence of lactic acid bacteria (viability and stability) in the GITs of hybrid abalone, *H. laevigata x H. rubra*, throughout their life stages (larvae, juvenile and adult) and screening them for their probiotic properties.

The result of present study results revealed that no LAB could be detected from the GITs of 30 abalone at the larval stage. These results might be related to the availability of nutrients, which varied during different life stages. For instance, abalone larvae are lecithotrophic organisms (having yolk sac for nutrition during dispersal); and they eat benthic diatoms after settlement and metamorphosis. Consequently, the nutrient compositions in the GIT larvae might not be able to support the growth of LAB. As described by LeBlanc et al. (2011), most of LAB members were categorized as auxotrophic bacteria which means having limited capacity in synthesizing nutrients such as amino acids from

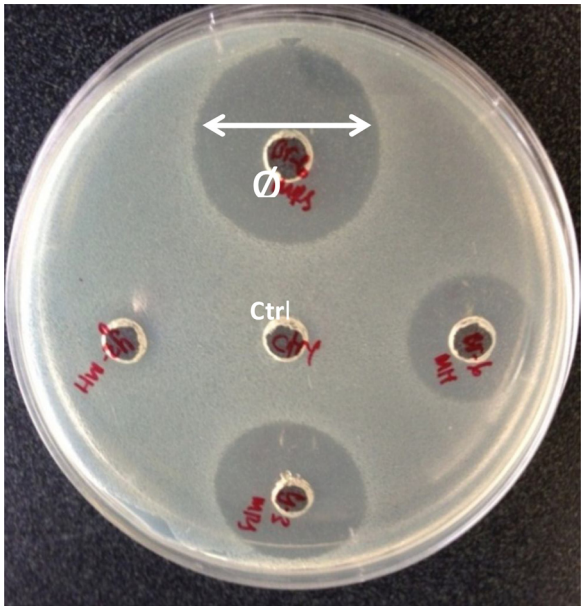

Fig. 3. Inhibition zones of CFSn extracted from the antimicrobial compound – producing bacteria on a Muller-Hinton agar plate seeded with L. *monocytogenes*. Ø is the diameter of the inhibition zone, and Ctrl is supernatant of sterilized MRS broth as the control.

Table 5
LAB isolates producing antimicrobial activity against aquatic and foodborne pathogens using an agar-well diffusion assay.

No	LAB	Indicator Pathogens							
		AH	VAng	VH	VAlg	VProt	Vpar	YR	LM
1	*Enterococcus faecium* MA002	–	–	–	–	–	–	–	++
2	*Eenterococcus lactis* MA056	–	–	–	–	–	–	–	+
3	*Leuconostoc mesenteroides* MA064	–	–	–	+	–	–	–	–
4	*Enterococcus lactis* MA068	–	–	–	+	–	–	–	++
5	*Enterococcus lactis* MA084	–	–	–	–	–	–	–	+

AH: *Aeromonas hydrophila,* **VAng***: Vibrio anguillarum,* **VH***: V. harveyi,* **VAlg***: V. alginolyticus,* **Vpro***: V. proteolyticus,* **Vpar***: V. parahaemolyticus,* **YR***: Yersinia ruckeri,* and **LM***: Listeria monocytogenes*. "+" was the diameter of inhibition zone: 10−14 mm, ++: 15−19 mm and +++: > 19 mm).

inorganic sources, therefore relied only upon external supply. Another possibility was the isolation method and culture media which were used in the present study as method and culture media profoundly influenced the result of culturable bacteria (Wackerbauer et al., 1981; Zhang et al., 2010). The present study used only one type of culture media (MRS), while according to De Man et al. (1960) the MRS supported the growth of only specific genera of LAB, including *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Latococcus*. In addition, a method used in the present study was only a culture-dependent technique. While Schmeisser et al. (2007) reported that microorganisms which can be cultured were less than 1% from the total microbial population, which means that the result of culture-dependent technique may underestimate the existence of LAB communities in the GIT of abalone larvae. Thus, based on these results, it is highly recommended to use a molecular approach for the detection of LAB in abalone larvae in future studies. In terms of culture media, it is recommended to use more different types of commercial media such as M17 or other modified media (Kang et al., 2016; Terzaghi and Sandine, 1975) in order to recover more genera of LAB.

Other study results showed that LAB strains were successfully recovered from the GITs of abalone at the juvenile and adult stage. It may suggest that LAB species colonized abalone GIT only in later ontogenetic stages. There were 44 LAB from the GIT of juvenile stage, and 49 LAB strains from the abalone at the adult stage. The appearance of LAB in the juvenile stage could be due to the abalone at the juvenile stage had started receiving artificial feed (pellet), which provided more nutrients required by LAB compared to the natural feeds. In terms of numbers, this study isolated more species than a previous study by Sarkono et al. (2010), only four strains which referred to only one species, *Lb. paracasei*. While in this study, a total of 93 LAB strains were found, 44 strains from juvenile and 49 isolates from the adult stage. The higher number of LAB obtained in the present study might be because more abalone sample were used. The present study used 90 abalone, while Sarkono et al. (2010) used only 20 abalone. Based on partial 16S rRNA sequences, these 44-LAB associated with juvenile abalone were identified as 10 LAB ribotypes which belonged to 5 genera, including *C. divergens, E. durans, E. faecium, E. casselliflavus, E. lactis, Lactobacillus curvatus, Lactococcus formosensis, Pd. Pentosaceus, Pd. lolii* and *Pd. acidilactici.* In addition, there were 8 LAB ribotypes which belonged to 3 genera found to be associated with GIT's of adult abalone, which were *E. faecalis, E. faecium, E. lactis, E. malodoratus, E. mundtii, Lb. curvatus, Lb. sakei* subsp. sakei, and *Leuconostoc mesenteroides*. These results confirm previous studies in which LAB are part of healthy microbiota in the GIT of aquaculture species. Additionally, the present study result can be used as initial information to support the development of indigenous probiotic candidates for hybrid abalone as well as prebiotic approach, especially at juvenile onwards.

4.2. Antimicrobial compound-producing LAB and antagonistic mechanisms

Five of 93 LAB strains (5%) showed antagonistic activity toward et least one targeted pathogen. In terms of percentage, the number was very small compared to a previous study by Klaenhammer (1988) who reviewed that 99 % of LAB have at least one bacteriocin-encoding gene. The different results might be due to several factors including the difference in the screening method and the number as well as the diversity of indicator pathogens. The present study used a well-agar diffusion assay, while Klaenhammer (1988) used a molecular approach. The present on the bacteriocin-encoding gene is not necessary means that these genes are all expressed or active. Blanchard et al. (2016), for instance, explained that many factors influenced the expression of certain genes including concentration and the availability of nutrients and energy in growth media. Similarly, other studies explained previously that temperature, culture equipment as well as the presence of bacterial inducers, contributed significantly to the expression of bacteriocin-encoding genes (Ali et al., 2015; Chanos and Mygind, 2016; Miller and McMullen, 2014; Perin et al., 2016). Besides, the number of screening assays used in the present study (only well diffusion assay) might also contribute to the less number of bacteriocin-producing LAB. Whereas Balouiri et al. (2016) reviewed that many different methods could be used to evaluate antimicrobial production such as microtiter plate assay, well-diffusion assay, disk-diffusion assay, and broth or agar dilution method, and each of these techniques might have different results. Thus, it is highly recommended to use more than one screening method in parallel in order to obtain a more comprehensive result. Furthermore, the present study used only eight bacterial pathogens, which was less compared to other studies (Dong et al., 2011).

There are diverse groups of antimicrobial compounds which have been reported from members of lactic acid bacteria including organic acids (Amin et al., 2016; Vazquez et al. 2005), hydrogen peroxide, bacteriolytic enzymes (Verschuere et al., 2000) and various classes of bacteriocins (Amin et al., 2020; Lin et al., 2013; Ringo, 2008; Verschuere et al., 2000). The antagonistic activity of organic acids was generally associated with a decrease in the pH values of extracted supernatant (De Vuyst and Vandamme, 1994; Presser et al., 1997), and being confirmed in the present study. Additionally, the inhibitory activity which remained active after the pH of CFS had been neutralized suggests that other antimicrobial substances including hydrogen peroxides and bacteriocin-like inhibitory substances (BLIS) are present. However, the formation of hydrogen peroxides was prevented since the LAB was cultured anaerobically. Therefore, the antimicrobial compound produced by LAB in the present study was possibly members of BLIS, although further studies are still required to confirm this speculative reason.

In conclusion, there was no cultivable LAB detected from the larval stage. LAB initially colonized GITs of hybrid abalone at the juvenile stage and became part of intestinal microflora afterward. In addition, the result also showed that LAB colonizing GIT of abalone tend to be less diverse as the animals were growing in terms of both genera and ribotypes. Furthermore, the screening assay indicated that five of the 93 LAB had inhibitory activity against at least one of eight bacterial pathogens, although studies to identify the antimicrobial compounds need to be further studied. Furthermore, *in vivo* studies on the probiotic applications and prebiotics approach need to be conducted for more

comprehensive results.

CRediT authorship contribution statement

Muhamad Amin: Conceptualization, Data curation, Writing - original draft, Visualization, Investigation. **Mark B Adams:** Supervision, Writing - review & editing. **Christopher M Burke:** Supervision, Writing - review & editing, Validation. **Christopher JS Bolch:** Supervision, Methodology, Software.

Declaration of Competing Interest

No conflict of interest.

Acknowledgments

The authors would like to thank all colleges in the Laboratory of Aquatic Microbiology, IMAS Launceston, and Laboratory of Microbiology, Human Life Science Faculty, University of Tasmania.

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