Isolation, Identification and Preliminary Characterization of Candidate Probiotic Bacteria from the Intestine of Domesticated Goldfish (*Carassius auratus*)

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ABSTRACT

Aquaculture faces challenges from the emergence of many different diseases. For many years, these diseases, especially those caused by bacterial pathogens, were often treated using antibiotics, but due to rampant use of these drugs, many antibiotic-resistant pathogens have emerged. Besides market rejection, aquaculture products with high amounts of antibiotic traces can, in one way or another, harm consumers, and this has become a serious issue pertaining to food safety. In recent years, research on the use of probiotics has shown them not only to control fish diseases but also improve growth performance of farmed fish. Therefore, this study was undertaken to screen for bacteria with probiotic properties from the intestine of domesticated goldfish (*Carassius auratus*). A total of 169 bacterial isolates were tested for antagonistic properties towards selected fish bacterial pathogens using the disc diffusion method. Five bacterial strains with high antagonistic activity were selected for identification and evaluation of their probiotic capability against *Streptococcus agalactiae*, *Aeromonas caviae*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Photobacterium damselae*. The bacterial strains included *Alcaligenes faecalis*, *Staphylococcus saprophyticus*, *Bacillus thuringiensis*, *Enterobacter cloacae* and *Skermanella stibiiresistens*. Of all the bacterial strains, *B. thuringiensis* showed a wide range of antagonistic activity towards fish pathogens, highest growth rate (0.34 %) with shortest doubling time (204 seconds) and survived through all the extreme environmental challenges (low acid, high bile and temperature). It seems that *B. thuringiensis* has more advantages than the other bacterial strains, and is assumed to have better probiotic potential. However, study to determine the beneficial effect of *Bacillus thuringiensis* in probiotic formulation requires further investigation.

Keywords: Antagonism, *Bacillus thuringiensis*, Food safety, Intestinal bacteria

INTRODUCTION

Aquaculture has become the fastest growing food production sector in the world. Despite the fact that aquaculture is the hope for future seafood security, it struggles with diseases (Carda-Dieguez *et al*., 2014). The global economic impact of bacterial diseases on aquaculture production is estimated to reach billions of dollars annually (Pridgeon and Klesius, 2012).

There have been various control strategies to combat these diseases but many are very expensive and yet not effective, because sick fish generally do not have appetite towards medicated food (Pridgeon and Klesius, 2012). Excessive application of antibiotics in aquaculture farms can make bacterial pathogens to become resistant, and hence pose a hazard risk to human health (Sharifuzzaman and Austin, 2017). Furthermore, once a disease caused by antibiotic-resistant bacteria

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occurred, it is hard to eradicate (Bondad-Reantaso *et al*., 2005; Pridgeon and Klesius, 2012).

In addition to antibiotics, fish diseases can be prevented by vaccination. In fact, vaccines are now available for important bacterial diseases (e.g., furunculosis, columnaris, vibriosis and yersiniosis) of farmed tilapia, amberjack, and Atlantic cod (Sommerset *et al*., 2005). However, vaccines are only effective to specific diseases and may not be able to protect against diseases for which the vaccines were not designed (Anderson, 1992).

An alternative way of protecting fish from diseases is to boost the immune system via the use of probiotics. Probiotics are living microorganisms which, when administered in adequate amounts, can confer health benefits to the host animals (Micheal *et al*., 2014). In recent years, the use of probiotics has become an integral part of fish health management, particularly in shrimp aquaculture (Nayak, 2010; Sharifuzzaman and Austin, 2017), due to its environmental-friendly nature. This strategy has been shown to reduce risk of disease outbreaks and improve health status, thereby enhancing production (Micheal *et al*., 2014).

Screening of potential probiotic bacteria for aquaculture was started several years ago (Carda-Dieguez *et al*., 2014). They are usually isolated from fish intestines (Hagi and Hoshino, 2009; Sumathi *et al*., 2012; Muthukumar and Kandeepan, 2015). However, many factors such as attachment site, stress factors, diets and environmental conditions can influence the colonization and function of probiotics in the gut environment of host fish (Nayak, 2010). In order to be beneficial, probiotics must not be virulent to the fish, should enhance growth rate, and should be able to reach the target organs of the host animals easily (Raja *et al*., 2015). Hence, probiotic candidates are often tested for their ability to withstand low pH, high concentrations of bile salts and heat. In addition, strong antagonistic properties towards pathogens and non-virulence to the host fish are among the primary criteria for selection of probiotics (Temmerman *et al*., 2003). To date, the development of probiotic potential of intestinal microbiota of freshwater fish is still in the infancy stage. Hence, this paper examines the antagonistic and probiotic properties of bacterial strains isolated from the intestine of domesticated goldfish (*Carassius auratus*) for future probiotic formulations of farmed fish.

MATERIALS AND METHODS

Fish dissection

A total of 20 individuals of domesticated goldfish (*Carassius auratus*) were purchased from Popular Aquarium Shop, Jalan Kionsom, Inanam Kota Kinabalu, Malaysia (5°59'31.0"N 116°08'15.1"E). The fish were kept in four transparent plastic bags filled with oxygenated freshwater in a 26-L cooler box (Coleman, USA) at 4 to 8 °C. Then, the fish were transported to the Borneo Marine Research Institute Laboratory of Universiti Malaysia Sabah (UMS).

Upon arrival at the laboratory, the fish specimens were humanely killed by overdosing with α-methyl quinolone (Transmore®, Nika Trading) and the total length of the fish was also measured using Vernier caliper. The dissection was started by making a small incision with a sterile scalpel blade at the fish anus. The incision was extended using sterile scissors along the abdomen towards the head. Then, the soft bones on both sides of the operculum were cut through and both thin muscle layers of the abdomen were pulled open to expose the inner organs. Finally, the intestine was pulled out from the abdomen using sterile forceps. Fat or any particles attached to the fish intestine were removed carefully before immersing in sterile PBS (pH 7.2) solution. After that, the intestine was blot-dried using a sterile paper towel and finally cut into pieces of approximately 0.5 cm each.

Bacterial isolation

The cut pieces of intestine were homogenized with a sterile mortar and pestle with 1 mL sterile PBS solution. One mL of homogenate was transferred into a test tube containing 9 mL of sterile PBS solution and diluted ten-fold in an additional nine test tubes. Subsequently, 100 µL of diluted solution from each tube was spread on tryptic soy agar

(TSA, pH 7.3) (Merck, Germany) plates using a sterile bent rod. All the TSA plates were then sealed using Parafilm® tape (Bemis, Malaysia), labelled accordingly and incubated overnight at 27 °C. This isolation process was conducted for 15 days. All the bacterial isolates were subjected to Gram staining according to the method suggested by Johnson and Case (2001).

Antagonism tests

Each bacterial isolate was also subjected to antagonistic properties tests against selected bacterial pathogens using disc diffusion method as suggested by Zaidan *et al*. (2005). Briefly, overnight pathogen cultures (Table 1) were first adjusted to optical density (OD) 0.2 at 600 nm wavelength before spreading on newly prepared TSA (pH 7.3) plates. Meanwhile, each bacterial isolate was cultured in a 50-mL culture flask with tryptic soy broth (TSB, pH 7.3) and incubated overnight. Then, 50 µL of each bacterial isolate was aseptically absorbed into three pieces of 9 mm diameter sterile disc (Whatman No.1, GE Healthcare, England) and aseptically placed onto the TSA plate freshly inoculated with test pathogens.

Blank disks were inoculated with 50 µL sterile TSB and placed aseptically on the pathogen-inoculated plates to serve as a control. All the TSA plates including controls were then incubated at 27 °C for 10 days. Zone of inhibition was measured using a Vernier caliper and recorded every 24 h.

Phenotypic characterization

The bacterial isolates that inhibited bacterial pathogens at diameter larger than 11 mm were considered to have good antagonistic properties and were selected for oxidase, motility and catalase tests (Chauhan, 2012). They were also screened for ability to grow on selective media including thiosulfate citrate bile salts sucrose agar (TCBS) (Merck, Germany), MacConkey agar (Merck, Germany) and Pseudomonas isolating agar (Merck, Germany), following the method of Pfeffer and Oliver (2003). Finally, the bacterial isolates were stored at 4 °C and -80 °C for short- and long-term storage, respectively.

Bacterial identification

The bacterial isolates were identified by 16S rDNA sequencing. Briefly, genomic DNA from the bacterial isolates was extracted using the CTAB-DTAB extraction method according to Nishiguchi and Doukakis (2002). Then, 2.0 µL (150 ng·µL^{-1}) of each bacterial genomic DNA was mixed with 1.0 µL (10 µmol) of forward primers, 16SJR33F (5'-GAACGCTGGCGGCAGGCCTAA-3') and 1.0 µL (10 µmol) of 16SJR1449R (5'-ACTCCCATGGTGTGACGGGCGG-3'), 0.5 µL

Table 1. Description of the bacterial pathogens used in this study.

Pathogen	Host	Origin	Strain	Reference
Streptococcus agalactiae	Tilapia (*Oreochromis niloticus*)	Malaysia	KT 869025	Laith *et al*. (2017)
Aeromonas caviae	NA	NA	ATCC 15468	Ruimy *et al*. (1994)
Aeromonas hydrophila	Japanese Eel (*Anguilla japonica*)	Japan	A 10	Kanai and Takagi (1986)
Aeromonas salmonicida	NA	NA	ATCC 33658	Ruimy *et al*. (1994)
Vibrio harveyi	Asian Seabass (*Lates calcarifer*)	Malaysia	JR 7	Ransangan and Mustafa (2009)
Vibrio parahaemolyticus	Shirasu food poisoning	Japan	ATCC 17802	Ruimy *et al*. (1994)
Vibrio alginolyticus	Horse mackerel fish	Japan	ATCC 17749	Ruimy *et al*. (1994)
Photobacterium damselae	Marine fish	Japan	ATCC 51805	Kimura *et al*. (2000)

(0.5 U) of Taq-polymerase enzyme (Promega, Madison, Wisconsin), 1.0 µL (1 mM) of dNTP mixture (Promega), 3.4 µL (3.4 mM) magnesium chloride (Promega), 10 µL of 5X PCR buffer (Promega) and topped up to 50 µL with sterile double-distilled water (Tan *et al*., 2014).

Then, the bacterial DNA was amplified using a thermocycler (Kyratec, Australia) for 1 cycle at 95 ºC for 1 min; 30 cycles for 1 min at 95 ºC, 1 min at 58 ºC, 1 min at 72 ºC; 1 cycle at 72 ºC for 5 min and finally stored at 4 ºC until further analysis. Subsequently, the amplicons were purified using AccuPrep® PCR Purification Kit (Bioneer Corporation, Korea) following the manufacturer's standard protocol. The amplicons were ligated into cloning vector pGEM-T Easy Vector System (Promega, USA) and transformed into chemically competent *Escherichia coli* JM109 (Promega, USA) according to the manufacturer's instructions.

The recombinant plasmid was extracted from transformant *Escherichia coli* and purified using the DNA-spin™ Plasmid DNA Purification Kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. Prior to sequencing, the DNA insert was verified by EcoR1 restriction analysis following the manufacturer's protocol (Biolabs, New England). Finally, all plasmids with correct DNA insert (1.4 kbp) were sent to AIT, Singapore Pte Ltd for bidirectional DNA sequencing using the M13 forward and reverse primers.

The homology search of the DNA sequences of the bacterial isolates in this study was carried out using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov).

Growth profile

To minimize the bias in the tolerance tests, bacteria at the log phase was used in tolerance tests for heat, bile and acid (Rozak and Colwell, 1987). The growth profile of bacteria was estimated over 36 h. First, the overnight bacterial culture was adjusted to optical density (OD) 0.2 at 600 nm wavelength using a spectrophotometer (Implen, Germany) before inoculating it in a TSB tube. Then, the bacteria were incubated at 27 ºC and the OD was measured hourly for a period of 36 h. The growth profile of bacteria was plotted (Figure 1). The maximum OD of each bacterium was considered as its maximum biomass yield (A). The specific growth rate (µ) of bacteria was calculated following Vine *et al*. (2004) as follows:

$$t_d = \frac{\ln 2}{\mu}$$

where µ denotes growth rate and td is the time taken by the bacteria to double its biomass during the growth period.

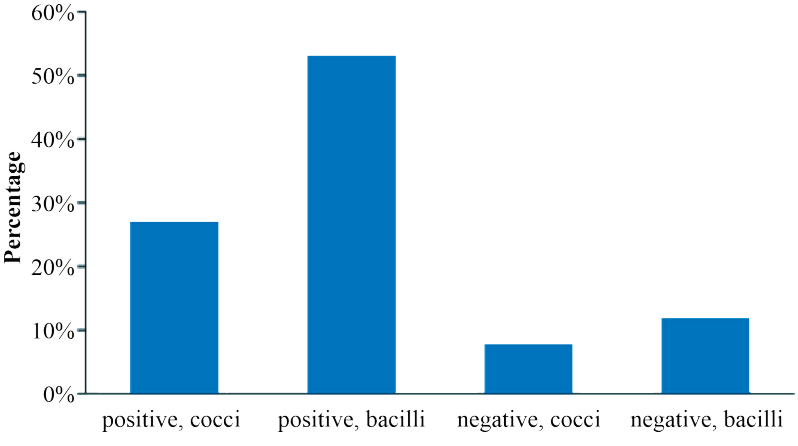

Figure 1. Gram staining composition of bacteria isolated from the intestine of domesticated goldfish (*Carassius auratus*).

Tolerance tests

The probiotic candidates must survive and grow in the fish gut in order to bring about benefits to the fish. To survive in the gut, probiotics must be able to tolerate wide ranges of bile concentration and acid level. Meanwhile, they must also be able to survive through the hot pellet-feed processing environment.

The heat tolerance test was conducted according to the method devised by Kim *et al*. (2001). Briefly, each bacterial isolate at log phase in TSB tubes was exposed to varying temperatures (18, 25, 37, 45, 50, 55, 58 and 60 ºC) for one hour. Subsequently, the bacteria cultures were spread on TSA plates using sterile cell spreader and incubated overnight at 28 ºC. Bacterial growth on TSA plates indicated that the bacteria were tolerant to heat, while no growth indicated that the bacteria were sensitive.

The bile tolerance test was carried out following the method of Buntin *et al*. (2008). Briefly, bacteria at log phase were first inoculated in 5 mL of freshly prepared TSB and incubated at 28 ºC for three hours. Afterwards, the bacterial culture was adjusted to optical density of 0.2 at 600 nm wavelength before being transferred to TSB tubes containing 0–1.0% bile salts (Sigma, USA) (w/v) and incubated for one hour. Finally, the bacterial cultures were spread onto TSA plates and incubated overnight at 27 ºC. Growth of bacteria on TSA plates after overnight incubation indicated tolerance, while no growth indicated sensitivity to bile salts.

The acid tolerance test was conducted by a modified method of Buntin *et al*. (2008). The test was started by inoculating a loop-full of log-phase bacterial culture into TSB containing 0.01–1% HCl (w/v) (Sigma, USA) and incubating at 27 ºC for 1 and 24 h. Then, 100 µL of the bacterial culture was spread onto freshly prepared TSA plates and incubated overnight. Growth on TSA plates after incubation indicated tolerance, while no growth indicated sensitivity to acid.

Statistical analysis

The growth performance of each bacterial isolate at 0, 2, 8, 12, and 36 h of incubation was analysed using the One-Way ANOVA (IBM SPSS Ver. 26) at 95 % confidence level employing Tukey's post hoc analysis to determine the growth differences among bacterial isolates. The test was considered significant when the P value is less than 0.05.

RESULTS

Bacterial composition

A total of 169 bacterial isolates were isolated from the intestines of goldfish specimens. They consisted of gram-positive bacilli (53.13 %), gram-positive cocci (27.08 %), gram-negative bacilli (11.98 %) and gram-negative cocci (7.81 %). It was also noted that bacillus bacteria were more abundant (65.11 %) than coccus bacteria (24.89 %).

Identification of bacteria

Blast analysis of the 16S rDNA sequences of the five selected bacterial isolates showed high DNA sequencing similarity to *Bacillus*, *Staphylococcus*, *Skermanella*, *Enterobacter* and *Alcaligenes* (Table 2).

Phenotypic characteristics

Phenotypic characteristics of the five selected bacteria are shown in Table 3. It was noted that all bacterial isolates were positive for catalase. However, only one bacterial isolate (*Alcaligenes faecalis*) recorded positive for the oxidase test. Three isolates (*Alcaligenes faecalis*, *Enterobacter cloacae* and *Skermanella stibiiresistens*) were motile, while two isolates (*Staphylococcus saprophyticus* and *Bacillus thuringiensis*) were not. In terms of survival on selective media, four isolates (*Alcaligenes faecalis*, *Bacillus thuringiensis*, *Enterobacter cloacae* and *Skermanella stibiiresistens*) grew on MacConkey agar, two isolates (*Staphylococcus saprophyticus* and *Skermanella stibiiresistens*) grew on TCBS, and only one isolate (*Bacillus thuringiensis*) grew on Pseudomonas agar.

Table 2. BLAST analysis 16S rDNA sequences of bacterial isolates.

Laboratory reference number	Bacterial strain	Identity similarity (%)	Length of amplicon	DNA reference (Accession number)
34.4.2	*Alcaligenes faecalis* subsp. *phenolicus* DSM 16503	99	1324 bp	MF 319604
47.5.1.1	*Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305	98	1326 bp	MF 319605
32.4.1.2	*Bacillus thuringiensis* serovar konkukian str. 97-27 chromosome	99	1329 bp	MF 319603
49.4.2	*Enterobacter cloacae* subsp. *cloacae* ATCC 13047	98	1324 bp	MF 319601
39.3.2	*Skermanella stibiiresistens* SB22 contig106	99	1326 bp	MF 319602

Table 3. Phenotypic characteristics of bacterial isolates based on triplicate observations.

Bacterial isolates	Phenotypic characteristics							
	Gram staining	Motility	Catalase	Oxidase	MacConkey	TCBS	PIA	TSA
Alcaligenes faecalis	-	+	100 %	100 %	100 %	0 %	0 %	100 %
Staphylococcus saprophyticus	+	-	100 %	33 %	0 %	0 %	0 %	100 %
Bacillus thuringiensis	+	+	100 %	33 %	0 %	0 %	67 %	100 %
Enterobacter cloacae	-	+	100 %	0 %	100 %	0 %	0 %	100 %
Skermanella stibiiresistens	-	+	100 %	0 %	100 %	0 %	0 %	100 %

Note: Phenotypic characteristics are given as percentage based on triplicate observations, except for Gram staining and Motility tests. TCBS: Thiosulfate Citrate Bile Sucrose; TSA: Tryptic Soy Agar; PIA: Pseudomonas Isolation Agar

Tolerance tests

Results of tolerance tests are given in Table 4. It was shown that *Alcaligenes faecalis* and *Staphylococcus saprophyticus* were not able to grow at temperatures higher than 37 °C. However, all bacterial isolates were able to survive in the presence of bile salts of up to 1% (g/v). The majority of the bacterial isolates were able to grow in a wide range of pH (2-9) except for two isolates (*Alcaligenes faecalis* and *Staphylococcus saprophyticus*), which were not able to survive at pH 2. Three isolates (*Bacillus thuringiensis*, *Enterobacter cloacae* and *Skermanella stibiiresistens*) were shown to survive through the extreme levels of temperature, bile salt and acidity.

Antagonism assay

Antagonistic properties of the selected bacterial isolates towards fish pathogens are summarized in Table 5. An example of a positive result of the antagonistic test is shown in Figure 2. It was noted that *Skermanella stibiiresistens* inhibited two bacterial pathogens, namely *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Photobacterium damselae*. *Alcaligenes faecalis* weakly inhibited *Streptococcus agalactiae* and *Aeromonas hydrophila* on the first day of incubation, but it strongly inhibited *Photobacterium damselae*. *Enterobacter cloacae*, on the other hand, inhibited *Vibrio parahaemolyticus* weakly but strongly inhibited *Photobacterium damselae*. Meanwhile,

Table 4. Tolerance of bacterial isolates towards extreme acidity, bile salt and temperature based on triplicate observations.

Bacterial isolate	Acid (pH)								Bile Salt (%)									Temperature (°C)						
	2	3	4	5	6	7	8	9	0.01	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	18	25	37	45	50	55	60
Alcaligenes faecalis	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Staphylococcus saprophyticus	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Bacillus thuringiensis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Skermanella stibiiresistens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Note: + indicates growth of probiotic candidate; - indicates no growth of probiotic candidate

Table 5. Antagonistic activity of selected bacterial isolates against bacterial pathogens recorded on the 1st, 5th and 10th days of incubation.

Pathogens	Bacterial isolates														
	Staphylococcus saprophyticus			*Skermanella stibiiresistens*			*Alcaligenes faecalis*			*Enterobacter cloacae*			*Bacillus thuringiensis*		
Days of incubation	**1**	**5**	**10**	**1**	**5**	**10**	**1**	**5**	**10**	**1**	**5**	**10**	**1**	**5**	**10**
Streptococcus agalactiae	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0
Aeromonas caviae	1	1	1	0	0	0	0	0	0	0	0	0	2	2	2
Aeromonas hydrophila	0	0	0	0	0	0	6	0	0	0	0	0	1	1	1
Aeromonas salmonicida	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4
Vibrio harveyi	0	0	0	10	10	10	0	0	0	0	0	0	9	9	9
Vibrio parahaemolyticus	0	0	0	8	8	8	0	0	0	9	5	0	11	11	11
Vibrio alginolyticus	0	0	0	6	6	6	0	0	0	0	0	0	11	11	11
Photobacterium damselae	7	7	8	5	5	5	7	7	6	5	5	5	4	4	4

Note: Zero value (0) represents no antagonistic activity of the bacterial isolate towards pathogens. The antagonistic activity was measured by the average diameter of clearing zone (mm) from triplicate experiments.

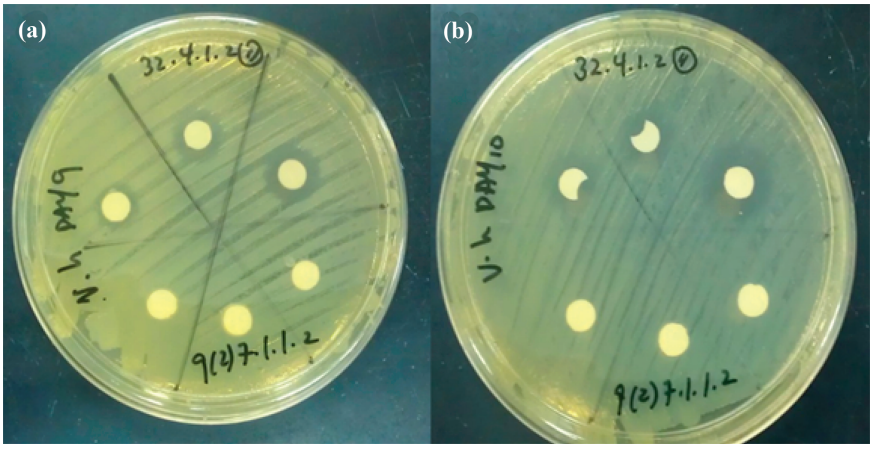

Figure 2. Antagonistic activities of *Bacillus thuringiensis* towards *Vibrio harveyi* recorded on the 9th day (a) and 10th day (b) of incubation, respectively.

Staphylococcus saprophyticus inhibited two bacterial pathogens, *Aeromonas caviae* and *Photobacterium damselae*. *Bacillus thuringiensis* showed great antagonistic activity against seven of the bacterial pathogens.

Growth profile

Results of growth performance of the five selected bacterial isolates conducted over a period of 36 h are given in Table 6 and depicted in Figure 3 whereas their growth profiles are shown in Table 7. At the initial incubation period (0 h), the growth of every bacterial isolate within and between groups was not significantly different [$F(4,10) = 0.00$, $p>0.05$]. At two-hour incubation, *Skermanella stibiiresistens*, *Alcaligenes faecalis* and *Enterobacter cloacae* began to grow significantly [$F(4,10) = 5.82$, $p<0.05$] from the other bacterial isolates (*Bacillus thuringiensis* and *Staphylococcus saprophyticus*). The growth of the bacterial isolates at 36-h of incubation continued to differ each other [$F(4,10) = 16.93$, $p<0.05$] whereby *Alcaligenes faecalis* produced a significantly higher biomass yield ($M = 3.6$, $SD = 0.17$) compared to the other bacterial isolates. *Skermanella stibiiresistens* ($M = 2.7$, $SD = 0.25$), *Enterobacter cloacae* ($M = 2.4$, $SD = 0.32$) and *Staphylococcus saprophyticus* ($M = 2.0$, $SD = 0.41$) also produced high biomass yields but not significantly different to each other. It was noted that all the bacterial isolates underwent log-phase growth during the first to sixth hour of incubation. Despite low maximum biomass yield, *Bacillus thuringiensis* recorded the highest specific growth rate (μ) of 0.34 % and exhibited the shortest doubling time (t_d) at 204 seconds.

Table 6. The optical density (OD) at 600 nm of each bacterial isolate recorded within the 36 h growth experiment.

Bacterial Isolate	Time (hour)									
	0	**1**	**2**	**3**	**6**	**8**	**12**	**13**	**35**	**36**
Bacillus thuringiensis	0.2	0.4	0.1	1.3	1.8	1.6	1.9	2	1.3	1.0
Enterobacter cloacae	0.2	0.7	0	2.3	2.7	2.6	3.2	3.3	2.7	2.4
Skermanella stibiiresistens	0.2	0.7	0	2.3	2.6	2.5	3.2	3.3	3.0	2.7
Alcaligenes faecalis	0.2	0.6	0	2.4	2.9	2.8	3.4	3.6	3.8	3.6
Staphylococcus saprophyticus	0.2	0.6	0	1.3	2.2	1.6	2.8	3.0	2.6	2.0

Table 7. Growth profiles of the bacterial isolates.

Bacteria	μ	A	t_d
Alcaligenes faecalis	0.0017	3.8	414
Staphylococcus saprophyticus	0.0013	3.0	540
Bacillus thuringiensis	0.0034	2.0	204
Enterobacter cloacae	0.0014	3.3	504
Skermanella stibiiresistens	0.0014	3.3	504

Note: Specific growth rate (μ); Maximum biomass yield (A) and Doubling time (t_d)

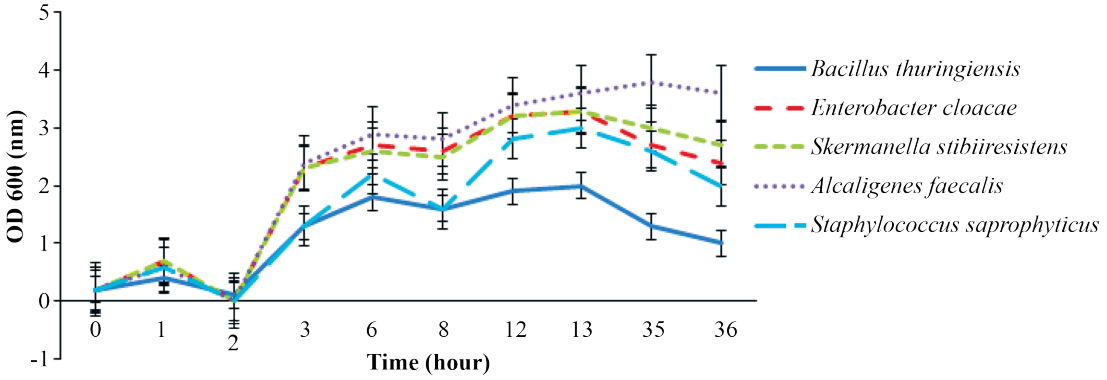

Figure 3. Growth performance of bacterial isolates recorded during 36 h of incubation.

DISCUSSION

The gut microbiota of goldfish (*Carassius auratus*) is varied but mostly composed of gram-positive bacilli. According to Ye *et al*. (2014), microbiota composition of the fish gut depends on types of food, gut morphology, digestion capability and physiological behaviour of fishes. Studies have shown that many gut bacteria have probiotic potential. Rawls *et al*. (2006) noted that the gut microbiota of zebrafish (*Danio rerio*) larvae reared both in conventional and microbe-free conditions are conserved across a broad species spectrum. They also noted that many of the gut microbiota have genes that play important roles in immunity and nutrition.

According to Ibrahem (2015), *Bacillus* spp., *Lactobacillus* spp., *Streptococcus* spp., *Pseudomonas* spp., *Vibrio alginolyticus* and *Aeromonas* spp., are amongst the potential probiotic bacteria that can be found in the fish gut. Nguyen *et al*. (2014) also reported that some members of *Alcaligenes, Bacillus* and *Enterobacter* from shellfish intestines could have probiotic potential. Moreover, Sugita *et al*. (1996) reported that some members of *Aeromonas* had strong antagonistic properties towards fish pathogens and could be good candidates as probiotics.

Antagonistic activity is the result of extracellular products that are produced by bacteria to inhibit the growth of other microorganisms, so that the producing bacteria are able to compete for space and nutrients. Extracellular products can include enzymes, complex slimes and hormones (Hagi and Hoshino, 2009). The production of these extracellular products depends on the available nutrients, temperature, pH and other microorganisms that co-exist in the same growing medium (Zaidan *et al*., 2005).

In the present study, it was noted that *Bacillus thuringiensis* showed antagonism against many of the test pathogens. In addition, it had the highest specific growth rate (μ), survived in extreme heat (high temperature), high bile concentration and low pH better than the other bacterial strains tested. *Bacillus thuringiensis* has been reported to produce a variety of extracellular products including phospholipases, proteases and chitinases (Alvarez and Loto, 2010). As shown in the antagonistic test, this bacterium appeared to effectively inhibit the growth of marine bacterial pathogens which included *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. It inhibited *Photobacterium damselae*, *Aeromonas salmonicida*, *Aeromonas hydrophila* and *Aeromonas caviae* weakly but showed no activity towards *Streptococcus agalactiae*.

Over the last 20 years, numerous studies have shown *Bacillus* spp. to have remarkable probiotic characteristics. These include the ability to boost immune systems of the host (Vaseeharan and Ramasamy, 2003), improve water quality, possess antagonistic effects on pathogens and naturally ingested by animals (Lalloo *et al*., 2007).

Bacillus thuringiensis is not harmful to fish. It forms crystals that can pass through the fish gut and reaches the fish intestine, where it benefits the fish with its probiotic properties (Du *et al*., 1994). Vaseeharan and Ramasamy (2003) also reported that *Bacillus* spores can be used as bio-control agents to minimize the occurrence of vibriosis in shrimp farms.

Alcaligenes faecalis was not able to survive at temperatures beyond 45 °C or in highly-acidic conditions (pH 2 and 3). However, it was tolerant to high bile concentration and maintained longer exponential growth, and hence would be able to produce high biomass. Annamalai *et al*. (2011) reported that *Alcaligenes faecalis* strain AU01 inhibited *Flavobacterium* sp., *Pseudomonas fluorescens*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Proteus* sp. However, our strain of *Alcaligenes faecalis* was only shown to strongly inhibit *Photobacterium damselae*, and was very weak against *Streptococcus agalactiae* and *Aeromonas hydrophila*. This bacterial strain is known to produce serine protease when cultured in protein-rich media such as tryptic soy agar. It degrades organic substances and is able to soften animal skin (Thangam and Rajkumar, 2000). According to Ju *et al*. (2016), when *Alcaligenes faecalis* is co-cultured with *Bacillus thuringiensis*, it produces a more potent protease that can hydrolyse intestinal and cuticle tissues of nematodes. However, Thangam and Rajkumar (2000) noted that the protease production in *Alcaligenes faecalis* is affected by pH. In situations when other factors are not limiting, production of extracellular products is often a function of time (Zahir *et al*., 2013). This may explain the ability of *Alcaligenes faecalis* to inhibit the growth of *Streptococcus agalactiae* and *Aeromonas hydrophila* on the first day of the antagonism test. The type of extracellular products produced by this bacterium are presumably bacteriostatic in nature.

Enterobacter cloacae survived through the different levels of bile salt, pH and heat. Biomass production and doubling time of this bacterium was also high compared to other bacterial isolates in this study. It was noted that *Enterobacter cloacae* inhibited *Vibrio parahaemolyticus* and *Photobacterium damselae*. Capkin and Altinok (2009) have shown that feed supplement containing *Enterobacter cloacae* does not only protect rainbow trout from diseases but also enhances its digestibility and utilization of feed. However, serious caution should be exercised when incorporating *Enterobacter cloacae* into probiotic formulations, as a strain of this bacterium was reported to cause disease in flathead grey mullet, *Mugil cephalus* (Sekar *et al*., 2008).

Skermanella stibiiresistens appeared to survive through low pH, high bile salt concentration and high temperature. It produced high biomass but had high doubling time. This bacterium was described as a highly antimony-resistant bacterium (Luo *et al*., 2012). The *Skermanella stibiiresistens* isolated in this study appeared to have antagonistic activity against marine pathogens including *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Photobacterium damselae*.

Staphylococcus saprophyticus had a short doubling time and grew well in high bile salt concentration, but was unable to survive in low pH (pH 2) or extreme temperatures (>45 °C). It is known to produce an extracellular enzyme complex that inhibits the growth of both gram-positive and gram-negative bacteria (Hovelius and Mardh, 1984). Hjelm and Lundell-Etherden (1991) reported that the extracellular product produced by *Staphylococcus saprophyticus* is in slime form. Our strain of *Staphylococcus saprophyticus* was shown to have bacteriostatic activity towards *Aeromonas caviae* and *Photobacterium damselae*.

Considering their antagonistic activity and ability to survive in extreme conditions of fish intestines (high bile salt concentration and low pH), three bacterial isolates in this study can be explored further for probiotic formulations: *Bacillus thuringiensis*, *Skermanella stibiiresistens* and *Alcaligenes faecalis*. Probiotics are expected to bring benefits by improving the survival rate, disease resistance, growth performance and immunity of a fish host towards pathogens (Ibrahem, 2015; Subharanjani *et al*., 2015). Other probiotics can benefit culture systems by improving the quality of water in which the fish are being cultured (Ige,

2013). However, it is unlikely to find a probiotic candidate that fulfils all these characteristics (Ibrahem, 2015) in a single source. Perhaps this is the underlying reason why probiotics are always in the form of a mixture of several bacteria from different sources (Gatesoupe, 2005; Ige, 2013).

In order to be successful, probiotics have to be effectively delivered directly into the fish digestive system, and this is particularly challenging in large-scale aquaculture operations. There are few delivery choices; the most common methods are immersion and through feeding. Nevertheless, orally administered probiotics still face challenges from extreme environmental conditions during the feed preparation, as well as low pH and high bile concentration in the stomach and intestine of fish. Hence, for a probiotic to be successfully delivered through feeding, it must have some advantageous features such as high specific growth rate, high biomass production, short doubling time and longer lag period (Vine *et al*., 2004). Above all, it must be highly tolerant to high temperature, high bile concentration and low pH conditions (Hagi and Hoshino, 2009), and of course, be harmless to the fish.

CONCLUSION

This study successfully characterized bacterial isolates from the intestine of domesticated goldfish that showed antagonistic and probiotic properties. Three bacterial strains which are likely to produce probiotic benefits when applied in aquaculture are *Bacillus thuringiensis*, *Skermanella stibiiresistens* and *Enterobacter cloacae*. However, more thorough investigation is needed to prove this perspective.

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