INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 21, No. 3, p. 82-88, 2022
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Rapid screening of potential probionts from the gut microbiota of climbing perch, *Anabas testudineus*

Christopher Marlowe A. Caipang[*1,2,3], Joel E. Deocampo Jr.[1], Rolando V. Pakingking Jr.[4], Jehannie T. Fenol[1], Francis B. Onayan[1]

[1]*Department of Biology, College of Liberal Arts, Sciences, and Education, University of San Agustin, Iloilo City, Philippines*

[2]*Center for Chemical Biology and Biotechnology, University of San Agustin, Iloilo City, Philippines*

[3]*Center for Educational and Institutional Research, University of San Agustin, Iloilo City, Philippines*

[4]*Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFDEC-AQD), Tigbauan, Iloilo, Philippines*

Article published on September 25, 2022

Key words: Aquatic, *Kurthia*, Microbiota, Ornamental fish, Probionts

Abstract

The intestinal microbial community has crucial functions for their vertebrate host. Several studies in fish showed that their gastro-intestinal tract harbors a diverse population of bacteria that supplies exogenous nutrients, enzymes, fatty acids and vitamins to their host. Most studies on probiotics involved their practical use for aquaculture, but are limited for the ornamental fish industry. Hence, this study aimed to screen the gut microbiota of a freshwater fish, *Anabas testudineus*, for potential probiotic candidates for the ornamental fish industry. Gut bacteria were obtained from the gut of climbing perch by plating of serially-diluted samples of the gut contents onto Nutrient Agar (NA). *In vitro* antagonistic activities of these gut bacteria against a fish bacterial pathogen, *Aeromonas hydrophila*, were determined by spot-on-lawn method. Isolates that had strong antagonistic activities against *A. hydrophila* were further characterized using standard staining and biochemical techniques. Rapid screening of the gut microbiota of climbing perch resulted in the identification of a promising probiont, *Kurthia gibsonii* through sequencing of its 16S rRNA gene. This bacterium is a member of the Planococcaceae family and is a Gram-positive, non-spore forming and rod-like bacterium. The isolate is yellowish in appearance and has a filamentous colony on nutrient agar. It exhibited catalase and amylase activities. Immersion challenge of freshwater ornamental fish with the bacterial isolate showed no mortality at 15 days after exposure. Taken together, the present study demonstrated that the gut microbiota of fish is a rich source of probiotic candidates that can be utilized during the culture of freshwater ornamental fish.

***Corresponding Author:** Christopher Marlowe ✉ cmacaipang@yahoo.com

Introduction

It is widely accepted that fish reared in artificial environments are more susceptible to disease-causing agents than in the wild due to erratic rearing conditions in the former. Most fish culturists would resort to the use of antibiotics as a solution for treating sick or diseased fish. Inappropriate and long-term use of antibiotics would also result in the emergence of drug-resistant bacteria, which are increasingly difficult to control and eradicate (Miranda and Zemelman, 2002). Hence, alternative methods of disease control and management were explored. One such method is the use of beneficial bacteria as probionts (Verschuere *et al.*, 2000; Irianto and Austin, 2002) for disease prevention in fish husbandry. A number of studies on the use of probionts in fish have resulted in better growth, higher disease resistance of the fish and to some extent contribute to good water quality.

The intestinal microbial community provides a variety of crucial functions for their vertebrate host (Ivanov and Litman, 2011). Several studies have demonstrated that the gastro-intestinal tract of fish harbours diverse population of bacteria that could be used as potential probionts (Hirimuthugoda *et al.*, 2006; Hagi and Hoshino, 2009; Lazado *et al.*, 2010). The intestinal microbiota also produces and supplies exogenous nutrients, enzymes, fatty acids and vitamins (Dhanasiri *et al.*, 2011; Lazado and Caipang, 2014). In addition, studies on microbial populations have opened new perspectives concerning the role and physiological functions of the secondary metabolites released by intestinal microorganisms. These chemical repositories can be utilized for numerous applications yet this distinct microbial pool is least prospected for the discovery of novel bioactive compounds. Some bacterial isolates from fish gut have been demonstrated to be producing compounds of particular interest such as sebastenoic acid, phytase, chitinase, among others (Lazado *et al.*, 2010; Lazado *et al.*, 2012; Sanchez *et al.*, 2012). As the ornamental fish industry in the Philippines is expanding (Muyot *et al.*, 2018), it is necessary that efficient health management strategies have to be implemented. The use of probionts as a health management tool would ensure that production is sustained and at the same keep the fish healthy during the rearing process. Hence this study aimed to identify probiotic candidates with potential use for the ornamental fish industry through bulk screening of the gut microbiota of a freshwater fish, *Anabas testudineus*.

Materials and methods

Collection of fish and screening of bacterial isolates

Mature climbing perch, were caught from a river by hook and line and transported to the Biology Laboratory of the University of San Agustin. The fish were starved for 48h to empty the gastro-intestinal (GI) tracts (Ray *et al.*, 2010). After starvation, the fish were immediately sacrificed by giving a sharp blow to the head and the ventral surface of each fish was thoroughly rubbed with 1% iodine solution for surface sterilization. The fish were dissected aseptically and their gastrointestinal tracts were excised carefully. Gut samples were processed for the isolation of adherent (autochthonous) bacteria following the as described by Dhanasiri *et al.* (2011). The gut segments were homogenized with 10 parts of sterilized pre-chilled 0.9% NaCl solution and ten-fold serial dilutions were prepared. Diluted samples (0.1mL) were poured aseptically on Nutrient agar (NA) plates and incubated at 28°C for 48 h to determine culturable heterotrophic autochthonous bacteria.

After a 48-h incubation period, four hundred fifty (450) distinct bacterial colonies were randomly picked and re-streaked on fresh NA medium for further testing on their in vitro antagonistic activities against *Aeromonas hydrophila*, a bacterial pathogen of freshwater fish. Inhibition of *A. hydrophila* was determined using the spot-on-lawn method following the procedures of Caipang *et al.* (2010). Bacterial isolates that exhibited zones of clearance on the agar plate with *A. hydrophila* were purified using fresh NA medium and kept for subsequent experiments. Pathogenicity of the bacterial isolates to ornamental fish was carried out using a bath challenge following the procedures described by Sasmal *et al.* (2005).

An overnight broth culture (approximately 10^7 CFU per m L) of each isolate was added to the culture tank (5 li) of molly, *Poecilia* sp., at a density of 10 fish/li. The final concentration of the bacterial inoculum in each rearing tank was at 10^3 CFU per m L. After 1 hour of exposure, each group of fish were immediately transferred to new 5-li container and observed for mortalities and pathological changes for two weeks.

Characterization of bacterial isolates

The bacterial isolates that were not pathogenic to molly were further subjected to morphological, physiological, and various biochemical tests following standard methods. Identification of the strains was primarily based on the phenotypic characters and biochemical properties described in the Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 2000).

To facilitate molecular identification of the isolates, bacterial genomic DNA was extracted using a commercial kit from an overnight culture of the isolates in 5mL Trypticase Soy Broth (TSB) following the procedures described by manufacturer (Purelink Genomic DNA Mini, Thermo Fisher Scientific, California, USA). 16S rRNA was amplified using the eubacterial universal primers (Forward: GAGAGT TTGATCCTGGCTCAG; Reverse: CTACGGCTACCT TGTTACGA) of (Bianciotto *et al.*, 2003) in a 25 µL PCR reaction consisting of 2 µL (10-15 ng) of DNA as the template, 2 µL of each primer (5 pmol), 2.5 µL of 10 PCR buffer, 1.5 µL of 2mm dNTP, 1 µL of 50mm $MgCl_2$ and desired volume using distilled water. Polymerase chain reaction amplification was carried out following the PCR conditions described by Caipang *et al.* (2010). The PCR products were cleaned and sent for sequencing (Macrogen, Korea).

Sequenced data were aligned and analyzed to find the closest homolog of the bacterial isolates using the publicly available data of NCBI GenBank (blast.ncbi.nlm.nih.gov). MEGA 7.0 (Tamura *et al.*, 2013) was used for aligning DNA sequences together with the reference sequences in the construction of the phylogenetic trees. Species identification was inferred using Neighbor-Joining (NJ) tree with 500 bootstrap replications (Tamura *et al.*, 2013).

Results and discussion

There were 39 isolates or 8.7% that showed inhibition against *A. hydrophila*. These isolates were checked for the presence of some enzymes including catalase (Barbosa *et al.*, 2005), protease (Pailin *et al.*, 2001), lipase (Iqbal and Rehman, 2015) and amylase (Alariya *et al.*, 2013) following procedures described previously. Table 1 shows the presence of these enzymes in the bacterial isolates. There were 18 isolates that possessed at least 2 of the beneficial enzymes; thus, were subsequently used for the pathogenicity tests in freshwater ornamental fish using molly, *Poecilia* sp., as the model organism.

Table 1. Enzymatic activities of the gut bacteria obtained from the gut of climbing perch.

Isolate Numb	Catalase	Amylase	Lipase	Protease	Isolate Numbe	Catalase	Amylase	Lipase	Protease
A1	X	P	X	X	D1	P	X	X	X
A2	P	P	P	X	D2	P	X	X	X
A3	P	P	X	X	D3	P	P	X	P
A4	P	P	X	X	E1	X	P	X	X
A5	P	P	X	X	E2	P	P	P	P
A6	P	P	X	X	E3	P	P	P	X
A7	P	P	X	X	E4	P	P	P	P
A8	P	P	X	X	E5	X	P	X	P
A9	P	P	X	X	E6	P	P	P	P
B1	P	X	X	X	E7	P	P	P	P
B2	P	P	X	X	E8	P	P	P	P
B3	P	X	X	X	E9	P	P	P	P
B4	P	P	X	P	E10	P	P	P	P
B5	P	X	X	P	E11	P	P	X	P
B6	P	P	X	X	E12	P	P	P	P
B7	P	X	X	X	E13	P	X	X	P
B8	P	X	P	X	E14	P	P	P	P
B9	X	P	X	X	E15	P	P	X	P
B10	X	P	X	X	E16	P	P	X	P
B11	P	X	P	X					

Bacterial isolates highlighted in red font were further tested of their pathogenicity in ornamental fish.

P – indicates presence of enzymatic activity, X – indicates absence of enzymatic activity.

Ornamental fish that were challenged with the 18 bacterial isolates had survival rates ranging 80-100% over an observation period of 2 weeks, indicating that all isolates were not pathogenic to fish (Table 2).

Moreover, there were no moribund fish and the dead fish did not exhibit any pathological changes that would likely indicate bacterial infection. Using the data on the spot-on-lawn assay, presence of beneficial enzymes and pathogenicity tests, we selected four (4) promising bacterial isolates that were further tested for phenotypic and biochemical characterization as well as molecular identification. The isolates B2, B5, B6 and B10 exhibited strong inhibitory activity against *A. hydrophila*, possessed beneficial enzymes and were not pathogenic to ornamental fish. Morphological characterization of the four probiotic candidates is shown in Table 3. All four isolates are rod-shaped, Gram-positive bacteria. Isolate B2 has milky color, filamentous margin and has a slimy texture. On the other hand, isolates B5, B6 and B10 have an undulate margin, opaque in color and have a matte texture. B2 isolate does not have endospores and is a non-acid fast bacterium, whereas the three other isolates have endospores and non-acid fast bacteria. Isolates B5, B6 and B10 were gelatinase- and oxidase-positive (Table 4). Moreover, arabinose activity was present in B2, B5 and B6 isolates.

Table 2. Survival of ornamental fish following bath challenge with the various gut bacterial isolates obtained from climbing perch.

Isolate Number	Survival (%)	Isolate Number	Survival (%)
A2	90	B10	100
A6	100	D3	90
A7	100	E7	100
A8	80	E8	100
B2	100	E9	100
B4	100	E10	100
B5	80	E14	100
B6	80	E15	100
B8	100	E16	100

Table 3. Morphological characterization of the gut bacterial isolates.

Characteristics	Isolate			
	B2	B5	B6	B10
Gram stain	+	+	+	+
Cell shape	Rod	rod	rod	rod
Luminescence	-	-	-	-
Colony Description				
1. Margin				
Filamentous	+			
Undulate (wavy)		+	+	+
2. Color				
Opaque or white		+	+	+
Milky (yellowish)	+			
3. Elevation				
Flat	+	+	+	+
4. Texture				
Slimy, moist	+			
Matte		+	+	+
5. Shape				
Filamentous	+			
Irregular		+	+	+
6. Size				
Endospore	-	+	+	+
Acid-fast	-	-	-	-

Molecular characterization of the isolates showed that the three isolates: B5, B6 and B10 are putative *Bacillus albus*. Surprisingly, isolate B2 showed high identity to *Kurthia gibsonii* as shown by the phylogenetic tree analysis (Fig. 1). The isolation of *Bacillus* spp from the gastro-intestinal tract of fish is

not new, considering that this bacterial group is commonly isolated from the gut of various species of fish as demonstrated in previous studies (Thankappan *et al*., 2015; Kavitha *et al*., 2018; Soltani *et al*., 2019; Kuebutomye *et al*., 2020). Studies on the identification or use of *Kurthia* spp as probiotics in fish are limited except for the testing of *in vitro* antagonistic activity of this bacterial species that was isolated from milk products (Chaudhary and Qazi, 2014; Chaudhary *et al*., 2021). It is interesting to note that various strains of *K. gibsonii* can be classified in any of these categories: pathogenic (Lozica *et al*., 2022), non-pathogenic with bioremediation properties (Wu *et al*., 2011; Sahadevan *et al*., 2016) or implicated in spoilage of milk products (Junior *et al*., 2019). Our future studies, will focus on more in-depth characterization of this bacterial isolate and explore the possibilities whether or not this can be utilized as probiotics for ornamental fish. We have to establish that this isolate is not a zoonotic agent and at the same time uncover the mechanisms of its probiotic actions in fish.

Table 4. Biochemical characterization of the gut bacterial isolates.

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
B2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
B5	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+
B6	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+
B10	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+

Activity for the following:
1: Galactosidase
2: Arginine dihydrolase
3: Lysine decarboxylase
4: Ornithine decarboxylase
5: Utilization of citrate
6: Production of hydrogen sulfide
7: Urease
8: Tryptophan deaminase
9: Production of indole
10: Detection of acetoin
11: Gelatinase
12: Fermentation of glucose
13: Fermentation of hexose
14: Fermentation of inositol
15: Fermentation of sorbitol
16; Fermentation of rhamnose
17: Fermentation of sucrose
18: Fermentation of melibiose
19: Fermentation of amygdalin
20: Fermentation of arabinose
21: Oxidase

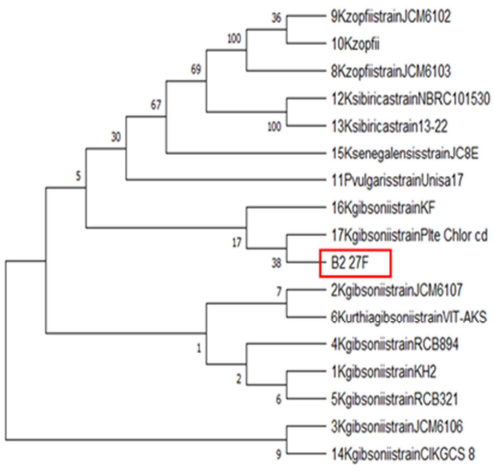

Fig. 1. Phylogenetic tree of the B2 isolate

Taken together, the results of this study showed that 1) the gut microbiota of freshwater fish is a good source of potential probionts that may be further developed for use by the ornamental fish industry, and 2) these potential probiotic candidates possess beneficial enzymes that are important to the host fish.

Acknowledgments

This work is partly supported by the University of San Agustin Professorial Chair Research Grant titled, "Gut Microbiome: A Potential Source of Probiotic Candidates for Ornamental Fish" awarded to CMA Caipang. The authors of this paper would like to thank the support provided by their respective institutions; namely, the University of San Agustin and SEAFDEC Aquaculture Department during the preparation of the manuscript.

Conflicts of interest

The authors declare that there is no known conflict of interest that could influence the work reported in this paper.

Ethics statement

No experimental procedures involving animals or human participants were carried out in the present study.

References

Alariya SS, Sethi S, Gupta S, Gupta BL. 2013. Amylase activity of a starch degrading bacteria isolated from soil. Archives of Applied Science Research **5,** 15-24.

Barbosa TM, Serra CR, La Ragione RM, Woodward MJ, Henriques AO. 2005. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Applied Environmental Microbiology **71,** 968-978. https://doi.org/10.1128/AEM.71.2.968-978.2005

Bianciotto V, Lumini E, Bonfante P, Vandamme P. 2003. '*Candidatus Glomeribacter gigasporarum*' gen. nov., sp. nov., an endosymbiont of arbuscular mycorrhizal fungi. International Journal of Systematic and Evolutionary Microbiology **53,** 121-124. https:// doi.org/10.1099/ijs.0.02382-0

Caipangcm A, Brinchmann MF, Kiron V. 2010. Antagonistic activity of bacterial isolates from intestinal microbiota of Atlantic cod, *Gadus morhua*, and an investigation of their immunomodulatory capabilities. Aquaculture Research **41,** 249-256.

Chaudhary A, Qazi JI. 2014. Probiotic antagonism of *Sphingomonas* sp. against *Vibrio anguillarum* exposed *Labeo rohita* fingerlings. Advances in Life Sciences **4,** 156-165. DOI: 10.5923/j.als.20140403.11

Chaudhary A, Qurat-ul-Ain Ahmad AM, Akram S. 2021. Antagonistic probioticity of novel bacterial isolates from Pakistan against fish pathogen *Pseudomonas fluorescens* in *Labeo rohita* fingerlings. Pakistan Journal of Zoology **53,** 1-14.

Dhanasiri AK, Brunvold L, Brinchmann MF, Korsnes K, Bergh Ø, Kiron V. 2011. Changes in the intestinal microbiota of wild Atlantic cod *Gadus morhua* L. upon captive rearing. Microbial Ecology **61,** 20-30. https://doi.org/10.1007/s00248-010-96

Hagi T, Hoshino T. 2009. Screening and characterization of potential probiotic lactic acid bacteria from cultured common carp intestine. Bioscience, Biotechnology and Biochemistry **73,** 1479-1483.

Hirimuthugoda NY, Chi Z, Li X, Wang L, Wu L. 2006. Diversity of phytase-producing marine yeasts. Ciencias Marinas **32,** 673-682.

Holt GJ, Krieg NR, Sneath PHA, Staley JT, Williams ST. 2000. Bergey's manual of determinative bacteriology. Ninth edition. Lippincott Williams and Wilkins, Philadelphia, USA.

Iqbal SA, Rehman A. 2015. Characterization of lipase from *Bacillus subtilis* I-4 and its potential use in oil contaminated wastewater. Brazilian Archives of Biology and Technology **58,** 789-797.

Irianto A, Austin B. 2002. Probiotics in aquaculture. Journal of Fish Diseases **25,** 633-642.

Ivanov II, Littman DR. 2011. Modulation of immune homeostasis by commensal bacteria. Current Opinion in Microbiology **14,** 106-114.

Júnior JR, De Oliveira AM, Silva FDG, Tamanini R, De Oliveira ALM, Beloti V. 2018. The main spoilage-related psychrotrophic bacteria in refrigerated raw milk. Journal of Dairy Science **101,** 75-83. https://doi.org/10.3168/jds.2017-13069

Kavitha M, Raja M, Perumal P. 2018. Evaluation of probiotic potential of *Bacillus* spp. isolated from the digestive tract of freshwater fish *Labeo calbasu* (Hamilton, 1822). Aquaculture Reports **11,** 59-69.

Kuebutornye FK, Lu Y, Abarike ED, Wang Z, Li Y, Sakyi ME. 2020. In vitro assessment of the probiotic characteristics of three bacillus species from the gut of Nile tilapia, *Oreochromis niloticus*. Probiotics and Antimicrobial Proteins **12,** 412-424.

Lazado CC, Caipangcm A. 2014. Atlantic cod in the dynamic probiotics research in aquaculture. Aquaculture **424,** 53-62.

Lazado CC, CaipangcmA, Gallage S, Brinchmann MF, Kiron V. 2010. Responses of Atlantic cod *Gadus morhua* head kidney leukocytes to phytase produced by gastrointestinal-derived bacteria. Fish Physiology and Biochemistry **36,** 883-891.

Lazado CC, CaipangcmA, Kiron V. 2012. Enzymes from the gut bacteria of Atlantic cod, *Gadus morhua* and their influence on intestinal enzyme activity. Aquaculture Nutrition **18,** 423-431. https://doi.org/10.1111/j.1365-2095.2011.00928.x

Lozica L, Maurić Maljković M, Mazić M, Gottstein Ž. 2022. *Kurthia gibsonii*, a novel opportunistic pathogen in poultry. Avian Pathology **51**, 26-33. https://doi.org/10.1080/03079457.2021.

Miranda CD, Zemelman R. 2002. Bacterial resistance to oxytetracycline in Chilean salmon farming. Aquaculture **212,** 31-47. https://doi.org/10. 1016/S0044-8486(02)00124-2

Muyot FB, Mutia MTM, Manejar AJA, Guirhem GL, Muñez MJ. 2018. Value chain analysis of marine ornamental fish industry in the Philippines. The Philippine Journal of Fisheries **25,** 57-74.

Pailin T, Kang DH, Schmidt K, Fung DYC. 2001. Detection of extracellular bound proteinase in EPS-producing lactic acid bacteria cultures on skim milk agar. Letters in Applied Microbiology **33,** 45-49. https://doi.org/10.1046/j.1472-765X.2001.00954.x

Ray AK, Roy T, Mondal S, Ringø E. 2010. Identification of gut-associated amylase, cellulase and protease-producing bacteria in three species of Indian major carps. Aquaculture Research **41,** 1462–1469. https://doi.org/10.1111/j.1365-2109.2009.02437.x

Sahadevan N, Mathew J, Radhakrishnan EK. 2016. Potential of *Kurthia gibsonii* MB 126 as a biocontrol agent against fusarium oxysporum wilt disease of tomato plants. Bharata Mata Journal of Multidisciplinary Studies **3,** 1-15.

Sanchez LM, Wong WR, Riener RM, Schulze CJ, Linington RG. 2012. Examining the fish microbiome: vertebrate-derived bacteria as an environmental niche for the discovery of unique marine natural products. PloS one **7,** e35398.

Sasmal D, Babu CS, Abraham TJ. 2005. Effect of garlic (*Allium sativum*) extract on the growth and disease resistance of *Carassius auratus* (Linnaeus, 1758). Indian Journal of Fisheries **52,** 207-214.

Soltani M, Ghosh K, Hoseinifar SH, Kumar V, Lymbery AJ, Roy S, Ringø E. 2019. Genus *Bacillus*, promising probiotics in aquaculture: aquatic animal origin, bio-active components, bioremediation and efficacy in fish and shellfish. Reviews in Fisheries Science and Aquaculture **27,** 331-379.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution **30,** 2725-2729.

Thankappan B, Ramesh D, Ramkumar S, Natarajaseenivasan K, Anbarasu K. 2015. Characterization of *Bacillus* spp. from the gastrointestinal tract of *Labeo rohita*- towards to identify novel probiotics against fish pathogens. Applied Biochemistry and Biotechnology **175,** 340-353.

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews **64,** 655-671. https://doi.org/10.1128/MMBR.64.4.655-671.2000

Wu J, Zhong S, Wang G, Li Y. 2011. Identification and activity of a bacterial strain for the biodegradation of microcystins. China Environmental Science **31,** 116-122.