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Isolation, Characterization and Identification of Gut Bacteria from Piscine Ectoparasite *Argulus bengalensis* (Ramakrishna 1951) and their Relationship with Haematophagy

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Abstract

Gut microbiome plays a pivotal role in health and nutrition of mostorganisms. Argulus bengalensis is a haematophagus ectoparasite of fresh water carp and its infection causes extensive damage to fish farms. Thepresent study aims at isolation, characterization and identification of the gut bacteria from Argulus bengalensis and to decipher its potential contribution in haematophagy of the parasite. The gut bacteria were isolated, cultured and identified based on analyses of its morphological, physiological and biochemical features as well as 16s rRNA analyses against the NCBI genetic database. Antibiotic sensitivity of the bacterial strains was tested. Haemolytic activity and ability of the isolates to produce anticoagulant substance were also tested to justify the hypothesis. Two isolates were identified to be similar type strains of Acenatobacter baumanii and Aeromonas hydrophila respectively which have accession number MW811800 and MW806655 respectively. Both bacteria were found sensitive to most antibiotics. Both Acenatobacter baumanii and Aeromonas hydrophila showed their haemolytic activities and able to secrete anticoagulant substances establishing their mutualistic association which increase the fitness of the parasitic host facilitating haematophagy. The outcome of the study thus may provide a scientific direction to develop novel methods to control of argulosis.

Keywords: *Argulus bengalensis; Haematophagy; Acenatobacter baumanii; Aeromonas hydrophila, Haemolysis, Anticoagulant*

1. Introduction

Symbiosis, sometimes known as "living together," primarily refers to a tight relationship between several species. The "endosymbiosis" is one of the many symbiotic connections, and it exhibits the most coherent form. In this connection, the symbiont inhabits the host, the partner it partners with. Symbiotic bacteria are typically found in species that have restricted diets, such as those that eat woody debris, vertebrate blood, or plant sap. The gut lumen, the hemocoel, or specialized cells called mycetocytes or bacteriocytes are frequently the locations of certain bacterial species in certain cases (Buchner, 1965). Such bacteria are generally found in the host insects, are passed down vertically from generation to generation (Pais et al., 2008; Hosokawa et al., 2010), and frequently cannot be grown in vitro. Many different insect species depend on microorganisms for their growth development and survival.These contributions in insects include the synthesis of necessary nutrients, generation of vitamins and sterols, digestion of meals like cellulose or proteins and determination of food consumption, and detoxification of foods (Douglas, 2009). There aren't many studies that describes

the elements and roles that these interactions play, despite the importance of these microbial linkages with insects. Insects those consume diets deficient in nutrients, frequently have bacterial endosymbionts. When their symbionts are experimentally removed, the host insects frequently experience stunted development, infertility, and/or death (Buchner, 1965; Douglas, 1989; Baumann and Moran, 1997).

All of the genera in the order Branchiura are parasitic, mostly on fish. According to Poly (2008), there are about 129 species of *Argulus* (Müller, 1785) that are known to be haematophagous (Swanepoel and Avenant-Oldewage, 1992; Gresty et al., 1993; Saha et al., 2011) and developing oligocythemic hypochromic macrocytic anaemia in the host (Datta et al., 2022a) and prominent primary stress responses (Datta et al., 2022b). The endosymbionts replenish essential vitamins for the survival of their hosts in the nutrient-limited diets of blood-feeding insects (Pais et al., 2008; Hosokawa et al., 2010). Haematophagy has been developed as a favoured method of ingestion in many tiny creatures, such as worms and arthropods, since blood is a fluid tissue rich in nourishing proteins and fats that can be ingested without requiring a great deal of effort. *Argulus* feeds by penetrating the skin with a lengthy stylet structure that resembles a spine. The parasite uses two secretions that are released from glands associated with the feeding apparatus as anti-haemostatic agents: a vasodialator (Marshall et al., 2008) and an anticoagulant (Saha et al., 2011). Despite their diversity, some other animals that only consume blood, such as leeches, hookworms, and various species of vampire bats, all secrete anti-haemostatic and fibrinolytic substances from their salivary glands to stop blood clotting while they are feeding and blood storage in the gut while digesting (Ribeiro, 1987; Fontaine, et al., 2011; de Araújo, et al., 2012; Kotál, et al., 2015; Chmelař, et al., 2016). There are several anti-coagulant and anti-thrombotic compounds found in blood-sucking insects. It is imperative to mention that development of a symbiotic relationship with bacteria in their gut, is consistent with haematophagy. By providing extracellular enzymes, the symbionts are hypothesized to either assist the parasite's anti-haemostatic mechanism in preventing blood from coagulating in their intestines or facilitate blood digestion. The symbionts occasionally provide dietary supplements by manufacturing vitamins. In their gastrointestinal systems, all vertebrates and many invertebrates have microbial communities (Trust and Sparrow, 1974; Lindsay and Harris, 1980; Lesel et al., 1986). These populations grow upon the food absorbed by the host animal and their digestive secretions. The bacterial flora of endogenous sources often possesses a considerable and diverse range of enzymatic abilities. It is reasonable to expect that an amount of the partner's metabolism may be disrupted by the enzyme mass stuck in the digestive system.

The symbionts undoubtedly have a part in certain elements of nutrition in the broadest sense, but their effects on morphology, reproduction, and digestion further complicate matters. The connection is so strong and intimate that frequently the processes involved resemble cell organelles more than they resemble those of independent bacteria. As we proposed in Ghoshal (2018), the objective of the current work is to identify and characterize the endosymbiotic bacteria and to ascertain their role in the survival of the parasitic *Argulus*. Due to its widespread damage, argulosis is a big worry for fish growers globally. Therefore, studying the physiological relevance of the symbionts is important to understand the link that may offer a clear hint to an alternate parasite management strategy.

The remainder of the essay is structured as follows. The results are presented structurally following an explanation of the study's materials and procedures. The next part covers the conclusions and wraps up the study.

2. Material and Methods Sample Collection

Carp remains from the "Barasagar Dighi" fish farm in Malda, India (24°58'08.86" N, 88°06'09.70" E), were examined for parasitic variants of the *Argulus bengalensis* species. *Cirrhinusmrigala*, one of its favoured hosts, was used to grow a breeding colony of the parasite in laboratory settings (Hamilton, 1822). Morphometric features of the parasite as described by Ramakrishna (1951) were used to identify the parasite.

Isolation of gut microbes from *Argulus bengalensis*

Ten numbersofadult *Argulus bengalensis*specimenswerekept onstarvationfor4 hourstocleartheir alimentary tract and allochthonous gut flora. Inside a laminar flow hood, the starved specimens were subjectedtosurfacesterilizationwith0.01%mercuricchloride.Thereafterthespecimenswere

autopsied under an inverted microscope inside the laminar hood to obtain their gut. Finally, the tissue was homogenized in a sterile condition. After five serial 1:10 dilutions, the homogenate was

employed as inoculum (Beveridge et al., 1991). In triplicate, 0.1 ml of the inoculum from each dilution was placed on tosterilized Tryptone Soya Agar (TSA) plates. The culture plates were incubated at30°C for 24 hours for obtaining aerobic or facultative anaerobic bacterial colonies. The well-separated colonies were streaked separately on TSA plates to obtain pure cultures

Microscopic, biochemical, and molecular characterization of the microbes

Microscopic characterizations of the selected microbial strains were done after Gram staining.According to Bergey's Manual of Determinative Biology (Holt et al., 1994), biochemical characterizations were carried out. The strains' capacity to flourish at pH levels ranging from 4 to 9 was also examined, NaCl concentrations of 2% to 9%, and temperature conditions of 10ºC to 55ºC. The fermentative behavior was determined for various carbohydrates (Glucose, Mannitol, Sucrose Rhamnose, and Xylose).

DNA isolation of the microbial isolates was done where bacterial culture was homogenized with 1 ml of extraction buffer and subsequently transferred to a 2ml microfuge tube. After mixing gently with an equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1), the homogenate was centrifuged for 15 minutes at 14000 rpm. The supernatant was collected after a second centrifugation using isoamyl alcohol (24:1) and mixed with an equal volume of chloroform. The DNA sample was centrifuged under the same conditions after being precipitated for 15 minutes at room temperature with the addition of 0.1 volume of 3M sodium acetate pH 7.0 and 0.7 volume of isopropanol. The DNA pellet was washed twice with 70% ethanol and then briefly with 100% ethanol, and then dissolved in TE (Tris-Cl, 10 mM pH 8.0, EDTA 1 mM). After that, the DNA pellet was air dried. To remove RNA 5µl of DNAse free RNAseA (10mg/ml) was added to the DNA. For 16S rRNA gene sequencing, two isolates (CM1MG1 and CM1MG2) were selected because they showed distinct features from other colonies. Using the primers 518F (5' CCAGCAGCCGGTAATACG 3') and 800R (5' TACCAGGGTATCTAATCC 3'), the 16S rRNA gene was amplified using PCR (Senthilraj et al., 2016). The approximately 1,400 bp purified PCR result was sequenced

The two sequences were obtained and put through BLAST analysis; phylogenetically related sequences were chosen from the NCBI Gene Bank and put through multiple sequence alignment; the aligned sequences were then cut to similar nucleotide lengths and put through phylogenetic tree construction (neighbor-joining) using MEGA 6. Partial sequences of 16S rRNA of the 2 isolates were deposited in the NCBI GenBank database to obtain accession numbers.

Antibiotic susceptibility test

The disc diffusion technique and susceptibility test discs (HiMedia, India) were used to assess antibiotic susceptibility in accordance with procedures outlined by the National Committee for Clinical Laboratory Standard (NCCLS,2012). The strains were classified as sensitive (zone diameter 20 mm), moderately sensitive (zone diameter 15–19 mm), or resistant (zone diameter 14 mm). The susceptibility of the isolates was evaluated using the antibiotics Chloramphenicol (30 g), Streptomycin (10 g), Tetracycline (30 g), Netilmicin (30 g), Ciprofloxacin (5 g), Doxycycline (330 g), Co-trimoxazole (25 g), and Nalidixicacid (30 g).

Detection of anticoagulant property

The capillary technique was used to determine the anticoagulant property. Non-heparinized capillary tubes in standard diameters were used for this. Fish blood samples were collected by severing the caudal peduncle of *Cirrhinus mrigala* following the method proposed by Hesser (1960) using tricaine methanesulfonate (MS-222; 50 mg/l) as an anaesthetic (Stetter 2001; Neiffer and Stamper 2009) and time count was initiated to enumerate the clotting time (Ct). Bacteria were cultured in nutrient broth media (30ºC, pH 7, 48 hours) centrifuged (4ºC, 4000 rpm,10 minutes) and mixed thoroughly with the blood sample by gentle shaking in a ratio of 1:1 within two 1.5 ml micro-centrifuge tubes. Un-inoculated nutrient broth kept under the same condition and mixed with blood in the same ratio was used as a control. Each of the blood mixes was immediately drawn in three capillary tubes, thereafter one end was sealed with plasticine. After every 5-second interval, a small part of the filled capillary tubes was broken and two fragments were separated gently. The end time point was noted as clotting time (Ct) as soon as the blood mix forms a continuous thread of clot between the broken ends of the tube (Hoffbrand and Steensma, 2019). The procedure was replicated five times to calculate the mean timing.

For further confirmation of Ct, an APTT test was carried out. A fish blood sample was prepared as per NCCLS Document H21-A3and mixed with the culture supernatant in a ratio of 1:1.The control was

the same as mentioned previously. The mixture was then discharged into an Automated Coagulometer (Synthasil APTT) by Instrumentation Laboratory, Italy, 2020; and the test was carried out as per operator manual provided by the company, and the clotting time was recorded.

Extra cellular enzyme assay

The bacterial isolates were evaluated for different extracellular enzyme production viz., amylase, protease, lipase, and cellulase. For the qualitative assessment of enzymes, selective agar media such as peptone-gelatin-agar media, starch-agar, carboxymethylcellulose (CMC)-agar, and tributyrin-agar-containing plates were utilized. Amylase (Bernfeld, 1955) and protease (Walter, 1984) activities were quantified based on the findings of a qualitative experiment and represented as units (U). It is possible to get a more thorough explanation of the quantitative measurement of these extracellular enzymes elsewhere (Bairagi et al., 2002).

Haemolysis assay

The hemolytic assay of bacterial isolates was performed following the procedure described by Vaughan *et al*. (2005), with minor modifications. The bacteria were spotted on sheep blood agar base plates (HiMedia) containing 5% sheep blood and incubated for 48 hours at 37°C. Bacterial colonies were classed as either haemolytic (having a greenish halo around the colony), non-haemolytic (having no halo around the colony), or hemolytic (having a clear zone surrounding the colony) based on whether or not they had a clear zone.

3. Results and Discussion

Isolation

On the plate, several autochthonous bacterial colonies were discovered. These colonies were distinguished by their colony morphology, such as form, size, color, margin opacity, elevation, etc. To produce pure culture for additional research, morphologically different and dominant colonies were chosen for repeated subculture by streaking on nutrient agar plate.

Microscopic, biochemical, and molecular characterization of the microbes

The outcomes of morphological, biochemical, and physiological characterization of the bacterial strains are depicted in Table 1a, 1b and 1c respectively. The two isolates viz. CM1MG1and CM1MG2 were Gram-negative, rod-shaped bacteria, and showed positive growth at ≤15ºC, 2–9% NaCl concentration (w/v), and between pH ranges of 4-9.The isolates were non-motile. Starch and gelatin were hydrolyzed by CM1MG2 only. Further CM1MG2 showed positive results for, the Voges-Proskauer, Indole, and Methyl red tests but CM1MG1had inverse results for these. CM1MG1 showed positive results for glucose, raffinose, galactose, and xylose but negative result for the rest whereas CM1MG1showed acid production from glucose, sucrose, lactose, and fructose, but negative for rhamnose, xylose.

Table 1a. Morphological characteristics of bacterial strains CM1MG1 and CM1MG2

Characteristics	CM1MG1	CM1MG2
Configuration	Round	round
Margin	Entire	entire
Elevation	Raised	raised
Pigment	White	white
Density	Opaque	opaque
Gram reaction	-ve rods	-ve rods
Spore	-ve	-ve
Motility	-ve	-ve

Table 1b. Growth performance of bacterial strains CM1MG1 and CM1MG2 at different culture conditions

Growth performance at different culture conditions								
Temperature (°C)	**CM1 MG1**	**CM1 MG2**	**pH**	**CM1 MG1**	**CM1 MG2**	**NaCl concentration (%)**	**CM1 MG1**	**CM1 MG2**
10	-	-	4.0	-	+	2.0	+	+
15	-	-	5.0	+	+	3.0	+	+
25	+	+	6.0	+	+	4.0	+	+
30	+	+	7.0	+	+	5.0	+	+
37	+	+	8.0	+	+	6.0	+	+
42	+	+	9.0	+	+	7.0	+	+
50	-	+				8.0	+	+
55	-	+				9.0	+	+

Table 1c.Biochemical characteristics of bacterial strains CM1MG1 and CM1MG2

Biochemical Tests			Acid productions from carbohydrates		
	CM1MG1	CM1MG2		CM1MG1	CM1MG2
Growth on MacConkey Agar medium	+	-	Glucose	+	+
Indole test	-	+	Fructose	-	+
Methylred test	-	+	Salicin	-	-
VogesProskauer test	-	+	Mannitol	-	+
Citrate test	-	+	Raffinose	+	+
Casein test	-	-	Sucrose	-	+
Starch hydrolysis	+	-	Rhamnose	-	-
Gelatin hydrolysis	-	+	Galactose	+	-
Nitrate reduction	-	+	Innositol	-	-
Catalase	+	+	Xylose	+	-
Oxidase	-	+	Maltose	-	+
Esculine Hydrolysis	-	+			
H_2S gas production	-	+			
Urease test	-	-			

Molecular characterization and phylogenetic tree construction

Based on the evolutionary study of the 16S rRNA gene sequence and the homology of the nucleotide sequence, it reveals that the strains CM1MG1 and CM1MG2 were 99.81% and 99.71% similar to the

type strains *Acenetobacter baumanii* (LN611355.1.) and *Aeromonas hydrophila* (NR_074841.1) respectively. Type strains for both the isolates had been retrieved from NCBI GenBank. The dendrograms show the evolutionary relationship between two chosen strains and their closely related type strains that were obtained from NCBI GenBank (Figure 1). Submission of the sequences of the isolates to NCBI GenBank provided the accession numbers MW811800 and MW806655 for the respective strains

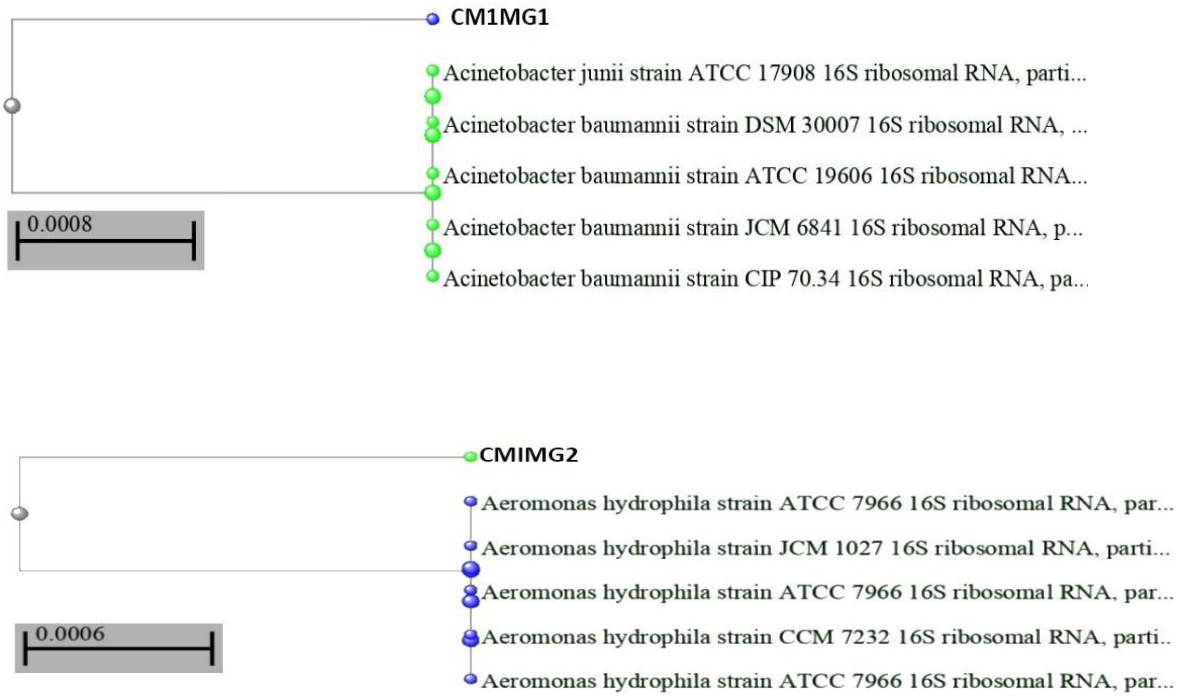

Figure 1: Dendogram of CM1MG1 and CM1MG2, isolated from *Argulus bengalensis* gutdisplaying their phylogeneticrelationshipstoothercloselyrelatedtypestrains based on NCBI algorithm.

Antibioticsensitivitytest

The results of the antibiotic tests are mentioned in Table 2.The CM1MG1 was found to be sensitive to antibiotics like tetracycline, ciprofloxacin and doxycycline, whereas moderately sensitive to co-trimoxazole and streptomycin. However, CM1MG2 was found sensitive to all the discs.

Table2: SensitivityofdifferentdrugsonCM1MG1 and CM1MG2

Chemical	DiscContent (µg)	CM1MG1	CM1MG2
Chloramphenicol	30	R	S
Streptomycin	10	R	M
Tetracyclin	30	M	S
Netilimycin	30	M	M
Ciprofloxacin	5	S	S
Doxycycline	30	S	M
Cotrimoxazole	25	M	S
Nalidixicacid	30	R	M

S= susceptible, M= moderately susceptible, R= Resistant

Anticoagulant assay

It has been estimated that the mean clotting time of the blood sample combined with culture supernatant is longer than that of the control blood sample. The blood combined with CM1MG1 and CM1MG2 exhibited mean clotting times of 15.8 and 21.6 seconds, respectively, compared to the control blood's mean clotting time of 11.8 seconds. The APTT test also revealed that the clotting time

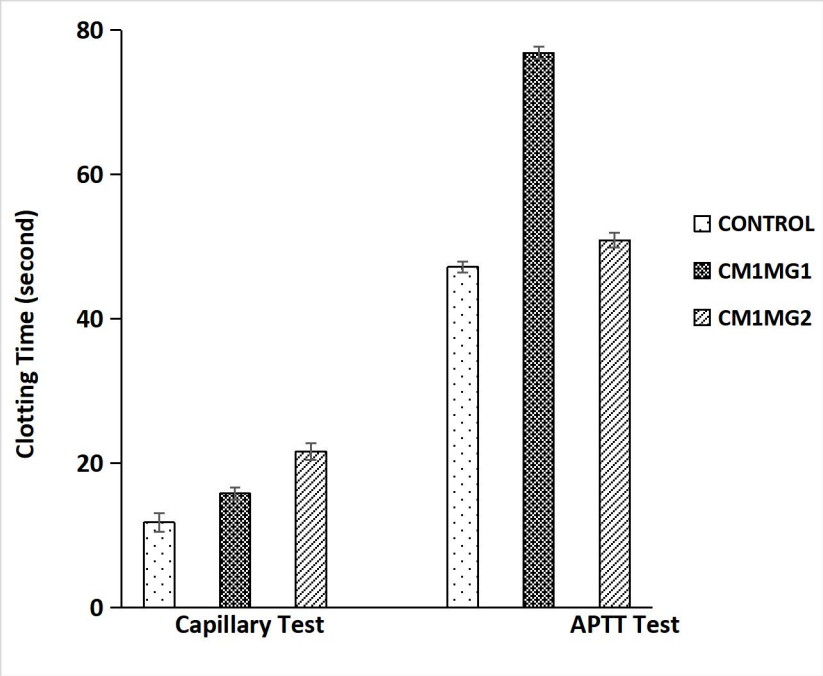

Figure 2: Clotting time of fish blood mixed with culture supernatant of CM1MG1 and CM1MG2 determined through capillary test and APTT test

Extra cellular enzyme assay

The lipase and cellulase activities were beyond the level of detection in both isolates. CM1MG1 exhibited amylase activity and it was 3.06±0.14 mg/ml/hr, whereas CM1MG2exhibited protease activity and it was 0.87±0.02µg/ml/min which have been presented in Figure 3.But the value of protease activity in CM1MG1 and amylase activity in CM1MG2 were not of much significance.

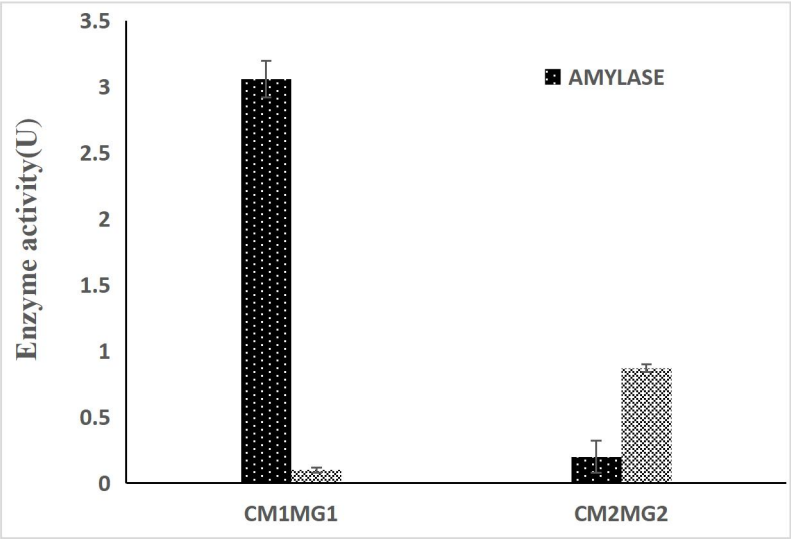

Figure 3: Enzymatic activities of bacterial strain CM1MG1 and CM1MG2

Haemolysis assay

Both the isolates were hemolytic as CM1MG1and CM1MG2 both showed a clear halo zone around the colonies grown on to sheep blood agar media plates, thus were β hemolytic in nature

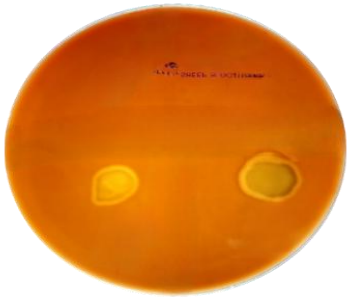

Figure 4a: β Haemolysis shown by CMIMG1

Figure 4b:β Haemolysis shown by CMIMG2

Figure 4: β Haemolysis shown on blood agar plate by the two isolates CM1MG1and CM1MG2 respectively

Symbiotic associations have been overwhelmingly emphasized on the role of endosymbionts and more specifically of gut microbiota in their host nutrition (Clark et al., 1992; Douglas and Prosser, 1992; Kaya and Gaugler, 1993). By secreting digesting enzymes and adding vitamins, the nutritional contributions of the symbiont frequently enable the affected host to thrive on less-than-ideal diets. For endosymbionts like *Buchnera*sp. these nutritional contributions are well documented (Douglas, 1998). But in many instances, the bacterial population in the gut could provide similar benefits

The *Argulus* sp. used in this investigation were kept on fasting for 4 hours and surface sterilized before isolating their gut microflora. As a result, it may be assumed that these isolates are considered as part of the autochthonous adherent microflora, as claimed by Ghosh et al. (2010). In addition, culture-based investigation of the leech gut microbiome revealed, *Aeromonas* sp. to be an exclusive and persistent symbiont of leech (Nelson and Graf, 2012). The study also shows that two bacterial isolates may survive in a wide pH range, supporting the use of the gut as an appropriate biotope. Almost exclusively free-living, *Acenetobacter* is frequently seen in the water or on the skin of animals (Doughari et al., 2011).The piscine host of *Argulus* sp. are ecologically equivalent of those bacterial flora. Moreover, argulids complete their life cycle in the same aquatic environment. Therefore, environmental sharing may facilitate the bacteria's connection with *Argulus*. Kar et al. (2008) claimed that carbohydrates such as mannose, xylose, raffinose, and cellulose may be used by gut bacteria to produce enzymes. Therefore, the results of carbohydrate consumption of both isolates' indicate that they are enzyme-producing gut bacteria that can function on a variety of substrates.

Interference with the bacterial growth may impose negative impact upon survival of *Argulus* spp. The results of the antibiotic susceptibility test may also provide a reference for potential treatment of fish against *Argulus* infestation. The inhibition of the clotting of blood is an important adaptation associated with haematophagy (Schofield et al., 1993; Gomes et al., 2005). Anticoagulant assay of the bacteria shows anticoagulant properties as the clotting time gets delayed in the presence of culture supernatant. Similar instances were reported by Bykowska et al. (1985) and El Akoum et al. (1987) about the production of substances possessing anticoagulant properties by various bacteria like *Staphylococcus epidermis* and *Myxococcus xanthus* respectively. As *Argulus bengalensis* predominantly feeds on tissue fluid and blood of the fish host (Saha et al., 2011) it may be apprehended that these bacteria facilitate in blood feeding and digestion. A positive result of protease and amylase activity of both the isolates respectively shows contribution in the digestion of blood.

Minard et al. (2013) revealed the contribution of *Acenetobacter baumanii* in digesting various components specific to blood in the mosquito gut. Whereas *Aeromonas* sp. is found as digestive tract symbionts of varied blood-feeding organisms including mosquitoes, medicinal leech, and the vampire bat (Pinus and Müller, 1980; Graf, 1999, 2000; Pidiyar *et al*., 2002).Taking into consideration, that the important step of blood digestion is the release of hemoglobin, the major blood protein, it was hypothesized that the gut bacteria may have a putative functional role in lysis of RBC. The hemolytic

ability of both isolates marks their potential contribution to blood digestion. A mutualistic relationship

4. Conclusion

Acenetobacter baumanii and *Aeromonas hydrophila* are compatible strains using the gut of *Argulus* as a biotope and establishing a mutual relationshipincreases the fitness of the parasitic host facilitating haematophagy. Interruption of this association may be useful for controlling *Argulus* sp. from aquaculture system.

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