ISSN: 2347-5129
IJFAS 2014; 2(1): 72-78

www.fisheriesjournal.com
Received: 24-07-2014
Accepted: 14-08-2014

Pinki Ghosh
Fisheries Laboratory Department of Zoology Visva-Bharati University, Santiniketan 731 235 West Bengal India

Arun Kumar Ray
Fisheries Laboratory Department of Zoology Visva-Bharati University, Santiniketan 731 235 West Bengal India

Correspondence:
Arun Kumar Ray
Fisheries Laboratory Department of Zoology Visva-Bharati University, Santiniketan 731 235 West Bengal

Effects of duckweed (*Lemna polyrhiza*) meal incorporated diet on enzyme producing autochthonous gut bacteria in fingerling mrigal, *Cirrhinus mrigala* (Hamilton)

Pinki Ghosh, Arun Kumar Ray

Abstract
Effects of duckweed (*Lemna polyrhiza*) meal based diet on growth performance and enzyme-producing autochthonous gut bacteria of mrigal (*Cirrhinus mrigala*) fingerlings were investigated in a feeding trial lasting for 90 days. *Lemna* leaf meal incorporated diet resulted in the best growth performance of mrigal fingerlings. Population levels of amylase and cellulase-producing bacterial strains was recorded in higher densities than the protease-producing strains in the group of fish fed *Lemna* meal diet. The isolated gut bacteria were qualitatively screened and quantitatively assayed for amylase, cellulase, and protease activities. The *Lemna* meal diet resulted in increased activities of amylase by CMF6, isolated from the proximal intestine (PI) and cellulase by CMH9, isolated from the distal intestine (DI). The results indicate diet-associated differences in the production of extracellular enzymes by the gut bacterial community.

Keywords: Duckweed, Diet, Growth performance, Fish gut bacteria, *Cirrhinus mrigala*

1. Introduction
Considering the importance of nutritionally balanced and cost-effective artificial diets for fish, there is an increasing research effort to evaluate the nutritive value of different non-conventional feed resources, including terrestrial and aquatic macrophytes [1, 2, 3, 4]. The aquatic weeds have been shown to contain substantial amounts of protein and minerals [5]. These weeds, which otherwise remain unutilized, and often make the water body unsuitable for fish culture, may be converted into valuable fish flesh through their incorporation as an ingredient in carp diets. *Lemna polyrhiza*, commonly known as duckweed, grows luxuriantly in freshwater bodies in the tropical and subtropical areas including India and Bangladesh [6]. These weeds, which otherwise remain unutilized and often makes the water body unsuitable for fish culture, may be converted into valuable fish flesh through their incorporation as a feedstuff in carp diets.
A wide range of microbes derived from the surrounding aquatic environment, soil/sediment and feed are found to colonize in the gastrointestinal (GI) tract of fish. The GI tract of fish consists of a very complex and dynamic microbial ecosystem that is very important from a nutritional, physiological and pathological point of view. These microbial populations also provide a level of protection against pathogenic visitors to the GI tract and aid host digestive function via the production of exogenous digestive enzymes and vitamins [7]. One of the most important aspects of ecology of the GI tract microbiota is the understanding of how dietary factors influence the GI tract microbiota [8]. The resident microbiota may provide exogenous enzymes and fermentation products to fish with various feeding habits, especially when their diet is rich in recalcitrant substrates such as fibre [9]. The interest of the possible role of gut microbiota in fish digestion emerged with the increasing proportion of plant protein sources introduced in fish feed to compensate for the shortage of fish meal [10, 11]. However, reports on the effect of plant protein sources on gastrointestinal microbiota in fish are scanty [8, 12, 13, 14]. The present study, therefore, aims to (a) assess the growth response, and (b) evaluate enzyme- producing bacteria isolated from the GI tract of the fingerling of an Indian major

carp, mrigal, *Cirrhinus mrigala* fed duckweed, *Lemna polyrhiza* leaf meal incorporated diet.

2. Materials and methods

2.1. Experimental diets

Two diets were prepared using fish meal or *Lemna polyrhiza* meal as the major protein source (Table 1). The sun-dried *L. polyrhiza* leaves were finely ground and passed through a fine meshed sieve to ensure homogeneity. The duckweed leaf meal contained 18.6% crude protein and 11.0% crude fibre on dry matter basis whereas, fish meal contained 58.5% crude protein and 3.9% crude fibre, respectively. Diets were made isonitrogenous (35% crude protein) and isolipidic (8.5%). Dry feed ingredients were mixed and the diets were prepared in pelleted form using 0.5% carboxymethylcellulose as a binder. The pellets were sun dried for a few days and crumbled prior to feeding.

Table 1: Composition (g/ kg dry weight) of the diets

Ingredients	g/ kg diet	
	Fish meal Diet	***Lemna* meal**
Fish meal	400	300
Lemna meal	-	400
Mustard oil cake	230	280
Rice barn	350	_
Cod liver oil	10	10
Vitamin Premix[a]	10	10

[a] Vitamin and mineral mixture (Vitaminetes forte, Roche Products India Private Limited, Mumbai, India)

2.2. Experimental design

The experiment was conducted in flow-through 90 l circular fibre-glass tanks. Mrigal (*Cirrhinus mrigala*) fingerlings, obtained from a local fish farm, were acclimatized to the laboratory conditions for 15 days and fed with a mixture of rice bran and mustard oil cake. The fingerlings (mean weight 5.7 ± 0.34 g) were randomly distributed at the rate of 15 fish per tank with three replicates for each treatment. Each experimental tank was supplied with unchlorinated water from a deep tube well with continuous aeration. All the fish were fed once daily at 1000 h at a fixed feeding rate of 3% body weight per day for 90 days. The quantity of feed given was readjusted every 15th day after weighing the fish. Weight gain (%), specific growth rate (SGR, %/day), feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated using standard methods [15].

2.3. Bacterial cultivations

Isolation of bacteria was done from the intestine of *C. mrigala* fingerlings both prior to and at the termination of the feeding trial. In each case, the test fish were starved for 36 h prior to sacrifice to clean the intestine. The fish were carefully placed aseptically within laminar airflow on ice slabs and their intestines were removed and cleaned with sterile physiological saline solution. The intestine was divided into proximal (PI) and distal intestine (DI) as described by Ringø and Strøm [16]. The digestas from the two regions of the gut were squeezed out. Thereafter, both the regions of the gut were cleaned, slit opened by a longitudinal incision, transferred to sterile Petri dishes, and thoroughly flushed with sterilized chilled 0.9% saline in order to remove non-adherent or allochthonous bacteria. The two regions of the alimentary tract were separately homogenized with 10 parts of chilled 0.9% sodium chloride solution [17]. Bacteria associated with gut were quantified as log total viable count (TVC) per g intestinal tissue using three different types of agar. The total numbers of resident or autochthonous aerobic bacteria were estimated with plate count agar (Tryptic soy agar, TSA). For isolation and enumeration of protease-, amylase- and cellulase-producing bacteria, the diluted gut homogenates were spread onto the surface of peptone-gelatin agar, starch-agar and carboxymethylcellulose (CMC)-agar media plates, respectively.

2.4. Enzyme-producing capacity of selected bacterial strains

The isolated strains from the PI and DI of the test fish were screened for the production of extracellular protease, amylase and cellulase on agar plates of the selective media, namely, peptone-gelatin agar, starch-agar, and carboxymethylcellulose (CMC) - agar, respectively [18]. Qualitative extracellular enzyme activities were assessed based on the measurement of a clear zone (halo) around the colonies as follows: + (low, 5-14 mm halo diameter), ++ (moderate, 15-24 mm halo diameter), and +++ (high, 25-35 mm halo diameter).

After primary qualitative screening, the selected strains were cultured in selective liquid medium for quantitative enzyme assay. The strains were cultured in 4% tryptone soya broth for 24 h at 37±1 ^{0}C and used as the inoculum. Liquid production medium of 20 ml was inoculated with 0.5ml of inoculum obtained from the seed culture and incubated for 48-96 h at the same temperature. The contents of the culture flasks were centrifuged (9,000×*g*, 10 m, 4 ^{0}C), and the cell-free supernatant was used for enzyme assay. The protein content of the crude enzyme extract was estimated according to Lowry *et al*. [19]. The quantitative assay of amylase, cellulase and protease was performed using the methods described by Bernfeld [20], Denison and Kohen [21] and Walter [22], respectively. Specific enzyme activity was expressed as a unit (U)/mg protein.

3. Results

The growth performance and feed utilization of *C. mrigala* fingerlings in terms of percentage weight gain, SGR, FCR and PER fed with fish meal and *Lemna* diet are presented in Table 2. The performance of fish in terms of weight gain (%), SGR FCR, PER was better with 30% *Lemna* meal in comparison to the diet containing 40% fish meal.

Table 2: Growth and feed utilization efficiencies in *C. mrigala* fingerlings fed experimental diets for 90 days

Mean values	Fish meal diet	*Lemna* diet
Initial weight (g)	5.74 ± 0.34	5.74 ± 0.34
Final weight (g)	13.7 ± 0.42	14.3 ± 0.45
Weight gain (%)	138.6 ± 0.10	149.13 ± 0.14
SGR (%/ day)	0.977 ± 0.02	1.022 ± 0.02
FCR	2.39 ± 0.08	2.32 ± 0.09
PER	1.36 ± 0.04	1.19 ± 0.03

The population of cultured aerobic bacteria in the GI tract of mrigal on TSA plate showed no change in the log total viable count (log TVC) in the PI and DI before the commencement of the feeding trial (Table 3). Enumeration

of specific enzyme-producing bacteria showed that the amylolytic and cellulolytic strains were present at the highest population level in the PI, whereas highest count of proteolytic strains was recorded in the DI (Table 3).

Table 3: Log total viable counts of aerobic bacteria in the GI tract of *Cirrhinus mrigala* before feeding trial.

Gut region	Log total viable counts /g intestinal tissue			
	Total bacterial count (TSA)	Amylase-producing bacteria	Cellulase-producing bacteria	Protease-producing bacteria
Proximal Intestine (PI)	6.02	5.13	3.30	4.18
Distal Intestine (DI)	6.03	4.65	2.35	5.16

The bacterial profile in the PI and DI of mrigal fingerling after feeding fish meal and *Lemna* meal diets is depicted in Table 4. The total count of bacteria on TSA plate was recorded highest in the DI in both the dietary treatments. The population of amylolytic, cellulolytic and proteolytic strains, however, exhibited higher densities in the PI of mrigal in both the dietary treatments except the cellulase producing strains in the fish fed fish meal diet. The population of amylase and cellulase-producing strains was recorded in higher densities than the protease-producing strains in the group of fish fed *Lemna* meal diet.

Table 4: Log total viable counts of aerobic bacteria in the GI tract of *C. mrigala* after feeding with fish meal and *Lemna* meal diets

Gut region	Diets	Log total viable counts /g intestinal tissue			
		Total bacterial count (TSA)	Amylase-producing bacteria	Cellulase-producing bacteria	Protease-producing bacteria
PI	Fish meal	5.78	5.24	4.22	5.10
	Lemna meal	7.94	7.47	6.31	5.51
DI	Fish meal	5.80	5.24	4.32	4.15
	Lemna meal	8.28	6.54	5.76	4.22

Extracellular enzyme production by bacterial strains isolated from the digestive tract of mrigal was assayed qualitatively both before and after feeding trial (Tables 5 and 6). Initial screening of bacteria for enzyme production showed that most of the strains isolated from the PI and DI grew and produced translucent zones on specific culture plates. Among these, strains CMF8 and CMF1 isolated from the PI displayed the largest translucent zones on starch-agar and peptone-gelatin agar plates, respectively before commencement of feeding trial (Table 5). On the other hand, strains CMH8 and CMH9 isolated from the DI exhibited the largest translucent zone on starch-agar plates only. Most of the bacterial strains isolated from the PI and DI of mrigal fed fish meal diet displayed the largest translucent zones on starch-agar and peptone-gelatin-agar plates whereas, most of the strains isolated from the GI tract of mrigal fed *Lemna* meal diet exhibited large translucent zones on CMC-agar and peptone-gelatin-agar plates (Table 6).

Table 5: Qualitative extracellular enzyme activity in the bacterial strains isolated from the GI tract of *C. mrigala* before commencement of feeding trial

Gut Region	Strain designation	Amylase Activity	Cellulase Activity	Protease Activity
PI	CMF1	+	+	+++
	CMF2	++	++	+
	CMF4	++	++	++
	CMF5	+	-	++
	CMF6	+	-	++
	CMF8	+++	++	+
DI	CMH2	+	-	+
	CMH4	+	-	+
	CMH7	+	-	+
	CMH8	+++	++	++
	CMH9	+++	++	+

+ (low, 5-14 mm halo diameter), ++ (moderate, 15-24 mm halo diameter), +++ (high, 25-35 mm halo diameter)

Table 6: Qualitative extracellular enzyme activity in the bacterial strains isolated from the GI tract of *C. mrigala* after feeding with fish meal and *Lemna* meal diets

Fish meal diet				
Gut Region	**Strain designation**	**Amylase Activity**	**Cellulase Activity**	**Protease Activity**
PI	CMF1	++	+	++
	CMF2	+	+	-
	CMF4	+++	++	++
	CMF5	++	+	+++
	CMF6	+++	++	+++
	CMF8	++	-	-
DI	CMH2	++	+	+
	CMH4	++	-	-
	CMH7	+	++	++
	CMH8	+++	++	++
	CMH9	+++	++	+++
***Lemna* meal diet**				
PI	CMF1	++	+++	+++
	CMF2	+	+	++
	CMF4	-	+++	-
	CMF5	+	+++	+++
	CMF6	+++	+	+
	CMF8	++	-	-
DI	CMH2	+	-	+
	CMH4	+	++	-
	CMH7	++	+	+
	CMH8	+	+++	+++
	CMH9	+++	+++	+++

+ (low, 5-14 mm halo diameter), ++ (moderate, 15-24 mm halo diameter), +++ (high, 25-35 mm halo diameter)

The same11 bacterial strains as those used for qualitative extracellular enzyme activity were also investigated for quantitative enzyme activity both prior to and after feeding trial (Tables 7 and 8). Before feeding trial, the highest specific activities of amylase and cellulase were exhibited by the bacterial strains CMF8 (16.87±0.63 U/mg protein) and CMH7 (1.19±0.021 U/mg protein) isolated from the PI and DI, respectively. The maximum specific activity of protease was observed in strain CMF6 (0.71±0.036 U/mg protein) isolated from the PI. Cellulase activity was not detected in the bacterial strains CMF6 and CMF8, isolated from the PI. Protease activity was also not detected in the strain CMF8 (Table 7). Distinct changes in the quantitative enzyme activities in the bacterial strains were noticed after feeding with fish meal and *Lemna* meal diets (Table 8).

Table 7: Quantitative extracellular enzyme activity in the bacterial strains isolated from the GI tract of *C. mrigala* before commencement of feeding trial

Gut Region	Strain designation	Specific amylase activity(U/ mg protein)*	Specific cellulase activity (U/mg protein)#	Specific protease activity(U/ mg protein)†
PI	CMF1	2.80 ±0.80	0.68 ±0.035	0.15 ±0.036
	CMF2	1.49 ±0.026	0.28 ±0.043	0.08 ±0.026
	CMF4	9.24 ±0.62	0.18 ±0.026	0.20 ±0.026
	CMF5	1.60 ±0.046	1.00 ±0.044	0.27 ±0.026
	CMF6	2.03 ±0.030	ND	0.71 ±0.036
	CMF8	16.87 ±0.63	ND	ND
DI	CMH2	8.59 ±0.33	0.57 ±0.01	0.25 ±0.026
	CMH4	4.98 ±0.026	0.56 ±0.032	0.2 ±0.10
	CMH7	1.40 ±0.030	1.19 ±0.021	0.13 ±0.015
	CMH8	14.18 ±0.26	0.23 ±0.055	0.46 ±0.020
	CMH9	10.93 ±0.26	0.32 ±0.030	0.06 ±0.006

Data are means ± SE of three determinations.

*U = µg maltose liberated/ml of culture filtrate/min

#U = µg glucose liberated/ml of culture filtrate/min

†U = µg tyrosine liberated/ml of culture filtrate/min

ND = Not detected

In the fish fed fish meal diet, highest specific activities of amylase, and cellulase were exhibited by the strains CMH8 (56.28 ±0.72 U/mg protein) and CMH7 (1.44 ±0.36 U/mg protein), respectively, isolated from the DI, whereas highest protease activity was recorded in the strain CMF6 (1.67 ±0.33 U/mg protein) isolated from the PI. The *Lemna* meal diet resulted in increased activities of amylase (58.68 ±0.95 U/mg protein, by CMF6, isolated from the PI) and cellulase (34.70 ±0.49 U/mg protein by CMH9, isolated from the DI).

Table 8: Quantitative extracellular enzyme activity in the bacterial strains isolated from the GI tract of *C. mrigala* after feeding with fish meal and *Lemna* meal diets

Fish meal diet				
Gut Region	**Strain designation**	**Specific amylase activity (U/mg protein)***	**Specific cellulase activity (U/mg protein)#**	**Specific protease activity (U/ mg protein)†**
PI	CMF1	7.60 ±0.083	0.52 ±0.20	0.24 ±0.021
	CMF2	1.56 ± 0.02	0.30 ± 0.03	0.09 ±0.03
	CMF4	15.32 ±0.6	1.22 ±0.33	0.36 ±0.20
	CMF5	3.36 ± 0.57	0.18 ±0.03	0.45 ±0.16
	CMF6	52.14 ±1.48	1.42 ±0.23	1.67 ±0.33
	CMF8	17.22 ± 0.62	ND	ND
DI	CMH2	9.24 ±0.32	0.59 ±0.01	0.30 ±0.03
	CMH4	5.98 ±0.04	0.69 ±0.03	0.22 ±0.1
	CMH7	2.67 ±0.52	1.44 ±0.36	0.18 ±0.01
	CMH8	56.28 ±0.72	0.54 ±0.02	0.63 ±0.21
	CMH9	16.06 ±0.46	0.81 ±0.54	0.82 ±0.26
***Lemna* meal diet**				
PI	CMF1	19.00 ±0.255	25.82 ±0.48	0.61 ±0.015
	CMF2	13.87 ±0.097	2.18 ±0.38	0.17 ±0.045
	CMF4	12.29 ±0.36	6.68 ±0.18	0.19 ±0.020
	CMF5	24.89 ±0.37	1.95 ±0.29	0.03 ±0.01
	CMF6	58.68 ± 0.95	1.95 ± 0.29	1.76 ± 0.03
	CMF8	17.82 ± 0.42	0.00 ± 0.00	0.00 ± 0.00
DI	CMH2	9.84 ± 0.33	0.62 ± 0.02	0.40 ± 0.04
	CMH4	9.48 ±1.25	4.49 ±0.59	0.04 ±0.011
	CMH7	3.46 ± 0.12	2.16 ± 0.10	0.21 ±0.03
	CMH8	12.21 ±0.55	20.5 ±0.29	0.10 ±0.035
	CMH9	4.31 ±0.53	34.70 ±0.49	0.55 ±0.08

Data are means ± SE of three determinations.
*U = µg maltose liberated/ml of culture filtrate/min
#U = µg glucose liberated/ml of culture filtrate/min
†U = µg tyrosine liberated/ml of culture filtrate/min
ND = Not detected

4. Discussion

It is evident from the present study that duckweed meal can be utilized as a feed ingredient in the diets for the mrigal fingerlings effectively. Growth performance, PER and FCR of mrigal fingerlings were better with 30% *Lemna* leaf meal incorporated diet than with the fish meal diet. Das and Ray [23] demonstrated the possibility of incorporation of dried *Lemna polyrhiza* as a feed ingredient for rohu, *Labeo rohita* fingerlings. Bairagi *et al.* [2] also recorded improved growth of *L. rohita* fingerlings fed 30% *Lemna* meal incorporated diet in comparison to the reference diet containing 40% fish meal. Shireman and Smith [24] considered duckweed (*Lemna* sp.) as a highly nutritious vegetable food for grass carp because of its tenderness and high protein content compared to other aquatic weeds. Fish do not possess the enzyme cellulase to hydrolyze cellulose, which is the main ingredient of plant cell walls and the possible presence of a persistent population of cellulolytic microflora in fish gastrointestinal tract is a subject of much controversy [25, 26, 27, 28, 29, 30]. Bairagi *et al.* [31] isolated certain carboxymethyl cellulase-producing strains from the GI tract of Indian major carps and Chinese carps. In the present study, the population of amylase and cellulase-producing strains were recorded in higher densities in the GI tract of the fish fed *Lemna* meal incorporated diet. Better growth of mrigal fingerlings with *Lemna* diet may, therefore, be attributed to the higher population of amylase and cellulose -producing bacteria in the GI tract of the fish.

Generally, bacteria are abundant in the environment in which fish live and it is therefore, rather impossible to avoid them being a component of their diet [32, 33]. The bacteria enter along with the diet of fish during ingestion may adapt themselves in the gastrointestinal tract and form a symbiotic association [34]. Within the digestive tract of fish large numbers of microbes are present [7], which is much higher than in the surrounding water indicating that the digestive tracts of fish provide favourable ecological niches for these organisms [35,36,37,38]. While the digestive tracts of endotherms are colonized mainly by obligate anaerobes [39], the predominant bacterial genera/species isolated from most fish guts have been aerobes or facultative anaerobes [31, 35, 36, 38, 40]. In the present study, attention has been focused on the aerobic gastrointestinal bacteria. In the present investigation, the population of amylase and cellulose -producing strains was recorded in higher densities than the protease-producing strains in the group of fish fed *Lemna* meal diet. Ray *et al.* [41] also detected a huge population of amylase-producing bacteria

in the GI tract of three Indian major carps, catla (*Catla catla*), mrigal (*Cirrhinus mrigala*) and rohu (*L. rohita*), where amylase production was considerably higher by the strains isolated from the proximal intestine of catla and mrigal. The results of the present study corroborate the earlier reports that during ingestion of diet the bacteria may adapt themselves in the GI tract and form a symbiotic association and contribute in the process of digestion through production of extracellular enzymes [34, 40, 42].

An understanding of the contribution of endosymbionts to digestion requires information on the relative importance of exogenous (produced by gastrointestinal endosymbionts) and endogenous (produced by the host) digestive enzymes [9]. In the present study, the selected strains isolated from both proximal and distal intestine were quantitatively assayed for cellulase, amylase and protease activities to ascertain their role in exogenous production of digestive enzymes. Increased activities of amylase and cellulase were noticed in the bacterial strains CMF6 and CMH9 isolated from the PI and DI, respectively of mrigal fed *Lemna* meal diet. In contrast to the cellulase activity, amylase production was considerable by the strains isolated from both the the PI and DI of mrigal, except the strains CMF1 and CMH9, isolated from the PI and DI, respectively. The most important group of exogenous enzymes in symbioses between terrestrial vertebrate herbivores and microorganisms are cellulases, which degrade the cell walls of vascular plants [9]. A number of studies have examined cellulase activity in the alimentary tracts of fishes, with mixed results. Much of the controversy concerning the source of cellulase activity in the intestinal tract of fish has arisen due to the inability to isolate cellulase-producing microorganisms from the intestinal contents and to document diet-related fluctuations in the level of cellulase activity [29].

The activity of carbohydrases in general, and of amylase in particular, differs from species to species, and appears to be related to their feeding habits [43]. Das and Tripathi [17] reported high amylase activity in the GI tract of grass carp, *Ctenopharyngodon idella*, which appeared to be the result of its omnivorous feeding habit. Mondal *et al.* [34] (2008) also detected a considerable population of amylolytic bacteria in the fish species with herbivorous and omnivorous feeding habits. In the present investigation, in contrast to the cellulase activity, exogenous amylase production was intense in both the PI and DI of mrigal fed *Lemna* meal diet. The study indicates that there is a distinct microbial source of amylase and cellulase in the GI tract, which helped the fish to digest the complex carbohydrate like cellulose.

5. Conclusion

The information generated from the present investigation indicates that the type of food can influence the autochthonous enzyme-producing microbial community in the GI tract of *Cirrhinus mrigala*. As evidenced in this study, higher population of amylolytic and cellulolytic bacteria was registered in the GI tract of mrigal fed *Lemna* meal incorporated diet when compared to the fish fed fish meal incorporated diet. However, further investigations are required to know about the metabolic pathways used by these microorganisms in the GI tracts of fish. Investigations are in progress to identify the most promising enzyme-producing bacteria by 16S rDNA sequence.

6. Acknowledgements

We are grateful to the University Grants Commission, New Delhi for financial support.

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