STUDIES ON BACTERIAL FLORA ASSOCIATED WITH CULTIVABLE CARP SPECIES

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CERTIFICATE-I

This is to certify that the thesis entitled "STUDIES ON BACTERIAL FLORA ASSOCIATED WITH CULTIVABLE CARP SPECIES" submitted for the degree of **Master of Fishery science** in the subject of Aquaculture of the Orissa University of Agriculture and Technology, Bhubaneswar is a faithful record of bonafide and original research work carried out by **Pratap Kumar Dash** under my guidance and supervision and that no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled "**STUDIES ON BACTERIAL FLORA ASSOCIATED WITH CULTIVABLE CARP SPECIES**" submitted by **Pratap Kumar Dash** to the Orissa University of Agriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the degree of **Master of Fishery Science** in the subject of **Aquaculture** has been approved by the students advisory committee after an oral examination on the same in collaboration with an external examiner

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LIST OF ABBREVIATIONS AND SYMBOLS

cfu	:	colony forming unit
cm	:	centimetre
E	:	East
ed.	:	editor
eds.	:	editors
et al.	:	and others
etc.	:	and so on
Fig.	:	Figure
Figs.	:	Figures
g	:	gram
lb	:	pound
i.e.	:	that is
ml	:	millilitre
m	:	metre
mm	:	millimetre
N	:	North
p	:	probability
pH	:	hydrogen ion concentration
psi	:	per square inch
sp.	:	species (singular)
spp.	:	species (plural)
viz.	:	namely
%	:	percent
<	:	less than
'	:	minute
°	:	degree
°C	:	degree celsius
"	:	second

CHAPTER - 1

INTRODUCTION

1. INTRODUCTION

Indian freshwater aquaculture is mainly carp-based and carp polyculture has attained the status of an industry in the recent years. Good seed, nutritionally adequate feed and proper water quality management are three basic requisites for successful aquafarming, among which feed alone contributes to more than 50% of the production cost. Though natural pond productivity sustains the fish production to a considerable extent, further intensification of management practices requires critical input of supplementary feeds (Alikunhi, 1952). Hence, the aspect of feeding has great importance in achieving high fish production levels under intensive management practices.

Low cost, easy availability, easy digestibility, easy acceptability and longer duration of water stability are the basic criteria for a good feed. Present palletised feeds, formulated, based on the nutritional requirements, used in carp culture are costly due to its high cost ingredients. So, in order to decrease the production cost and economising the carp culture, effort are made at incorporation of a wide variety of low cost, easily available ingredients from plants and unconventional sources with varying degrees of success (Pattanaik and Das, 1979; Niamat and Jafri, 1984; Nandeesha *et al.*, 1986, 1989a; Patra and Ray, 1988).

One of the problems in use of ingredients from plant sources is their digestibility. High content of cellulose and crude materials limits their digestibility. Processing of the feed material to render them more digestible is an important consideration in the use of an array of feed ingredients for the purpose. Bacteria play a vital role in transforming these ingredients from unusable to usable form.

Among the physical, chemical and biological process of processing,the use of bacterial additives for lignocellulosic decomposition has received due attention by the researchers.

In case of ruminants, crude fibrous materials can be converted to useful energy, because of presence of a compartment in the fore part of the digestive tract, where bacteria break down these substances. In case of fish, in spite of lack of such a compartment, some fishes like tilapia and carps can utilise higher plant material as food, and the presence and characteristics of a bacterial flora in the digestive tract are known in these fishes (Trust and Sparrow, 1974; Hamid *et al.,* 1979; Lesel, 1981). Several enzyme activities may also be due to presence of a specific bacteria, some of which have been isolated in various species of fish. The nutritional importance of bacteria has been given due consideration in recent years (Ayyappan *et al.*, 1990b). The bacterial synthesis could have economic implications, as information about the rate of bacterial synthesis of nutrients in cultured species and associated influencing factors would help to reduce the amount of raw nutrients in commercial diets (Lovell, 1981).

The heterotrophic bacteria, the link between biotic and abiotic components, also play an important role in nutrient recycling and maintenance of water quality. Production efficiency of aquafarming systems is, in addition to other factors, influenced by microbiological parameters of the water, sediment and microbiological profile of the farmed animals. Hence, fishes and other aquatic life forms are prone to environmental hazards. Environmental stress may upset the balance between potential pathogen and their hosts to create disease conditions.

Bibliographical analysis of microflora associated with fishes indicates that most of the works in this field have been dealt with marine fishes (Shewan, 1961; MacFarlance *et al.*, 1986; MacCormack and Fraile, 1989-90). The limited

information available with regard to freshwater fish pertains to microbiological studies in aquaculture and relating to public health or fish health (Fang *et al.*, 1989; Ogbondeminu *et al.*, 1991; Ogbondeminu and Okoye, 1992; Ogbondeminu, 1994). Microbiological studies indicating nutritive role of gut microflora are scanty (Trust and Sparrow, 1974; Henebry *et al.*, 1988; Moriarty, 1989; Henson, 1990).

Considering the importance of analysis of the gut microflora of cultivable carp species with reference to their feeding habits and environments, an attempt was made to assess the bacteriological profile of the carp species with reference to digestive enzyme activities. The investigation on the heterotrophic microflora associated with carps (catla, rohu, mrigal and grass carp) and the gut enzyme profile was carried out at the Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar during July-December, 1995. The objectives of the study were:

i. Quantitative evaluation of heterotrophic bacterial loads of body surface, gills and gut of carps;

ii. Qualitative evaluation of the digestive enzyme activities of different regions of the gut and

iii. Characterisation of bacterial flora of gut of carp species.

For accomplishing stated objectives, the literature on food and feeding habits of carps, supplementary feeding, digestive enzyme studies and bacterial flora of fishes are reviewed in the chapter REVIEW OF LITERATURE. The experiments were carried out as per the procedures listed in MATERIALS AND METHODS and the results of the experiments are represented in the chapter RESULTS. Based on available information, the results were discussed in DISCUSSION to interpret the observations and inferences stated in CONCLUSIONS. The gist of the investigation has been presented in SUMMARY.

CHAPTER - 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Indian major carps and exotic Chinese carps are the principal cultivable candidate species for freshwater polyculture practices in India. Successful fish farming is essentially based on good seed supply, appropriate feed and proper water quality management. Feed accounts for more than 50% of the production cost in intensive carp culture systems. While efforts are made to increase the production of fish food organisms through fertilization, cost-effective artificial feeds are formulated for achieving the designed food conversion ratios. A knowledge of the digestibility of feed with relation to digestive enzyme as well as gut microflora assumes importance in this context. Considering the importance of this aspect and lack of relevant information, the present study on the gut microflora of carps with reference to enzyme profile was proposed and the different aspects were reviewed below.

2.1 FOOD AND FEEDING HABITS OF CARPS

Food and feeding habits of carps differ markedly in different stages of life. Certain microscopic crustacean groups and rotifers form the 'main food' (as defined by Schaperclaus, 1933) of spawn and fry of Indian major carps with phytoplankton forming the 'emergency food' (Alikunhi, 1952) because of easy digestibility. Feeding habits of adult fishes vary according to the amount and type of food present in a particular environment, depending on the availability of food supply. Herbivores and carnivores show definite peak periods in feeding, while omnivorous fish show little variation throughout the year.

Catla, the fastest growing species of the Indian major carps, is included in the group of surface plankton feeding fish (Hora and Pillay, 1962; Jhingran, 1991). Jhingran (1991) described the food items of catla fingerlings as waterfleas, planktonic algae and some vegetable debris while that of adults as crustaceans, algae, plants, rotifers and insects. Natarajan and Jhingran (1961) found the natural food of juvenile and adult catla based on their percentage of occurrence in the gut to be crustacean, algae, macrovegetation, rotifers, insects, protozoa and decayed organic matter. Molluscan and polyzoan larvae were also found in case of juveniles.

Rohu, a natural inhabitant of the rivers of north and central India, is a column feeder and planktophagic. Vegetable debris and microscopic plants form the bulk of food item for fingerlings, while adult fishes subsist mainly on vegetable debris, microscopic plants and detritus (Jhingran and Pullin, 1985; Jhingran, 1991). Jhingran and Pullin, 1985) reported that rohu fingerlings 100-250 mm long subsist on unicellular and filamentous algae (15%), decaying vegetation (55%), rotifers and protozoans (2%) and crustaceans (8%).

Mrigal is a bottom feeder and omnivorous in nature. Decayed plant and animal matter, algae, detritus, etc. are the food items for both fingerlings and adult fishes (Jhingran, 1991). It is a detritus eater, with a narrow range of variety, subsisting mainly on decayed vegetation (Jhingran and Pullin, 1985). Utilization of plant matter is much better in mrigal and rohu than catla (Jhingran and Pullin, 1985).

Grass carp, otherwise known as 'White Amur' in Russia brought from Hongkong in December 1959 (Alikunhi and Sukumaran, 1962), is now well accepted in polyculture practices throughout India. It is a freshwater fish and can tolerate a slightly wider range of physico-chemical characters of water (Singh *et al.*, 1967).

Grass carp is almost a exclusively herbivorous fish. It initially feeds on plankton but quickly changes to a diet composed entirely of macrophytes (Chang, 1966). Verigin *et al.* (1963) studied the food selectivity and daily ration of grass carp in cages and reported *Potamogeton pectinatus* to be the most preferred food but *Vallisneria spiralis* and *Myriophyllum* sp. of average liking. Grass carp feeds on protozoans, rotifers and nauplii at 7-9 mm, small cladocerans (*Moina, Daphnia, Chydorus*) and cyclops at 10-12 mm and further graze on cladocerans, copepods and small benthic animals at 13-17 mm (Ling, 1967; Bardach *et al.*, 1972). At a length of about 2 cm, the grass carp begins to feed on macrophytes but retains some potential to utilise the animal feed throughout the life. Sobolev (1970) compared the percentage of plant materials in the gut contents at different ages upto 45 days old grass carp. Aquatic macrophytes become a regular constituent of the diet when fish reach length range of 55 mm. *Hydrilla* and pond bank grasses were the major items after fish reach 87 mm total length. Initial preferred vegetable foods are small tender varieties such as filamentous algae, the moss *Fontinalis*, charales (*Chara, Nitellia*) and the anthophytans *Lemna, Spirodella, Potamogeton, Elodea Callitriche, Puspalam* and Najas (Opuszynski, 1972, 1979; Edwards, 1974, 1975; Sutton, 1977). Soft plants such as *Lactuca sativa, Lemna minor* and *Glyceria fluitans* over others such as *Juncus, Hottonia, Potamogeton, Carex* and *Typha* are also reported to be preferred by fingerlings of size range 22-32 g (Fischer, 1968). Fingerlings also feed upon animal components such as ephemeropteran nymphs, plecopteran nymphs, oligochaetes, chironemid larvae the gastropod, common carp hatchlings, mayflies, odonatan nymphs, toad tadpoles, insects, cladocerans and dipterans under certain conditions (Edwards 1973; Kilgen and Smitherman, 1973; Willey *et al.*, 1974; Singh *et al.*, 1976; Forester and Avault, 1978). Extensive works done on the plant selectivity of grass carp with fingerlings and juveniles reported hundreds of species of macrophytes/ macrovegetation (Avault, 1965; Edwards, 1973, 1974, 1975; Prabhavathy and Sreenivasan, 1977. Younger grass carp eat tender, socculent, less fibrous part of

plants, while larger one consume more species and tougher ones as compared to smaller grass carp. There exists a seasonality in the feeding pattern based on different conditions such as temperature of water and spawning season (Nikolsky, 1963). Several other factors such as conditions and the fat content also influence the feeding (Gorbach, 1971, 1972).

2.2 SUPPLEMENTARY FEEDING

The inherent capacity of pond to produce natural feed has its own limitation (Alikunhi, 1952). In view of higher stocking density, supplementary feeding is necessary to enhance the growth and survivality of carps where natural food availability is limited. Kawamoto (1987) described the usefulness of supplementary feeds in augmenting the production levels. Several works have been carried out on the supplementary feeding in fish culture (Ling, 1967; Shell, 1967; Hickling, 1971; Khan, 1971).

Commonly used supplementary feeds for carp culture practices comprise rice bran/wheat bran and locally available oil cakes in the ratio of 1:1. This supplementary feeds were further fortified with vitamins and minerals to obtain better food conversion efficiency (Das, 1960).

Several other ingredients were also incorporated to meet the nutritional requirements of fish for higher production rates using pelletized feed containing fish meal, rice bran, groundnut oil cake, rice flour and mineral mixture. Verghese *et al.* (1976) obtained 50% higher production as compared to conventional feed containing rice bran and groundnut oilcake (1:1).

Chakraborty *et al.* (1973) experimenting on performance of various feed mixtures *viz.*, mustard oil cake - rice bran mixture, groundnut oil cake - wheat bran mixture, silkworm pupae, soybean and prawn waste in spawn rearing found

soybean giving the best result in *Catla catla* and silkworm pupae and groundnut oil cake-wheat bran mixture in *Cirrhinus mrigala* and *Labeo rohita* respectively.

Considering the nutritive value and high palatability incorporation of fish meal in fish feeds has been found to be beneficial by several workers (Lovell *et al.*, 1974; Dabrowski and Kazak, 1978; Seneriches and Chiu, 1988; Swamy *et al.*, 1988).

Protein being the cost-contributing dietary component, its economic utilization has been studied particularly for replacement of fish meal and other animal protein sources (Devraj *et al.*, 1981; Bhat, 1986; Mohanty and Swamy 1986; Nandeesha *et al.*, 1986, 1989b). However, high cost short and uncertain supply, poor quality and diversified use of fish meal necessitated the screening of unconventional source of protein from plant origin such as algal meals, leaf protein concentrate(LPC), aquatic weeds and by-products of animal processing industries. Forming the secondary grade feed material these may contribute upto half the available protein in the fish diet; the remainder comprised of high grade fish meal (Tacon and Jackson, 1985).

Mohanty and Swamy (1986) substituted blood meal for fish meal to work on the aspect of dietary cost. They found significant growth in rohu when a diet containing 10% blood meal was fed. Nandeesha *et al.* (1986) studied the growth response of catla, rohu and common carp fed with three diets based on slaughter house waste, silkworm pupae and fish meal. A higher conversion rate was obtained in common carp fed with a feed containing silage as a substitute for fish meal (Venugopal and Keshavanath, 1987). Higher growth rates were obtained by complete substitution of fish meal by worm meal and 5% sardine oil (Nandeesha *et al.*, 1988). There are reports of incorporation of sericulture wastes (Nandeesha *et al.*, 1989) and viscera of fish and goat (Jadhav and Rao, 1991) in fish feeds.

Pattanaik and Das (1979) reported the usefulness of incorporating dried powder of nymphoides and *Spirodela* mixed with rice bran as fish feed. Das and Singh (1989) suggested the use of biogas effluent to be a substitute in carp diet. Ayyappan *et al.* (1991) obtained higher weight increment in case of rohu and mrigal fed with diets containing 10% *Spirulina* powder. Gupta and Ahmad, (1966) and Singh and Bhanot (1970) used powdered green algae in fish feed. Pattanaik and Das (1979) suggested the use of powdered duck weeds in supplementary feed for feeding fishes at different ages. Water hyacinth leaf meal is reported to be a cheap protein for use in fish feed (Niamat and Jafri, 1984; Pattanaik *et al.*, 1989).

Observations have shown that the Indian major carps, rohu and mrigal can utilize some of the aquatic weeds to a limited extent (Alikunhi, 1957). Patra and Ray (1988) evaluated the use of aquatic weed *Hydrilla verticillata* by rohu and obtained encouraging results as compared to feed comprising mustard oil cake and rice bran.

Not only the type of supplementary feed but also the amount to be given is important for economising the production. Feeding schedules in terms of weight of stocked animals are prescribed differently at different ages. In this context, for rearing carp spawn to fry, Alikunhi (1957) opined that the feeding schedule for first five days, 6th to 10th day and 11th to 15th day should be double, three times, and four times the weight of initially stocked spawn for day respectively. Hora and Pillay (1962) recommended it as equal, double and three times of the weight of initially stocked spawn respectively.

A comprehensive understanding of the complete nutritional requirements of fishes and further formulation of balanced diets are essential to realise the production potential of fish species. Protein requirements of carps have been studied by several workers and several ranges have been suggested, based

on stages of fish, temperature of water and sources of protein used in the experiments (Dabrowski, 1977; Sen *et al.*, 1978; Renukaradhya and Verghese, 1986; Singh *et al.*, 1987; Singh and Bhanot, 1988; Swamy *et al.*, 1988; Mohanty *et al.*, 1990). Also, the protein requirement varies according to feeding habit of fish. It decreases in case of herbivorous and omnivorous fishes in manured ponds, which ranges from 25 to 30% against 30-40% protein required by the carnivorous fishes like salmon and trout (Pandian, 1987).

Mitra *et al.* (1977) found effective result in incorporation of lipid source in supplementary feed.

2.3 DIGESTIVE ENZYMES

Digestion is the process by which feed in the digestive tract is broken down into simple compounds capable of passing through the intestinal walls to be absorbed in the blood stream. Proteins are hydrolysed into free amino acids, carbohydrates are broken down into simple sugars and fats into fatty acids and glycerols. The ability of an organism to digest a particular food depends on the presence and quantity of appropriate digestive enzymes (Smith, 1980).

Seasonal variations in digestive enzyme activities have been found in fish. Ananichev (1959) found the maximum activities of digestive enzymes to coincide with periods of intensive food intake. Onishi *et al.* (1976) observed daily rhythm in digestive enzyme activity in carp.

Digestive enzyme patterns are related to natural feeding habit of fishes (Hsu and Wu, 1979; Jonas *et al.*, 1983, Ghosh and Saigal, 1984; Bitterlich, 1985; Ray, 1988; Sabapathy and Teo, 1993) and type of diet (Das and Tripathi, 1991).

The variations in enzyme activity may be correlated with patterns of seasonal food intake as also that of temperature (Hofer, 1979a,b). Each enzyme has its highest activity at a certain optimum temperature (Kitamikado and Tachino, 1960b; Chiu and Benitez, 1981). Morishita *et al.* (1964) compared the activities of the digestive enzyme of a number of fish species and found that those of salmonids were more active at lower temperature than those of the warmwater fish studied.

Activities of digestive enzymes of microplanktophagic silver carp and macroplanktophagous big head were investigated with reference to pH by Bitterlich (1985). Trypsin and amylase in both the species had a pH optimum at 8.3 and 7.0 respectively. The optimum pH of the digestive enzymes of homogenised common carp intestinal extract was found to be 10 (Kitamikada and Tachino, 1960; Jonas *et al.*, 1983).

2.3.1 Proteolytic enzymes

In fish, protease secreting organs include gastric mucosae, intestine, pyloric caeca, etc. with pancreas being the principal protease secreting organ. Bondi and Spandonf (1953, 1954) found a high proteolytic activity of pancreatic extract of common carp but low activity of intestinal extract in breaking down protein (casein). Major proteolytic enzymes are pepsin, trypsin, chymotrypsin, pepsinogen, elastase, carboxy peptidase, etc. The activity of fish pepsin is about 150 times higher than that of mammalian pepsin (Ananichev, 1959). The relative activity of chymotrypsin and trypsin may differ in different fish species. Cohen (1981) and Jonas *et al.* (1983) found a higher activity of chymotrypsin than trypsin in common carp and silver carp but in sheat fish (*Silurus glanis*) the activity of trypsin was about four times that of chymotrypsin.

The trypsin activity is proportional to the body size and inversely proportional to the amount of food in the digestive tract (Reimer, 1986). Kawai and

Ikeda (1972) and Shcherbina *et al.* (1976) reported adaptive changes in the activity of proteolytic enzymes in *Cyprinus carpio* in relation to type of diet. Mukhopadhyay *et al.* (1978) found that the total protease activity increased significantly in fishes maintained on a 50% protein diet from those maintained on a 25% protein diet. Higher protein percentage practically showed no further stimulation of the enzyme above 50% protein.

All the intestinal proteolytic enzymes are active at pH range of 6-11. Bondi and Spandorf (1953, 1954) stated that in common carp, proteolytic enzyme secreted by pancreas was more active at the pH range of 7 and 8.2, but Cohen (1981) showed that the maximum activity of these enzymes was reached at pH 8.0 to 8.5. Das and Tripathi (1991) recorded optimum protease activity between pH 7.6 and 8.4 in both fingerlings and adult grass carp. Mukhopadhyay *et al.* (1978) found optimum protease activity in intestine of *Clarias batrachus* at pH 8.0.

Regarding the effects of temperature, Bondi and Spandorf (1953) and Kitamikado and Tachino (1960b) observed maximal proteolytic activity in common carp and rainbow trout at 38-40°C. Kuzmina (1990) found that the temperature functions of intestinal protease in various representative of various ecological feeding types is fairly similar and independent of substrate while in stomach,it is a function of type of hydrolysing substrate. To study the effect of exogenous enzymes present in feed, Ragyanszki (1980) studied the proteolytic enzyme activity in carp fry fed on rotifers and formulated diets and compared these activities with those found in starved fish.

2.3.2 Lipolytic enzymes

Major lipolytic enzymes are lipases and esterases. Triglycerides of long chain fatty acids are the substrates for lipases whereas esterases act on simple esters of low molecular weight. Major sites of lipase secretion are stomach,

pyloric caeca and intestine. Patankar (1973) concluded that the carnivore *Osteoletus ruber* showed higher lipase activity than the plankton feeder *Opisthopterus tardor,* the herbivore *Labeo rohita* and the omnivore *Sarotherodon mossambicus* and also that there was a correlation between the food habits of the fish and their stomach esterase activity levels.

Lipase activity in fish has been observed to be higher than in mammals (Ananichev, 1959). According to Noaillac-Depeyre and Gas (1974), the absorption of lipid occurs essentially in the fore-gut and first mid-gut portion of the intestine.

Demonstrating lipase activity in a number of fish, Kapoor *et al.* (1975) assumed the presence of lipase in digestive tract of all fishes. Goel (1975) found a strong lipase activity in the pancreas of *Cirrhinus mrigala*.

Al-Hussaini (1949) observed the occurrence of lipase in cyprinids and the activity was in descending order from anterior to posterior intestine. Dhage (1968) found that the lipase activity was more concentrated in the anterior intestine than in the posterior region in *Cirrhinus mrigala* and *Labeo rohita*, but was totally absent in the entire gut of *Catla catla.*

2.3.3 Carbohydrases

Major carbohydrases are amylase, glucosidases, invertase, lactase, cellobiase, etc. Amylase is responsible for starch hydrolysis and is found in most omnivorous fish such as cyprinids (Sarbahi, 1951; Bondi and Spandorf, 1954) and herbivorous fish such as grass carp (Das and Tripathy, 1991).

Researchers differ in their opinions regarding the presence of amylase in carnivorous fish (Kitamikado and Tachino, 1960; Nagayama and Saito, 1968). Chiu and Benitez (1981) reported the presence of amylase in milkfish.

Limited amylolytic activity in carnivorous fishes could be explained by more localised amylase secretion. Fish (1960) concluded that the amylase secretion in carnivore Perch (*Perca* sp.) is restricted to pancreas only whereas in case of tilapia, it is throughout the intestine. Major sources of amylase are stomach, pyloric caeca, intestine and pancreas. Sabapathy and Teo (1993) found no significant differences in amylase activity between oesophagus, stomach, intestine and pyloric caeca. Studying on carbohydrate digestion in rainbow trout, Spannhof and Plantikow (1983) found that amylase activity in the intestinal juice was higher when soluble starch was ingested than crude potato starch. Amylase is absorbed to crude starch thereby decreasing the activity. They further found that the total amylase activity was increased by 15 times after 6 hours of feeding. There is a negative correlation between carbohydrate content of the feed and its digestibility (Falge *et al.*, 1978; Kitamikado *et al.*, 1964; Ushiyama *et al.*, 1965).

Amylase is secreted by the entire intestine in India major carps, *Catla catla, Labeo rohita* and *Cirrhinus mrigala* and its activity is high towards the proximal and (Dhage, 1968) Kawai and Ikeda (1971) observed higher activity of amylase and maltase in juvenile carp compared to that of adults. Das and Tripathi (1991) reported higher amylase activity in grass carp fed with natural food relative to that in fish fed with *Lemna minor* or LPC diet.

2.4 BACTERIAL FLORA

Fish production efficiency of an aquatic farming system is the result of an interplay between the fish and its environment. Heterotrophic bacterial communities serve as a link between the abiotic and biotic components of an ecosystem. Relevant work in the area pertain to bacterial coenoces with reference to hydrobiological conditions in streams, lakes and ponds in Europe and America in Temperate zone and Japan in subtropics (Jones, 1971; Rao and Burnison, 1976; Barton and Lock, 1979; Guthke, 1980; Streichsbier, 1982, Sugita *et al.*, 1985c).

Information from Indian freshwater ecosystems are available in recent years (Adoni, 1975; Jana *et al.*, 1980; Jana and Roy, 1983; Jana and Patel, 1984, 1986; Ayyappan *et al.*, 1987, 1990a, 1992; Varadaraj and Ayyappan, 1989; Patralekh, 1991; Shantithirnumani and Chandrika, 1992; Dash, 1993).

Studies concerning the ecology and physiology of heterotrophic bacteria in freshwater ecosystem are meagre (Kawai *et al.*, 1975; Ram *et al.*, 1982; Sugita *et al.*, 1987; 1992; Ogbondeminu and Okoye, 1992; Barat and Jana, 1990; Jana and Barat, 1992).

Bacterial load of an aquatic system is influenced by physico-chemical conditions of water and vice-versa. There exists a positive correlation between the temperature regime and bacterial population (Rai and Hill, 1980, 1982; Mazumdar and Dickman, 1989). Reports on microbial populations with reference to nutrient concentrations in different water bodies pertain to those of Murray and Hudson (1984), Sepers *et al.* (1984), Sugita *et al.* (1987), Insam and Domsch (1988) and Kirchmann *et al.* (1990). Positive correlations were found between bacterial counts and phytoplankton communities (Schmidt, 1970; Tanaka and Kodota, 1980; Andersson, 1983; Gantar, 1985) and an inverse relation between bacterial population and zooplankton communities (Kuznetsov, 1952).

The bacterial populations of sediments are always higher than those of over-lying water due to higher rate of mineralization and transformation of nutrients at the sediment-water interface (Schornik and Ram, 1978; Ram *et al.*, 1980; Ayyappan, 1987; Crissafi and Maugaeri, 1988; Ayyappan *et al.*, 1987, 1990a, 1991a, 1992). Ogbondeminu *et al.* (1991a) reported the development of heterotrophic bacterial community in outdoor pond treated with fermented animal wastes (cow manure) and concluded that the treatment of a pond to stimulate plankton production with fermented cow manure at a dose of 2.28 kg/20 m^3 cause 100 fold increase in heterotrophic bacterial populations.

2.4.1 Bacterial flora associated with fishes

Information regarding microbiological aspects of poikilotherms like fishes has been mostly with regard to contamination spoilage and faecal pollution indicator organisms (Geldreich and Clarke, 1966). Various aspects of microflora associated with fishes studied include changes of flora during storage (Shewan, 1961) and effect of catching method or handling on microflora leading to deterioration (Colwell, 1962; Gillespie and MacRae, 1975).

The microflora of fish eggs are similar to that of water (Bell *et al.*, 1971; Igarshi *et al.*, 1989). Ogbondeminu (1994) investigating the commensal bacterial flora associated with incubating eggs of *Clarias anguillaris* reported that the non-fertilized, aseptically stripped eggs were apparently sterile, while incubating fertilized eggs had a viable count of $1.2 \times 10^5 \pm 1.2 \times 10^2$ cfu/g. In the case of unhatched dead eggs the count was $5.0 \times 10^6 \pm 1.2 \times 10^5$ cfu/g. Yoshimizu *et al.* (1980) analysed the microflora of the embryo and fry to know the exact time of establishment of normal microflora.

With a view to evaluate the sanitary quality of untreated domestic waste-water aquaculture system, Ogbondeminu and Okoye (1992) identified some fish and human potential bacterial pathogens which necessitated sanitary precautions and adequate and proper processing of harvesting fish from such system to be acceptable for human consumption. All the organs (muscle, skin, gill and intestine) of fish reared in such environment harboured bacteria ranging from 10^4 to 10^9/g of specific organs. The densities of coliform and faecal streptococci had counts varying from $10^2 \times 100$g and 10^2 to 10^5/100g respectively.

Okpokwasili and Alapiki (1990) examined the bacterial population of the skin of tilapia (*Oreochromis niloticus*), water, sediment and fish feed in a Nigerian freshwater fish pond. They reported aerobic heterotrophic bacterial load

to be higher in feed ($5.6 \pm 0.4 \times 10^6$ cfu/ml) and sediment ($8.4 \pm 1.6 \times 10^5$ cfu/ml) than in the water column ($4.2 \pm 1.0 \times 10^3$ cfu/ml). However the bacterial load of the skin of diseased fish was higher than that of healthy fish. Majority of the isolates in all the samples were gram-positive bacteria and Bacillus was the predominant genus identified. Other genera included *Lactobacillus, Corynebacterium, Streptococcus, Pediococcus, Staphylococcus, Aeromonas, Aerococcus, Escherichia, Micrococcus, Actinomyces, Proteus, Vibrio, Citrobacter* and *Flavobacterium* at different frequencies. The last three genera were present on the skin of diseased fish but not on that of the healthy fish.

The fish surface microflora is influenced by several factors such as methods of handling fish and their precapture environment (Colwell, 1962; Horsley, 1973). There were 10^2 to 10^3 viable heterotrophic bacteria per cm^2 on skin and a similar numbers per ml in water (Horsley, 1973). There are limited reports regarding the microflora of skin of fishes and most of them are dealt with marine fishes. (Gillespie and MacRae, 1975; Austin, 1983, 1985). With regard to freshwater, Allen *et al.* (1983) reported the presence of *Acinetobacter, Aeromonas, Alacaligenes,* coryneforms, *Enterobacter, Escherichia,Haffnia, Pseudomonas, Serratia, Vibrio, Yersinia, Agrobacterium, Arthrobacter, Bacillus, Bordetella, Cytophaga, Erwinia, Flavobacterium, Flexibacter, Klebsiella, Micrococcus, Moraxella* and *Staphylococcus* in temperate freshwater. Okafor and Nzeakor (1985) isolated *Micrococcus, Lactobacillus, Acinetobacter, Klebsiella, Aeromonas, Pseudomonas, Enterobacter, Flavobacterium, Moraxella* and coryneforms from freshly caught *Clarias lazera* from Oguru River, Nigeria.

The mucus of the gills, gut and skin of fish contain lysozyme and immunoglobulins providing for the defence mechanism against bacteria (Peleteiro and Richards, 1985; Lindsay, 1986).

Esteve and Garay (1991) analysed the aerobic heterotrophic microflora associated with *Anguilla anguilla* reared in freshwater and also the ambient environment. *Pseudomonas* was found to be the most frequently encountered genus. *Moraxella* species and *Acinetobacter* species were abundant in the water medium and *Aeromonas* species in elvers. Potential fish pathogens such as *P. fluoroscens, Vibrio anguillarum* and *Plesiomonas shigelloides* were generally observed in culture waters and healthy fishes.

Studies on skin bacterial flora of marine fishes refer to those of Gillespie and MacRae (1975) , Gilmour *et al.* (1976), Austin (1982) and MacCormack and Fraile (1989-90).

Both marine and freshwater fishes support quite high populations of a wide range of bacterial genera on their gills. Reviewing the microbiology of seawater fish, Shewan (1961) recorded *Pseudomonas, Achromobacter, Flavobacter* and *Vibrio* species in descending order of frequency on gills of marine fish from North sea and Norwegian waters, whereas only *Bacillus* and *Micrococcus* were isolated from gills of fish from warmer waters of India. Horsley (1973) reported the gill microflora of Atlantic salmon in marine and freshwater. *Vibrio* spp. were isolated only from samples of marine water whereas *Aeromonas* spp. were predominant in freshwater samples. Similar microflora were also isolated from the corresponding water.

Trust (1975) demonstrated the difference between the gill microflora of marine and freshwater fishes. In this study the mean bacterial load ranged from 6×10^2 to 2.2×10^6 number/g of gill weight. Mudarris and Austin (1988) found the highest aerobic heterotrophic bacterial count on the gill of healthy turbot, *Scophthalmus maximus* to be 7.0×10^5 per gram wet weight of gills. Ogbondeminu and Okoye (1992) estimated bacterial population in the gills of tilapia (*Sarotherodon*

galilaeus) to be 2.0×10^4 and 5.0×10^6/g weight of gill before and after raising in a waste water aquaculture system for ten months respectively. Those of *Cyprinus carpio* were 1.0×10^2 and 3.3×10^2 per gram respectively.

2.4.2 Gut microflora

Characteristics of bacterial microflora in fish can be modified by many environmental factors (Shewan, 1961; Wood, 1967; Horsley, 1977). Liston (1956,1957) and Georgala (1958) showed the seasonal variations of this microflora. Many aerobic and anaerobic bacteria found associated with fish could be derived from their environment (Margolis, 1953; Shrivastava and Floodgate, 1966; Horsley, 1973) and diet (Trust, 1971). Trust (1971) reported that a commercial food for fish contained *Enterococcus* and members of enterobacteriaceae, including species *Salmonella*. The presence of enterobacteriacea in fish feed indicates a potential danger to person engaged in pisciculture and to human and animal population. The intestinal microflora also may be indigenous to fish (Sera *et al.*, 1972).

Bibliographical analysis of bacterial taxa identified in digestive tract of fish showed that the same aerobic and facultative anaerobic groups are always found with gram-negative forms being the dominant forms. *Vibrio, Achromobacter* and *Pseudomonas* and to a less extent *Flavobacterium, Micrococcus* and *Bacillus* were the bacteria most frequently encountered among marine fishes. In freshwater, the dominated microflora are *Enterobacteria, Aeromonas, Bacillus*, coryneform bacteria, *Flavobacterium* and *Achromobacter*. Quantitatively the microflora fluctuates between 10^3 to 10^8/g of digestive reservoir (Lesel, 1981).

Shewan (1961) reviewed the microflora of marine fishes. Bacterial flora associated with sea water, skin, stomach and intestine of newly caught Antarctic fish (*Notothenia neglacta*) was studied by MacCormack and Fraile (1989-

90). The stomach flora showed variable results between samples with *Vibrio* being almost the exclusive genus. Despite extreme environmental conditions the Antarctic fish showed an intestinal indigenous microflora very similar to warmer fish.

Newman *et al.* (1972) isolated aerobic microflora from the intestinal contents of blue fish, *Pomatomus saltatrix*, the most abundant being *Vibrio, Pseudomonas*, enterobacteriaceae and *Achromobacter* with *Flavobacterium, Micrococcus* and *Bacillus* found to a lesser extent.

The bacterial populations in the digestive tract of deep sea fishes (Macrouridae) were reported to vary in the range of 10^3-10^6/g and the microflora of fed fish and unfed fish were dominated by *Pseudomonas* and *Bacillus* respectively (Ralijaona *et al.*, 1983). Ohwada *et al.* (1980) examined the bacterial population, generic composition and the barotolerant characteristics of selected bacterial isolates of fish collected at higher ocean depths. *Vibrio* sp. or *Photobacterium* spp. and yeast were the major components of gut flora of deep sea fishes, followed by *Pseudomonas, Achromobacter* and *Flavobacterium* spp. As regards barotolerant characteristic, isolates collected from intestine were more barotolerant than those from stomach and the gut microflora of benthic forms were adopted to increased hydrostatic pressure.

MacFarlane *et al.* (1986) studied the gut flora of striped bass (*Morone sexatilis*) from estuarine and coastal marine environments. Opportunistic fish pathogens, especially *Aeromonas hydrophila* were found to be predominant in both the environments. Other isolates from both environments includes *Vibrio*, pseudomonads, flavobacteria, *Alcaligenes* and enterics. *Micrococcus, Bacillus, Corynebacterium* and *Acinetobacter* were found to a lesser extent. The bacterial load in estuarine samples was hundred to thousand times higher than those from coastal marine samples.

A study undertaken to examine the variations in the intestinal microflora of tilapia reared in freshwater and sea water, reported *Aeromonas* or *Vibrio* and *Aeromonas* being frequent in the intestines of fishes reared in freshwater and seawater respectively (Sakata *et al.*, 1980). Sakata *et al.* (1984) identified *Vibrio, Aeromonas* and *Pseudomonas* as the dominant genera of the aerobic intestinal microflora of tilapia, *Sarotherodon niloticus* collected from different locations.

Sugita *et al.* (1983) isolated aerobic and anaerobic bacteria from the guts of seven types of fishes from different sites along a river in Japan and also from ambient waters, sediments and aquatic insects. The total viable counts of the gut contents were 10^5 to 10^8/g dominated by enterobacteriaceae and *Vibrio-Aeromonas* group in all samples. The bacterial profile of gastro-intestinal tract was similar to those of water, sediment and aquatic plants and insects suggesting the origin of flora of gastro-intestinal tract of fish from their environment.

Sugita *et al.* (1985c) noted the changes in total viable counts and varieties of bacteria in the water of carp rearing tank before, during and after the addition of fish. Total viable counts and varieties of group of bacteria detected in the water increased after the introduction of carp. The *Vibrio-Aeromonas* group and *Bacteroides* type-A which were major components in the intestinal tracts of carp, increased after introduction and decreased after removal of carp from the tank. Muroga *et al.* (1987) investigated aerobic bacterial flora in the intestine of farmed red seabream and black seabream at larval and juvenile stages in relation to microflora of ambient water and diet. They found the total bacterial number to be 7.4×10^4 and 3.4×10^4/ fish for red seabream and black seabream respectively against 3.1×10^4 cfu/ml for rearing water, 2.1×10^2 cfu/g for live diet and 1.2×10^4/g for artificial diets. *Vibrio* and *Pseudomonas* were the most dominant intestinal bacteria with *Pseudomonas* and *Moraxella* in water and *Pseudomonas* in the live diet. However *Vibrio* was found in both water and live diets.

Sugita *et al.* (1985b) studied the occurrence and distribution of enterobacteria in the gastrointestinal tract of freshwater fish and their environment in Tama River, Japan and isolated enteric bacteria from the fish guts, aquatic insects, aquatic plants, water and sediments. They concluded that most of the enteric bacteria originated from the environment or fish diet. The intestines of 94 freshwater fish samples comprising *Cyprinus carpio, Oreochromis niloticus, Sarotherodon galilaeus* and *Clarias anguillaris* were examined by Ogbondeminu (1993) for presence of enteric bacteria. The bacteria species were similar to those encountered in water samples. The enteric bacterial isolates comprised *Escherichia coli, Citrobacter, Enterobacter, Proteus, Salmonella, Serratia* and *Klebsiella*. Due to the presence of these diverse enteric bacteria in the aquaculture ponds they suggested strict hygienic procedures to be followed during handling and processing of fish from the culture system to prevent the transfer of potentially pathogenic bacteria to human.

Sakata *et al.* (1980b) found that the intestinal microflora of freshwater fish including gold fish, *Tilapia* and ayu to be containing the obligatory anaerobes. Sugita *et al.* (1985a), while studying the microflora in the intestinal contents of common carp, grass carp and tilapia (*Sarotherodon niloticus*) found *Aeromonas hydrophila, Bacteroides* type-A, *Citrobacter freundii, Pseudomonas* and *Micrococcus* as predominant bacteria in carp intestine. The flora of grass carp in intestine were predominated by *Bacteriodes* type-A, *Bacteroides* type-B, *Plesiomonas shigelloides* and *Aeromonas hydrophila*. They found that tilapia intestine was predominated by obligate anaerobes.

Characteristics of bacterial flora can be modified by several environmental factors. There is a logarithmic relationship between temperature and bacterial growth (Ingraham, 1962; Wilson and Miles, 1975), which does not seem to be seen with regard to digestive microflora of fish living at different temperature.

Lesel and Peringer (1981) studied the effect of temperature on bacterial population of skin and digestive tract and showed that the optimal temperature for microflora development was 19.5°C. Gerald (1985) studied the characteristics of bacterial flora of the digestive tract of *Clarias lazera* at 17°C, 22°C, 28°C and 32°C. This study envisaged qualitative increase of bacterial flora according to the temperature in spite of individual variability. These results could be further clarified by Lesel and Rouel (1987) drawing regression line of the flora established as a function of temperature. Arrhenius' equation provides for description of a satisfactory relationship between temperature and numerical abundance of the flora.

The effect of pH of water on microbial flora of fishes was studied by Eichler and Pope (1984) and found that there was no significant difference in quantitative microflora and generic composition.

Shivokene and Trypshene (1985), studying on abundance and biomass of microorganisms in the digestive tract of pond fish (*Cyprinus carpio, Ctenopharyngodon idella* and *Tinca tinca*) with respect to their age, habitat conditions and dietary features, showed that feeding intensity, diet composition, fish species and age are the major factors of the number of bacteria. Highest number of bacteria were found on the anterior and mid sections of the digestive tract showing the bacteria to participate in food decompositions.

2.4.3 Nutritional aspects of gut microflora

The nutritional aspects of fish gut microflora are being investigated in the recent years with regard to trophic role of the bacterial communities as well as their role in the process of digestion. Cellulase activity was found to occur in the stomachs of 17 out of 22 fish species examined and was apparently due to the production of this enzyme by gut microflora (Stickney and Shumway, 1974).

Minami *et al.* (1972). Stickney and Shumway (1979) isolated chitin decomposing bacteria from digestive tract of ayu (*Plecoglossus altivelis)* carp (*Cyprinus carpio)* and rainbow trout (*Salmo gairdneri*). Trust *et al.* (1979) studied the ability of bacteria from the intestine of grass carp to break down cellulose. Shiranee *et al.* (1993) studied the role of gut and sediment bacterial flora in the nutrition of cultured pear spot (*Etroplus suratensis*) and found that the gut microflora was predominated by amylolytic forms. Considerable percentage of isolates also exhibited protease and lipase activity both in gut and sediment.

Significant increase in number of bacteria from fore-gut to mid-gut, followed by significant decrease in mid-gut in the study of Henebry *et al.* (1988) suggested that bacterial concentration may increase and then be digested providing high quality protein for silver carp. Comparative study of natural and processed foods by Ayyappan *et al.* (1990b) indicated that the significance of microbial communities as trophic components of filter-feeding fishes showed possibilities of their better utilization for carp rearing through medium enrichment and diet incorporation.

Some more studies pertaining to nutritional role of gut flora include those of Trust and Sparrow (1974), Henson (1990) and Moriarty (1989).

CHAPTER - 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The studies were carried out at the fish farm and laboratories of the Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, Orissa, India (Lat. 20°11'06"-20°11'45"N,Long. 85°50'52"-85°51'35"E)during July to December, 1995. They pertained to bacteriological studies associated with carp raised at farm and their gut enzyme profiles. The fishes (catla, *Catla catla*; rohu, *Labeo rohita*; mrigal, *Cirrhinus mrigala* and grass carp, *Ctenopharyngodon idella*) were sampled three to seven times each, depending on the availability, for bacteriological studies. Simultaneously, the samples of water and sediment were also collected for bacteriological studies. They were followed by laboratory experiments and qualitative gut digestive enzyme assays (protease, lipase, amylase and invertase) for obtaining activities of such enzymes at different regions of the digestive tract of each species (fore-gut, mid-gut and hind-gut).

3.1 SAMPLING SOURCES

In order to assess the bacteriological loads associated with the carps, the following sampling sources were selected: (i) Fish pond water, (ii) Fish pond sediment, (iii) Fish body surface, (iv) Fish gill and (v) Fish gut.

For enzymatic assays in the digestive tract, the fore-gut, mid-gut and hind gut of the fish were sampled.

3.2 SAMPLES

All the four species of fish were collected from polyculture fish ponds of CIFA fish farm. The size ranges of fishes sampled for microbiological and enzymatic studies were as follows.

Species	Size range of fish (g)	
	For Bacteriological Studies	For Enzymatic Studies
Catla	10.3-230.0	14.7-235.4
Rohu	5.5-200-0	4.8-198.5
Mrigal	7.3-145.4	8.0-201.5
Grass carp	9.7-383.0	15.1-22.9

3.3 BACTERIOLOGICAL STUDIES

Water samples were collected in sterile stoppered glass bottles (autoclaved at 121 °C and 15 lb psi) under aseptic conditions. The pond sediment was collected in sterile petridishes (autoclaved at 121 °C 15 lb psi) using a sterile spatula under aseptic conditions. The water and sediment samples collected were immediately brought to the laboratory for further processing.

3.3.1 Preparation of samples

All fishes sampled from the ponds were transported immediately to the laboratory in sterile plastic bucket containing the water of the pond from where fishes were collected. The fishes were killed in the laboratory and processed immediately. Dry sterile cotton swabs were used for collecting the bacterial film on the body surface of fishes. In case of table-size fishes, the surface swabs of fish from an area of 10 cm^2 were collected. The swabs thus collected were placed into presterilised stoppered glass tubes containing 10 ml of sterile 0.85% physiological saline.

The samples of gills were obtained aseptically by tearing out the gill rakers and filaments, after opening the operculum aseptically with a pair of sterile forceps. The samples were further macerated in sterile porcelain mortar and pestle. 0.1 g of macerated gill was introduced into a pre-sterilised stoppered glass tubes containing 9.9 ml of 0.85% sterile physiological saline.

For collection of gut samples, the ventral surface of the fish species was first thoroughly washed with cotton wool dipped in alcohol to disinfect the surface. The fishes were dissected under aseptic conditions and the total gut was sectioned and macerated as in the case of the gills. 0.1g of the macerated materials was placed into a presterilised stoppered glass tubes containing 9.9 ml of sterile 0.85% physiological saline.

3.3.2 Bacteriological enumeration

The standard dilution plate count technique was employed for the enumeration of aerobic heterotrophic bacteria (Norris and Ribbons, 1970; Olah and Vasarhelyi, 1970; Collin and Lyne, 1976) using nutrient glucose agar (1.5%) as the medium (Beef extract 3g; Peptone 5g, Sodium chloride 8g, Agar 15g, Distilled water 1000 ml, with pH 7.3 ± 0.2). Serial dilutions upto 10^{-3} for water, 10^{-2} or 10^{3} for surface swabbed samples and 10^{-5} or 10^{-6} for other samples (gill, gut and sediment) were prepared with sterile saline blanks under aseptic conditions. 0.1 ml of samples from each dilution was inoculated on to the agar medium in sterile petridishes using pour plate method in replicates. The plates were incubated at the prevailing room temperatures in a biological incubator (BSE, model 1984) for 48 hours. The colony-forming units (cfu) in the petridishes were counted and from the means in different dilutions, average counts were derived. The results are expressed as no x 10^3 /ml water, no. x 10^2 or no. x 10^3/cm^2 body surface and nox10^5/g wet weight of the sediment/gills/gut.

3.3.3 Isolation and Identification

Following incubation at room temperature for 48 hours, representative colonies from the petridishes were taken on to agar slants after recording the morphological characteristics such as colour, shape, margin, elevation, transparency, etc. The slants were further incubated at room temperature for 48 hours. After obtaining the growth in slants they were stored in refrigerator at 4°C.

Gram-staining was done with 18-24 hours old bacterial culture using crystal violet and safranine as primary and secondary stains respectively. The morphological characters of cell were studied under oil immersion in a microscope (Reichert polyvar 2). The motility of isolates was checked observing the movement in motility test medium and manitol motility agar medium. The isolates were further subjected to biochemical tests. The identification was based on Bergey's Manual of Systematic Bacteriology (1984, 1986).

3.4 ENZYME ASSAYS

Qualitative enzyme assays were performed to assess the activity levels of certain enzymes (protease, lipase, amylase and invertase) in different regions of the alimentary canal (fore-gut, mid-gut and hind-gut). The procedures followed were based on the works of Saigal *et al.* (1974) and Ghosh *et al.* (1977), with detailed procedure described below.

The fish samples were collected from the ponds where samplings were done for bacteriological studies and made to starve under laboratory condition for 48 hours so as to empty their gut. After starvation, the fishes were killed and dissected on ice and the alimentary canal was cleaned, removed and thoroughly washed externally with chilled distilled water. The alimentary canal was marked into three parts *viz.*, fore- gut, mid-gut and hind-gut. Each portion of the alimentary canal was macerated with chilled glycerine (80%) to make an emulsion of the extract. The macerated mixture was centrifuged at 3000 rpm for 30 min. The supernatant fluid was collected for enzyme assays.

3.4.1 Protease

For detection of protease, gelatin was used as substrate. The substrate after addition to the extract was incubated at 25°C for 24 hours. The liquification of gelatin after incubation indicated the gelatin hydrolysis by protease present in the extract.

3.4.2 Lipase

Lipase was detected taking olive oil as substrate. The gut extract and olive oil mixture (1:1) was incubated at 38°C for 24 hours. After incubation, the pH indicator, bromothymol blue solution (pH 6.0 to 7.6; yellow to blue) was used as indicator. The presence of lipase could be assessed through the development of yellow colour after addition of indicator to the extract-substrate mixture, indicating an acidic change in the container.

3.4.3 Amylase

Amylase was qualitatively assayed taking starch as substrate. One ml of extract of each region was mixed with 10 drops of starch (1%) solution and the mixture was incubated at 35°C for 24 hours. After incubation a few drops of iodine solution (Iodine-1g, potassium iodide 2g and distilled water 100 ml) were added to the mixture to detect starch hydrolysis, indicated by the development of blue colour.

3.4.4 Invertase

Invertase presence was detected using sucrose as substrate. One ml of sucrose solution (2%) was mixed with one ml of extract and the mixture was incubated at 35°C for 24 hours. Invertase activity was tested using Benedict's qualitative reagent as per the colour changes indicated on the reagent bottle.

In each of the above tests, control tests were performed using boiled gut extract. Few drops of toluene were added to each test container to prevent fungal or bacterial growth.

The pattern of marking in this qualitative assay was as follows: Very strong (+ + + +), Strong (+ + +), Moderate (+ +), Absent (-).

3.5 STATISTICAL ANALYSES

In order to find out significant variations in mean viable counts of bacteria associated with body surfaces, gills and guts between species, the values were subjected to tests analysis of variance (Fisher and Yates, 1982; Sukhatme and Amble, 1989).

CHAPTER - 4

RESULTS

4. RESULTS

The results of the studies on microflora associated with body surfaces, gills and guts of carps and their gut digestive enzyme activities assessed through bacteriological and laboratory experiments are presented in this chapter.

4.1 BACTERIOLOGICAL STUDIES

4.1.1 Bacterial populations

The variations in total heterotrophic bacterial populations in water and sediment of pond from which fishes had been sampled are presented in Table 1. The bacterial counts of water medium were in the range of 1.25-2.55 x 10^3/ml and that of sediment were 1.23-2.50 x 10^5/g with mean viable count of 1.76 x 10^3/ml and 1.99 x 10^5/g respectively. The sediment samples showed higher bacterial loads than the water medium.

The variations in total heterotrophic bacterial loads of body surface, gills and gut of catla, rohu, mrigal and grass carp are presented in Tables 2,3,4 and 5 and Figs. 1,2,3 and 4 respectively. The mean viable counts of the above species are presented in Table 6.

Among the three sampling sources of catla, the gut harboured the highest bacterial load with mean viable count of 7.06 x 10^5/g while body surface had the least mean viable count (2.67 x 10^3/g) (Fig. 1). The variations of heterotrophs on the body surface were in the range of 0.10-10.00 x 10^3/cm^2 and those of gill and gut were 0.71-2.00 x 10^5/g and 3.28-15.00 x 10^5/g respectively (Table 2).

Table 1: Variations in heterotrophic bacterial populations in water and sediment media of fish pond

Samples	Water (no. x 10^3/ml)	Sediment (no. x 10^5/g)
1	2.55	2.25
2	1.25	1.23
3	1.50	2.50

Table 2: Variations in heterotrophic bacterial populations of the body surface, gills and gut of *Catla catla*

Samplings sources	1	2	3	4
Body surface (no. x 10^2/cm^2)	1.00	2.10	3.70	100.00
Gills (no. x 10^5/g)	1.22	0.71	1.43	2.00
Gut (no. x 10^5/g)	3.28	4.41	5.54	15.00

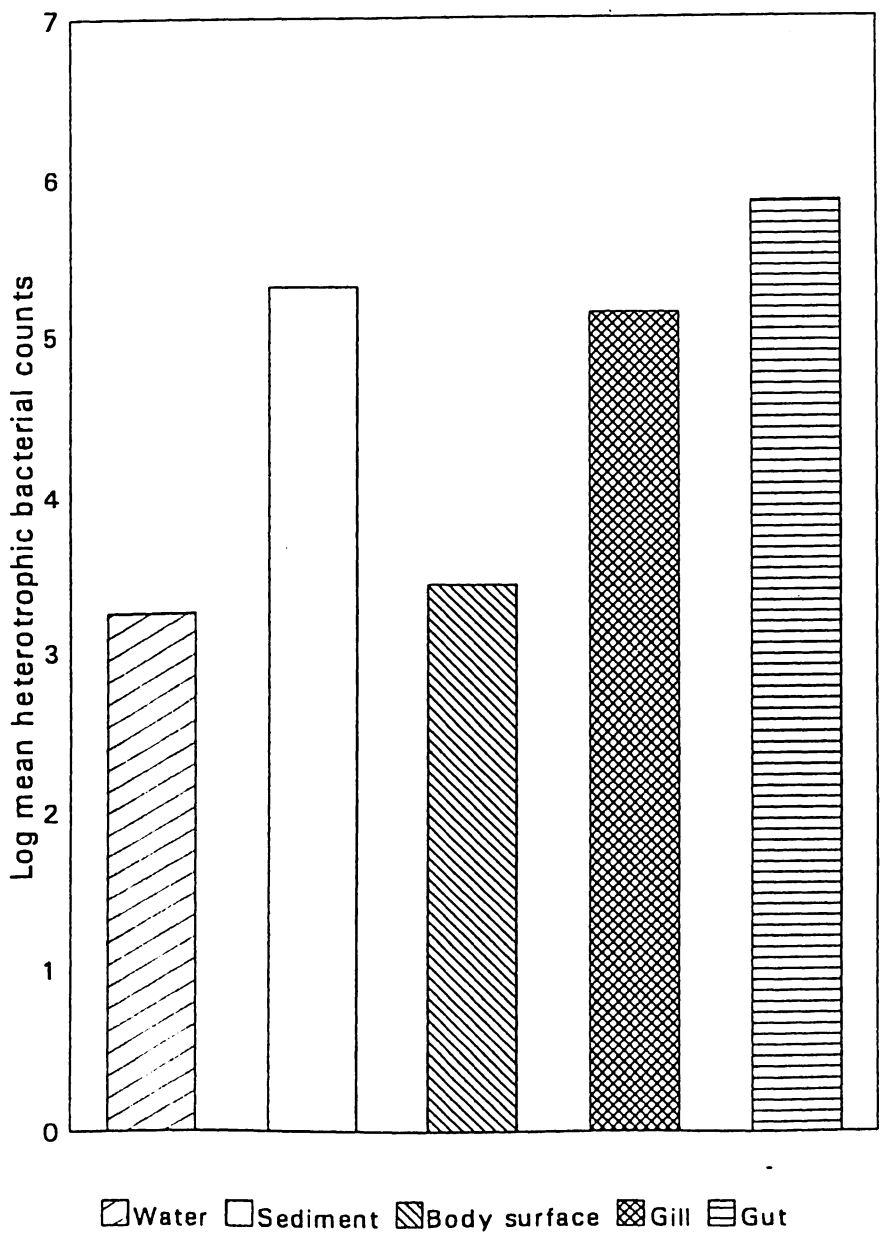


Fig. 1. Mean heterotrophic bacterial population counts associated with *Catla catla*.

The variations in the bacterial populations on the body populations on the body surface of rohu, *Labeo rohita* ranged from 4.96 to 28.10x10^3/cm^2. The bacterial counts from gills were in the range of 1.36-7.00 x 10^6/g with highest mean viable counts of 40.97 x 10^5/g. The changes of bacterial biomass in gut were in the range of 0.87 - 37.04 x 10^5/g. Contrast to catla gills of rohu supported highest bacterial proliferation than gut (Table 3 and Fig.2).

Similar patterns of bacterial growth, as in case of rohu, were observed in mrigal though to a lower magnitude (Table 4 and Fig. 3). The bacterial populations on the body surface were low (mean 4.45 x 10^3/cm^2) ranging from 2.22-6.67 x 10^3/cm^2. High range of variations of 1.5-29.55 x 10^5/g were seen in gill heterotrophs while gut microflora ranged from 0.16-5.26x10^3/g with mean viable count of 19.80 x 10^5/g and 2.38 x 10^5/g respectively.

The variations of bacterial counts associated with grass carp body surface, gills and gut are presented in Table 5 and Fig. 4. The densities of total heterotrophic bacteria (THB) were high in samples of gut with wide variations (0.46-38.00x10^5/g) and mean viable count of 9.76 x 10^5/g. The loads of total heterotrophic bacteria of body surface and gills varied from 0.34-11.67 x 10^3/cm^2 and 0.86-7.75 x 10^5/g respectively recording respective mean populations of 3.52 x 10^3/cm^2 and 3.34 x 10^5/g comparatively lower values than gut.

Mean heterotrophic bacterial populations of body surfaces, gills and gut of different carp species are presented in Figs. 5,6 and 7 respectively. Among body surfaces of all species studied the surface of rohu showed bacterial counts in the range of 4.96-28.10x 10^3/cm^2 with mean viable counts of 18.33 x 10^3/cm^2. The mean load was lowest on catla body surface (2.67 x 10^3/cm^2) with considerable variations (1.00-100.00 x 10^2/cm^2). The variations were minimal on the body surface of mrigal (2.22-6.67 x 10^3/cm^2) (Fig.5).

Table 3: Variations in heterotrophic bacterial populations of the body surface, gills and gut of *Labeo rohita*

Samplings Sources	1	2	3	4	5	6	7
Body surface (no. x $10^3/cm^2$)	-	-	-	18.05	22.22	28.10	4.96
Gills (no. x $10^6/g$)	-	-	-	1.36	7.00	6.63	1.40
Gut (no. x $10^5/g$)	0.87	1.25	1.32	10.25	33.23	37.04	21.30

Table 4: Variations in heterotrophic bacterial populations of the body surface, gills and gut of *Cirrhinus mrigala*

Sampling Sources	1	2	3	4	5	6	7
Body surface (no. x $10^2/cm^2$)	-	-	-	3.47	4.01	2.22	6.67
Gills (no. x $10^5/g$)	-	-	-	29.55	22.10	26.06	1.50
Gut (no. x $10^5/g$)	0.45	0.39	0.16	2.29	3.36	5.26	4.75

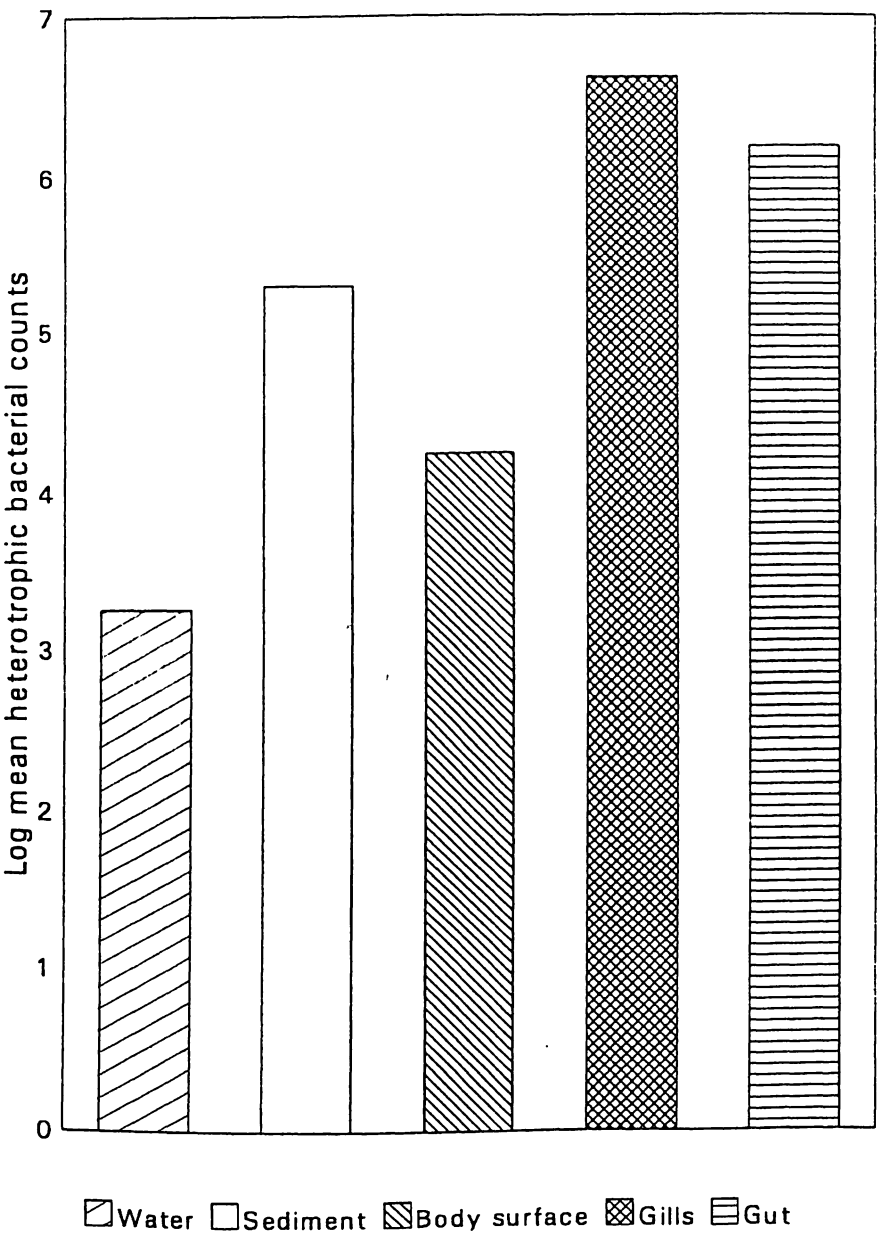


Fig. 2. Mean heterotrophic bacterial population counts associated with *Labeo rohit*a.

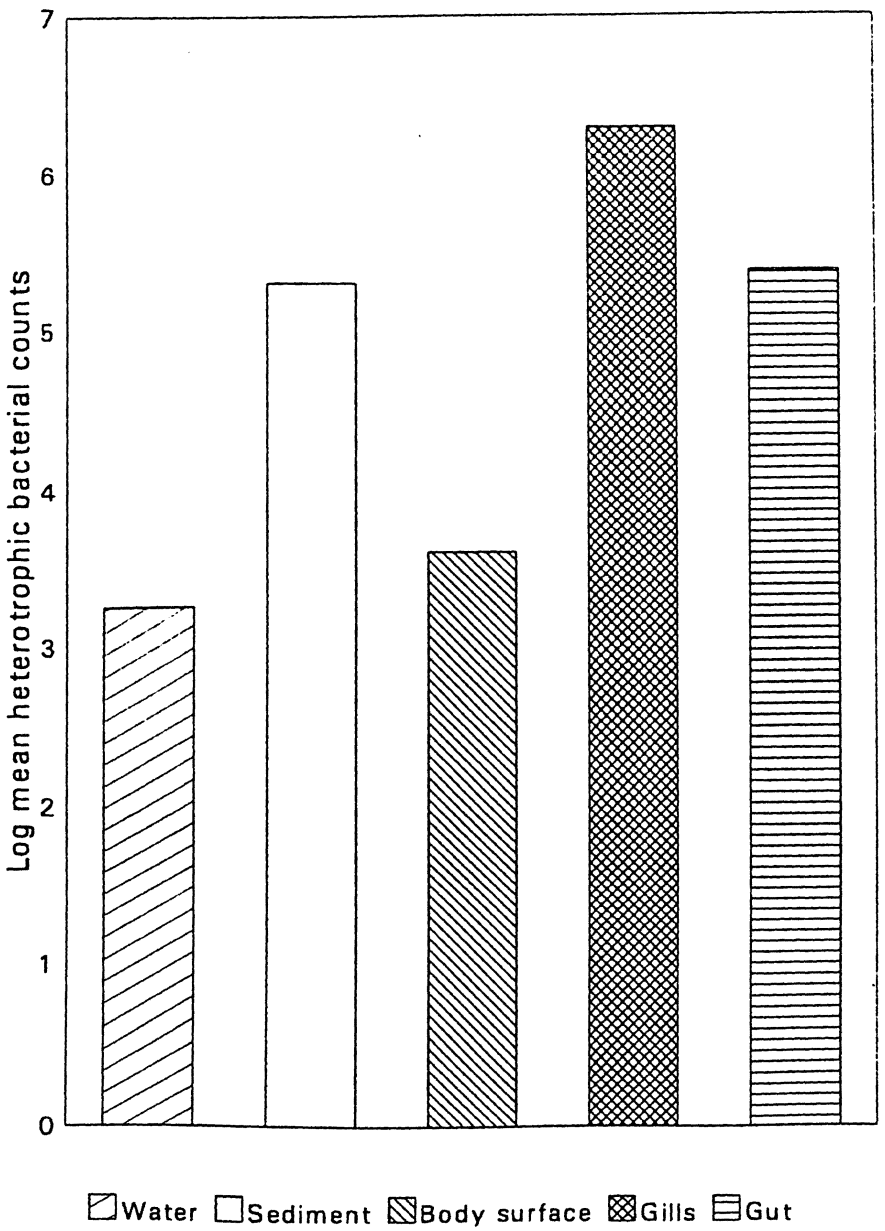


Fig. 3. Mean heterotrophic bacterial population counts associated with *Cirrhinus mrigala*.

Table 5: Variations in heterotrophic bacterial populations of the body surface, gills and gut of *Ctenopharyngodon idella*

Sampling Sources	1	2	3	4	5	6	7
Body surface (no. x $10^3/cm^2$)	-	-	-	0.34	0.51	1.56	11.67
Gills (no. x $10^5/g$)	-	-	-	2.66	0.86	7.75	2.10
Gut (no. x $10^5/g$)	0.46	0.68	2.43	8.13	7.91	11.78	38.0

Table 6: Variations in mean heterotrophic bacterial populations of body surface, gills and gut of carp species

Sources Species	Body surface (no. x $10^3/cm^2$)	Gills (no. x $10^5/g$)	Gut (no. x $10^5/g$)
Catla	2.67	1.34	7.06
Rohu	18.33	40.97	15.03
Mrigal	4.09	19.80	2.38
Grass carp	3.52	3.34	9.76

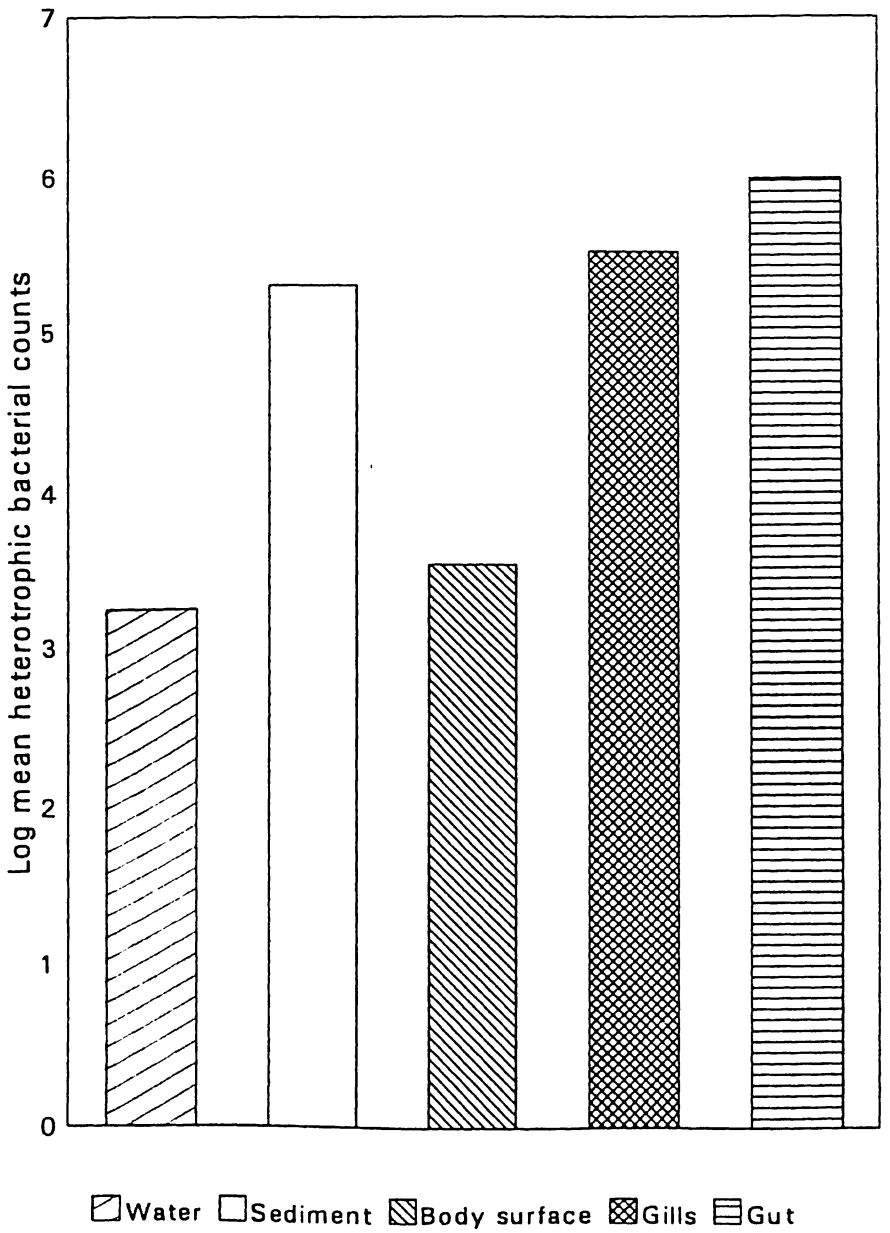


Fig. 4. Mean heterotrophic bacterial population counts associated with *Ctenopharyngodon idella*.

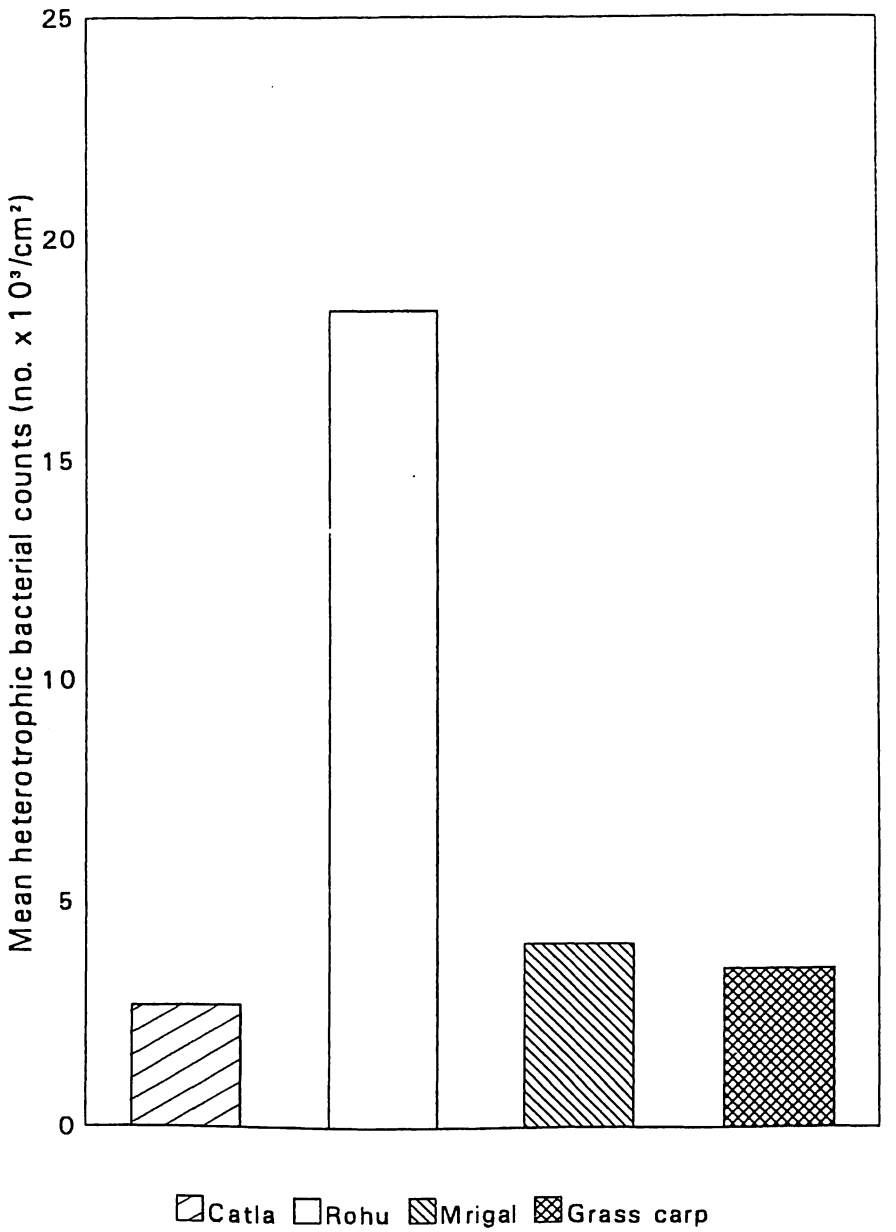


Fig. 5. Mean heterotrophic bacterial population counts of body surfaces of carp species.

The bacterial counts from gills recorded a similar trend of variations as that of body surface with highest mean value of 40.49 x 10^5/g in rohu and lowest in catla (1.34 x 10^5/g) with respective variations of 1.36-7.00 x 10^6/g and 0.71-2.00x10^5/g. The variations in mrigal and grass carp were 1.50-29.55x$10^{5/g}$ and 0.86-7.75x10^5/g respectively (Fig.6).

With regard to gut heterotrophic microflora, mrigal supported the lowest bacterial growth (mean 2.38 x 10^5) with minimal variations. Highest mean load (15.03 x 10^5/g) was found in rohu with considerable variations. The variations were highest in gut of grass carp being in the range of 0.46-38.00 x 10^5/g (Fig.7).

Of all the sources in fish species, highest bacterial colonisation was observed on the gill of rohu (40.97 x 10^5/g) with a variation of 1.36-7.00x10^6/g). The lowest bacterial growth was recorded on the body surface of catla. The variations in the bacterial populations were lowest in gills of catla (0.71 to 2.00 x 10^5/g).

Among body surfaces of four species, there were no significant differences in mean bacterial populations between catla, mrigal and grass carp. However, there was significant difference in mean values between rohu and other three species of carps with regard to body surface ($P < 0.05$). In case of gills no significant differences were found in the mean bacterial populations between species, except rohu and catla ($P < 0.05$) as well as rohu and grass carp ($P < 0.05$). As regards gut heterotrophic bacterial populations significant difference was observed between rohu and mrigal ($P < 0.10$).

4.1.2 Generic Composition

A total of 319 isolates were collected from different sources during the study period comprising 36.99% gram-negative bacilli followed by 25.71%

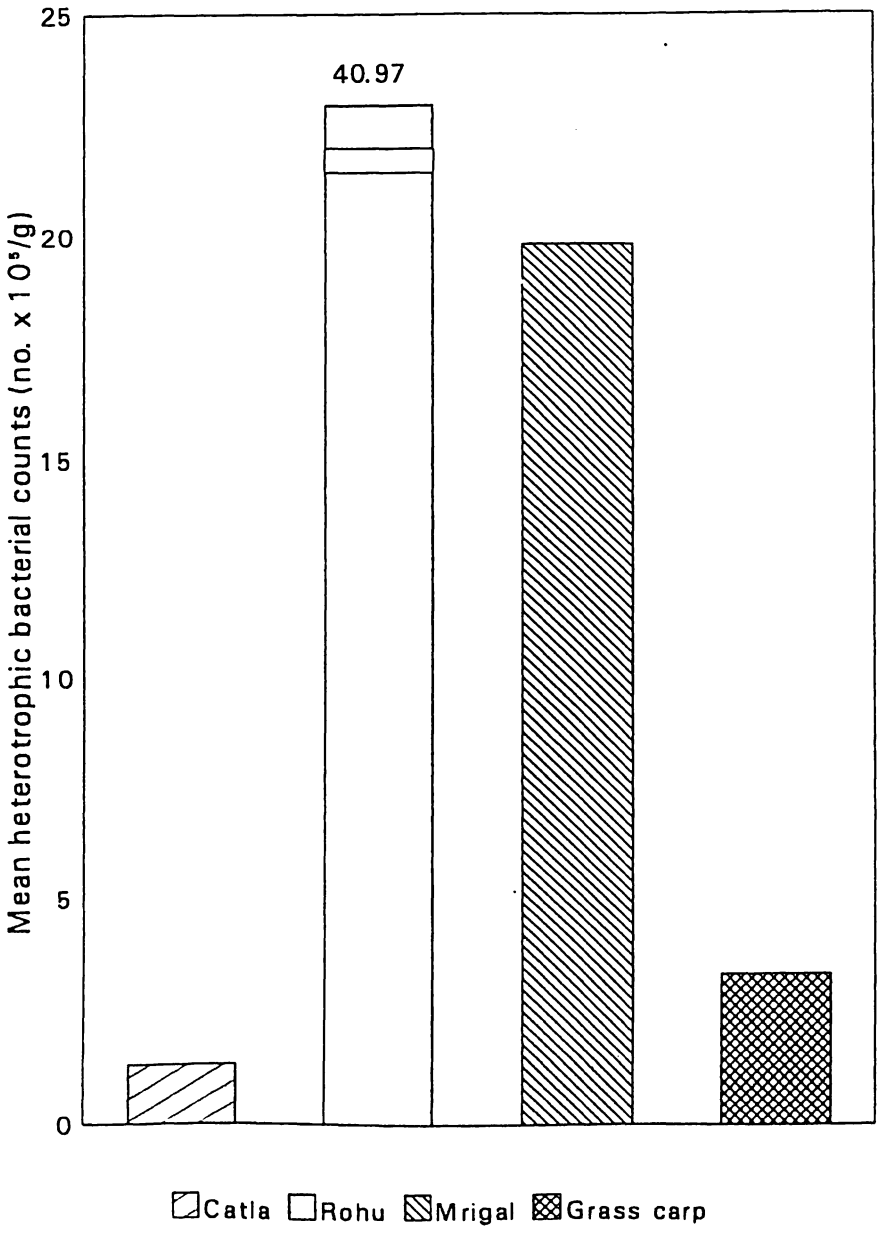


Fig. 6. Mean heterotrophic bacterial population counts of gills of carp species.

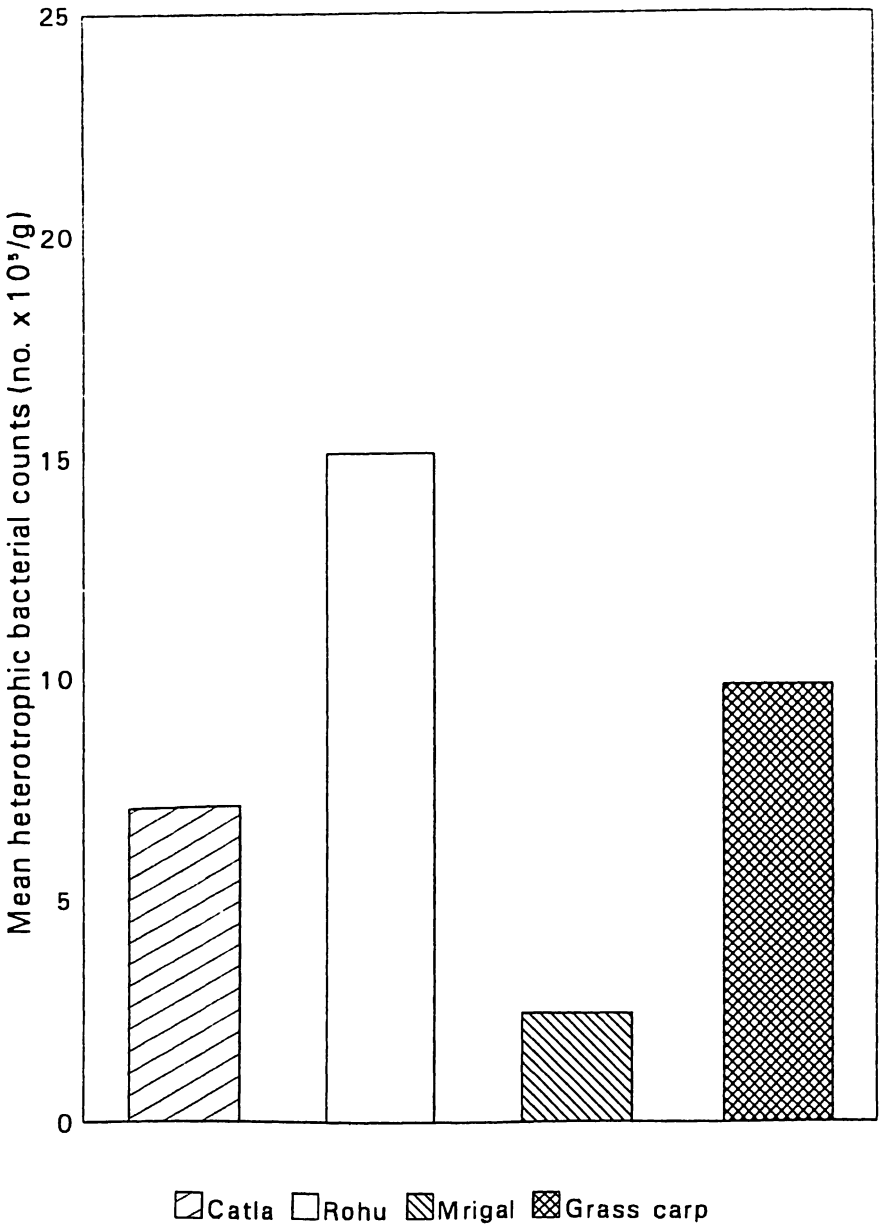


Fig. 7. Mean heterotrophic bacterial population counts of gut of carp species.

gram-positive bacilli, 15.36% gram-positive coccobacilli, 14.42% gram-positive cocci and 7.52% gram-negative coccobacilli (Table 7, Fig. 8). Gram-negative bacilli were observed to be dominating in all the sources except sediment where gram-positive and negative bacilli were equally frequent. Gram-positive bacilli constituted the second largest group in all the sources, except body surfaces of fish where gram-positive cocci were more frequent. Gram-positive cocci were absent in sediment. Among all the sources of fishes gram-positive coccobacilli were more in number than gram-negative coccobacilli. In case of gill microflora, both gram-positive coccobacilli and gram-positive cocci were equally frequent. Gram-negative coccobacilli were least in all the three sampling sources of fish analysed. The percentage compositions of different groups of heterotrophs based on gram staining and morphological features, isolated from different sources are presented in Table 7 and Figs. 9 and 10A .

The heterotrophic bacterial isolates of gut were identified upto genus level. The generic composition of the bacterial stains isolated from the gut are presented in Table 8 and Fig. 10B. The gram-negative bacilli and coccobacilli were represented by the genera *Enterobacter* (11.03%), *Pseudomonas* (11.03%), *Aeromonas* (5.88%), *Serratia* (5.88%), *Alcaligenes* (4.41%), *Proteus* (3.68%), *Plesiomonas* (2.94), *Acinetobacter* (1.47%), *Flavobacterium* (0.74%), and *Salmonella* (0.74%). Gram-positive bacilli and coccobacilli comprised of *Bacillus* (30.15%), *Kurthia* (5.88%), *Listeria* (2.2%), *Sporosarcina* (1.47%) and *Sporolactobacillus* (0.74%), Gram-positive cocci were mainly composed of *Micrococcus* (9.56%) with few number of *Planococcus* (2.94%). *Bacillus* was the predominant genus in the gut, alongwith *Enterobacter, Pseudomonas* and *Micrococcus* found to a considerable extent. Among the enterobacteriaceae, mportant genera were *Enterobacter, Serratia* and *Proteus*.

Table 7: Numerical representation of heterotrophic bacterial strains in different sources during the study period

Sources Types of bacteria	Water	Sediment	Body surface	Gills	Gut	No. of isolates
Gram-positive bacilli	4	6	16	24	32	82
Gram-positive coccobacilli	4	1	8	12	24	49
Gram-positive cocci	1	0	17	12	16	46
Gram-negative bacilli	6	6	18	32	56	118
Gram-negative coccobacilli	2	1	7	6	8	24
No. of isolates	17	14	66	86	136	319

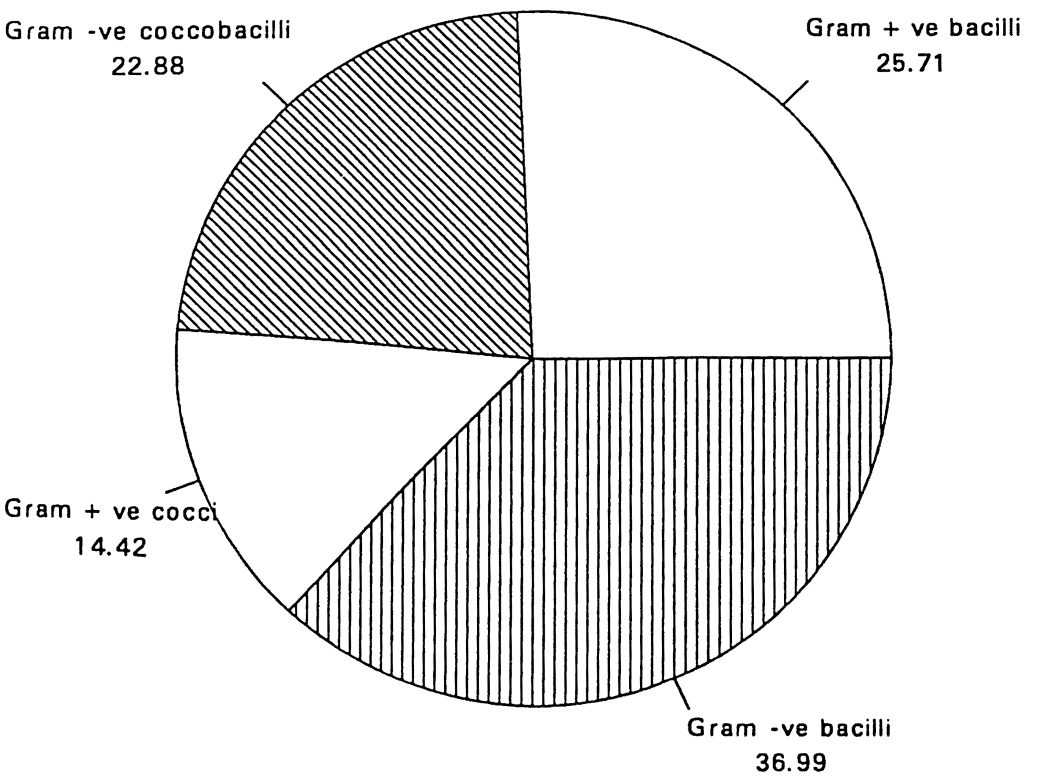
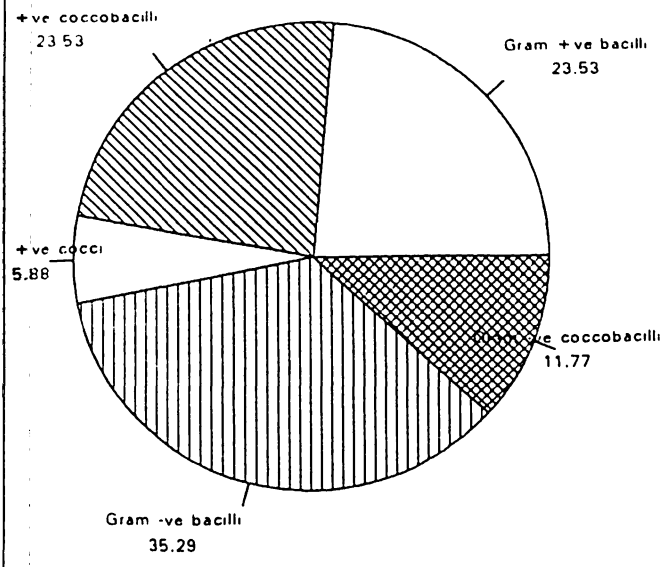
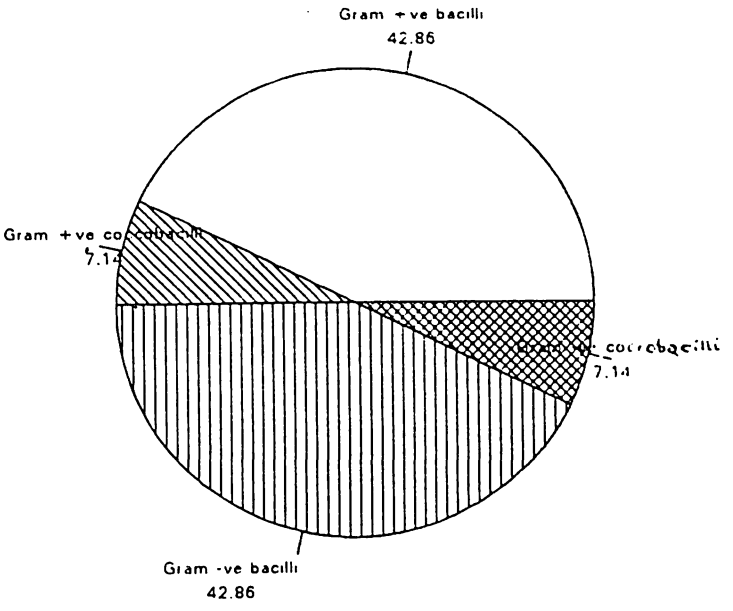


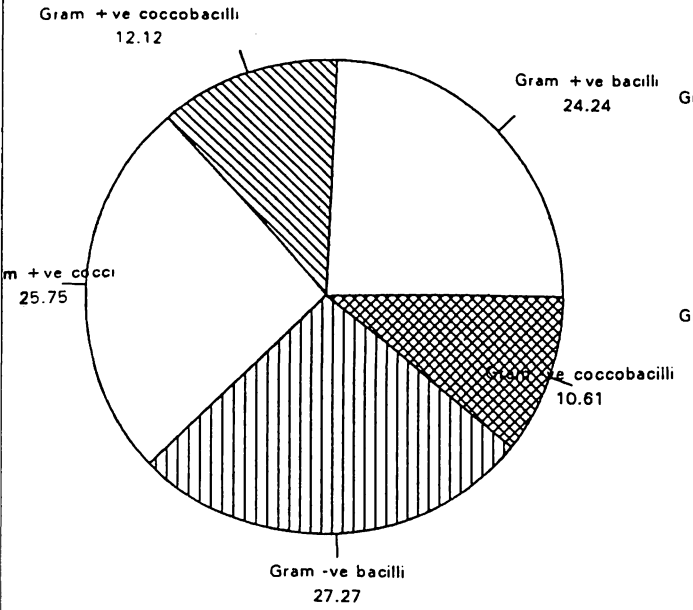
Fig. 8. Percentage composition of different groups of heterotrophic bacteria isolated during the study period



A. Water



B. Sediment



C. body surface

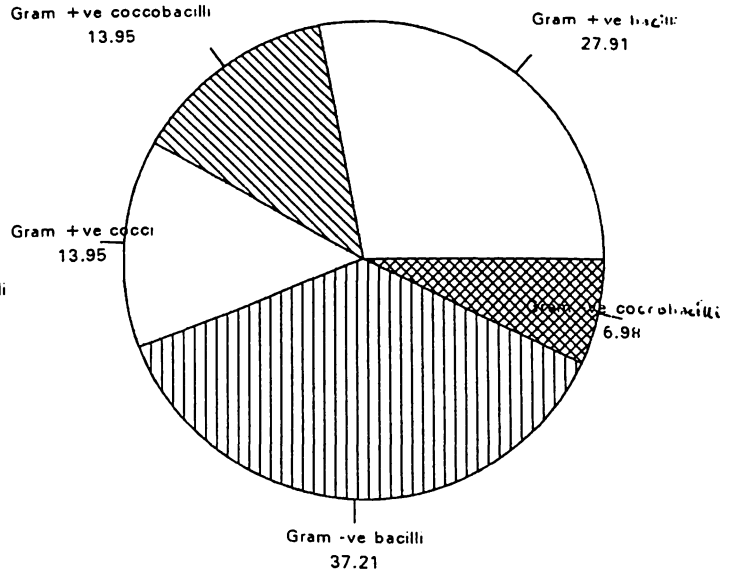


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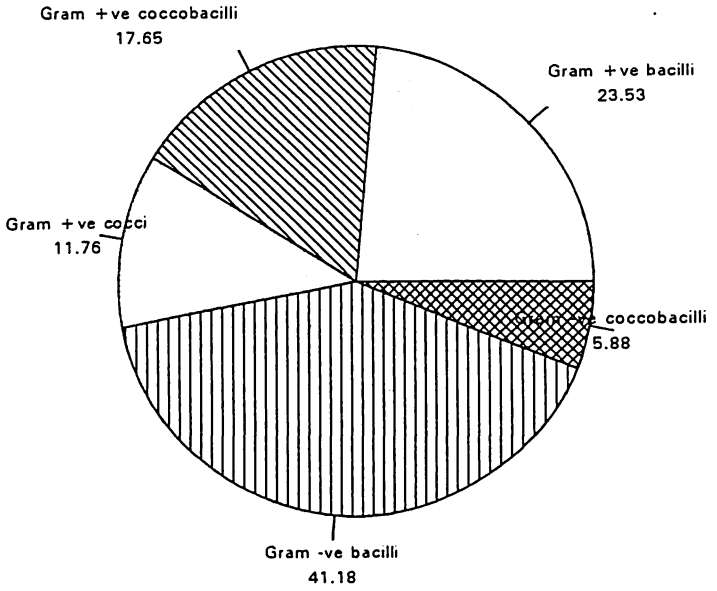
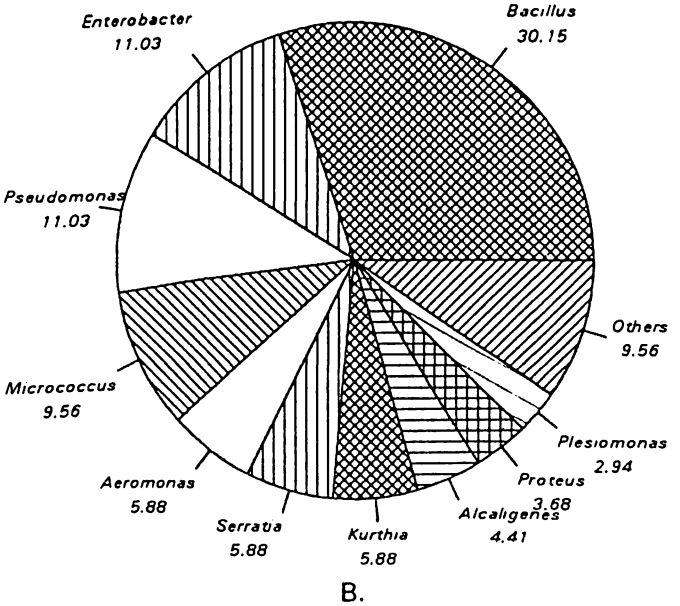
Fig. 9. Percentage composition of different types of heterotrophic bacteria isolated from water, sediment, body surface and gills of carp species.

Table 8: Generic composition of gut microflora of carp species

Genus	No.of isolates	percentage
Bacillus	41	30.15
Enterobacter	15	11.03
Pseudomonas	15	11.03
Micrococcus	13	9.56
Aeromonas	8	5.88
Serratia	8	5.88
Kurthia	8	5.88
Alcaligenes	6	4.41
Proteus	5	3.68
Plesiomonas	4	2.94
Planococcus	3	2.20
Listeria	3	2.20
Acinetobacter	2	1.47
Sporosarcina	2	1.47
Sporolactobacillus	1	0.74
Flavobacterium	1	0.74
Salmonella	1	0.74
Total	136	100



A.



B.

Fig. 10. Percentage composition of different groups and genera of heterotrophic bacterial strains of the gut of carp species.

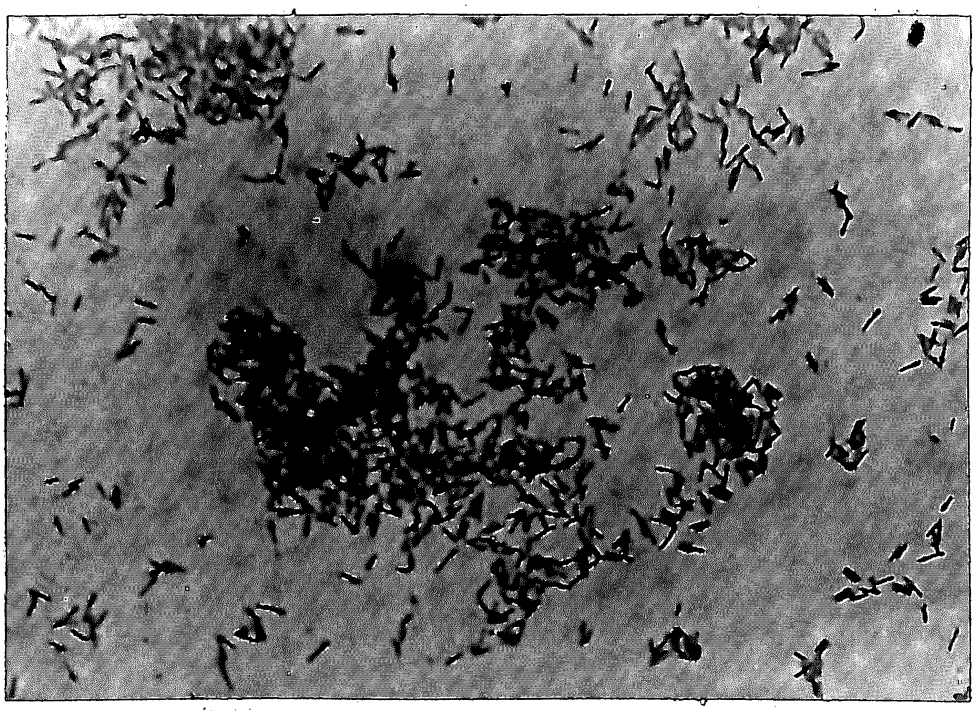


Plate 1. Gram-positive bacilli *(Bacillus* sp.) (x 1000)

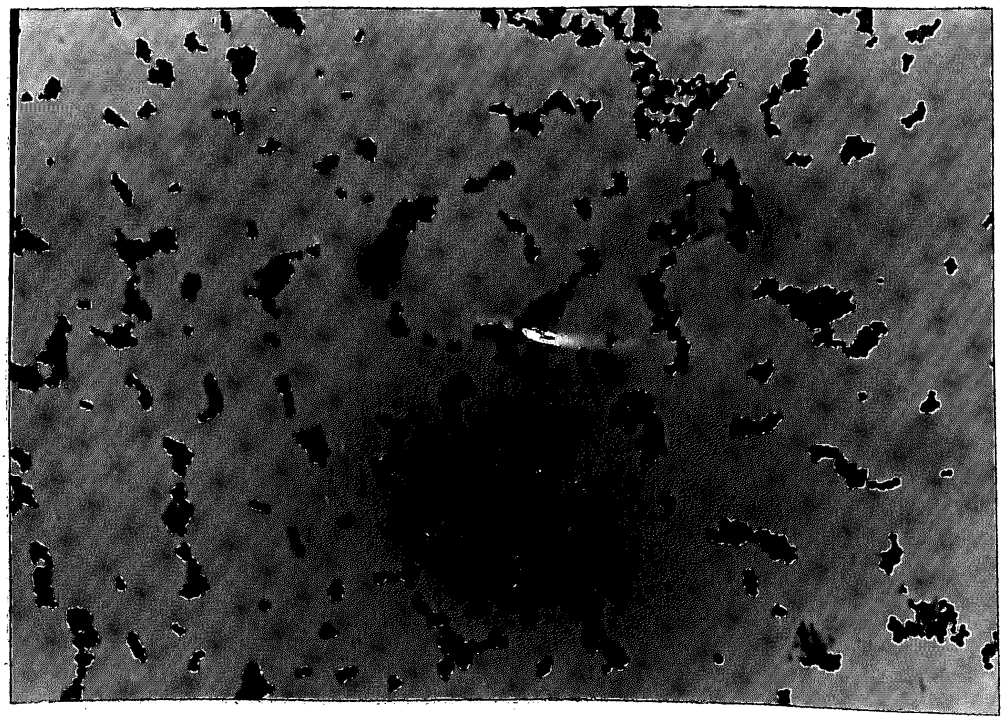


Plate 2. Gram-poistive cocci (*Micrococcus* sp.) (x 1000)

4.2 ENZYME ASSAYS

4.2.1 Catla

The protease activity in catla was strong in the fore-gut and there was a decreasing trend from the anterior to posterior gut. Amylase also showed a similar pattern as protease. Lipase activity, though found in the entire gut, did not show a regular pattern. It was moderate in both fore-gut and hind-gut but weak in the mid-gut. Invertase activity was low in the gut of catla and only found in the hind-gut of the alimentary canal. (Table 9).

4.2.2 Rohu

Rohu showed a strong activity of protease throughout the gut. Lipase and amylase were found to have uniform activities throughout the gut to a moderate extent. The presence of invertase was noticed only in the proximal part of the gut (fore-gut and mid-gut) to a low extent (Table 10).

4.2.3 Mrigal

Protease showed similar-patterns of fluctuations that of catla in a decreasing order from anterior to posterior gut. Moderate activity of lipase was observed in fore-gut and mid-gut followed by weak activity in hind-gut. Strong amylase activity and weak invertase activity were observed throughout the gut (Table 11).

4.2.4 Grass carp

Grass carp showed moderate activity of protease in the entire gut. Very strong amylase activity and weak lipase activity were observed throughout the tract. Invertase activity was observed in a decreasing order through the length of the gut (Table 12).

Table 9: Gut enzyme profile of *Catla catla*

Enzymes	Fore-gut	Mid-gut	Hind-gut
Protease	+ + +	+ +	+
Amylase	+ + +	+ +	+
Lipase	+ +	+	+ +
Invertase	-	-	+

Table 10: Gut enzyme profile of *Labeo rohita*

Enzymes	Fore-gut	Mid-gut	Hind-gut
Protease	+ + +	+ + +	+ + +
Amylase	+ +	+ +	+ +
Lipase	+ +	+ +	+ +
Invertase	+	+	-

Table 11: Gut enzyme profile of *Cirrhinus mrigala*

Enzymes	Fore-gut	Mid-gut	Hind-gut
Protease	+ + +	+ +	+
Amylase	+ + + +	+ + +	+ + +
Lipase	+ +	+ +	+
Invertase	+	+	+

Table 12: Gut enzyme profile of *Ctenopharyngodon idella*

Enzymes	Fore-gut	Mid-gut	Hind-gut
Protease	+ +	+ +	+ +
Amylase	+ + + +	+ + + +	+ + + +
Lipase	+	+	+
Invertase	+ + +	+ +	+

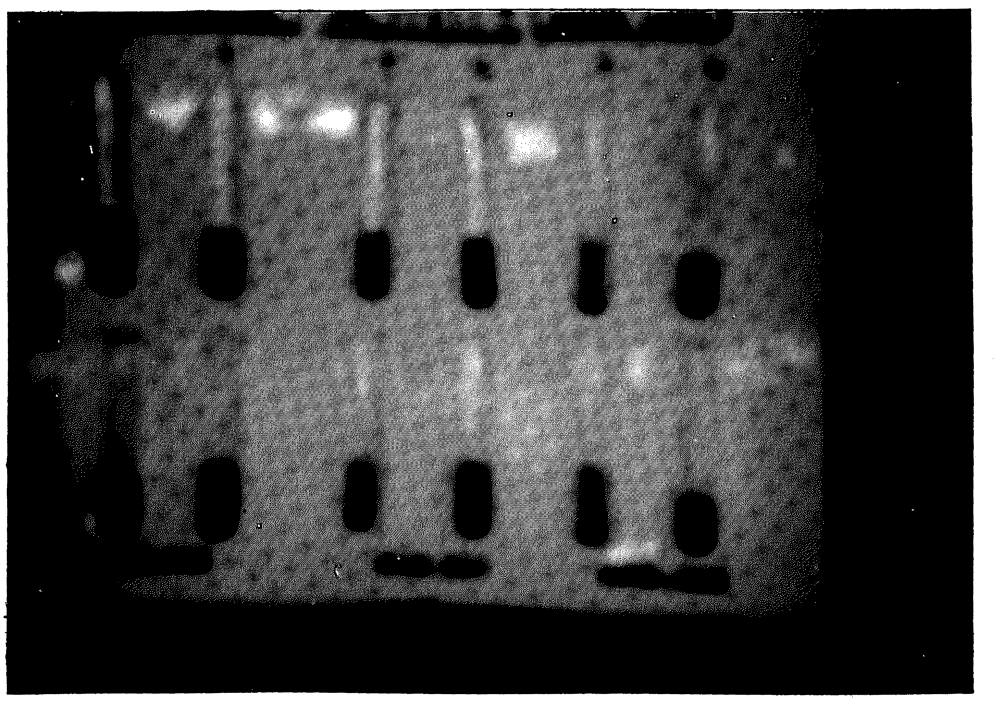


Plate 3. Test for amaylase activity in gut of carp species

CHAPTER - 5

DISCUSSION

5. DISCUSSION

The results of the present investigation are discussed in the light of the earlier studies and salient findings of the present study.

5.1 BACTERIOLOGICAL STUDIES

5.1.1 Bacterial populations

5.1.1.1 Water and sediment

The bacterial population counts of sediment samples were observed to be higher than those of water medium. This is attributed to higher substrate availability and higher rates of mineralisation and transformation of nutrients at the sediment-water interface as also observed by Schornik and Ram (1978), Kona and Tezuka (1979), Ram *et al.* (1980), Ayyappan (1987), Crissaffi and Maugaeri (1988), Ayyappan *et al.* (1991a,1992) and Sugita *et al.* (1992).

5.1.1.2 Body surface

As regards the body surface, the bacterial loads in the four carp species were in the range of $10^3/cm^2$ agreeing with the observations of Horsley (1973). Okpokwasili and Alapiki (1990) also observed similar values on the body surface of tilapia (*Oreochromis niloticus*). However, they recorded significantly higher loads on the skin of diseased fishes. Higher bacterial counts of the order of 10^6 to $10^9/cm^2$ were observed in the fishes raised in waste water aquaculture systems by Ogbondeminu and Okoye (1992). From these studies as well as present results, it is clear that bacterial populations on the body surface of fishes are influenced by the microbial loads of the ambient waters.

The differences in the bacterial loads on the body surfaces of catla, rohu and mrigal were perceptible, considering their different ecological niches. The heterotrophic bacterial populations in the water medium of pond ecosystem showed a reducing vertical profile from surface to bottom waters (Ayyappan *et al.*, 1990a, 1991a). In the present study, catla, grass carp and mrigal showed similar patterns of bacterial loads according to their corresponding ecological niches. Rohu, a column feeder, however showed significantly higher bacterial load on its body surface ($p < 0.05$). This may be due to the colonisation of bacterial flora on the body surface of rohu on the slime layer and further active multiplication and adaptation to the conditions of the water column where the fish largely inhabits.

5.1.1.3 Gills

The gill heterotrophic bacterial loads, as hypothesised, were higher as compared to the water medium owing to the continuous filtration of the water by the gills. The variations in microbial loads among the four carp species also showed similar pattern as that of body surface. The bacterial loads in the gills observed in the present study were similar to those observed by Mudarris and Austin (1988). Geldreich and Clarke (1966) demonstrated that bottom feeding fish have higher level of coliforms, faecal coliforms and streptococci than surface-feeding and predaceous fish. Ogbondeminu and Okoye(1992) observed that common carp, *Cyprinus carpio* harboured significantly higher bacterial growth than tilapia, *Sarotherodon galilaeus*.

5.1.1.4 Gut

In all the four species of carps, gut harboured higher bacterial loads than that of sediment. Higher bacterial loads in the gut of fish than sediment samples were observed by Mary (1977), Trust *et al.* (1979) and Henebry *et al.* (1988). This has been attributed to proliferation of bacterial cells on the ingested food materials.

Gut bacterial counts were always more numerous than bacterial counts in water medium surrounding the fish as also observed in grass carp by Trust *et al.* (1979). Higher bacterial load was due to ingestion of food material that support greater bacterial biomass per unit weight and also concentration of bacteria inside the gut (Odum, 1968; Mariorty, 1976).

The levels of bacterial loads in gut of all four species of carps were higher than those of body surface as observed by Ogbondeminu and Okoye (1992). However, they found higher total viable counts in the cases of *Cyprinus carpio* raised in waste water systems Ogbondeminu *et al.* (1991b) however, recorded higher bacterial load on skin than gut of *Oreochromis niloticus* reared in out-door concrete ponds. These results suggest that, the gut bacterial flora is not merely influenced by the ambient water conditions but to a great extent by the feeding spectrum and ingested feed and further colonisation in the intestine. These are in agreement with the observations of Sakata *et al.* (1980a) and Sugita *et al.* (1985) that intestinal microflora are derived from the diet or environment as also suggested by Ogbondeminu *et al.* (1991b) and become established on development of favourable ecological conditions.

Statistical analyses indicated that there were no significant differences in loads of heterotrophic bacteria among carp species except rohu and mrigal. The highest gut bacterial load in rohu may be attributed to the detritophagic feeding nature of rohu (Ayyappan, 1991; Jhingran, 1991). Ayyappan *et al.* (1990) evaluated the trophic significance of microbial communities as trophic components of filter feeding fishes, showing the possibilities of their better utilisation through medium enrichment or diet incorporation. The highest specific growth rate was observed in case of rohu provided with daily bacterial inoculation and nutrient enrichment. These results suggest that rohu utilise the bacterial cells to a great extent than other species of carps.

5.1.2 Generic Composition

In the present study gram-positive bacteria were dominant in the generic composition of bacterial flora, followed by gram-negative bacteria in all the sources, as also reported by Shiranee *et al.* (1979). Austin *et al.* (1979) and Sugita *et al.* (1985a,b) reported gram-negative bacteria prevailing in different water samples of marine and freshwater ecosystems. Lesel (1979), while reviewing the bacterial microflora of fish, reported that gram-negative bacteria were the prevalent in the gastro-intestinal tract of fishes.

Considering all five groups of heterotrophs, based on gram reaction and morphology, gram-negative bacilli dominated followed by gram-positive bacilli. On the body surface, however, prevalence of relatively higher number of gram-positive cocci was observed. Jones and Godinho Orlandi (1981) reported the dominance of gram-positive bacilli in the sediment of freshwater ecosystem. The present study showed the occurence of gram-positive bacilli with about similar representation of gram-negative bacilli.

Characterisation of gut bacterial isolates indicated that *Bacillus* was the dominant genus followed by enterobacteriaceae, *Pseudomonas, Micrococcus, Kurthia, Alcaligenes, Plesiomonas, Planococcus, Listeria, Acinetobacter, Sporosarcina, Sporolactobacillus* and *Flavobacterium*. Among enterobacteriaceae, the important genera were *Enterobacter, Serratia* and *Proteus*. Non-enteric gram-negative rods were dominated by *Pseudomonas* followed by *Alcaligenes*. *Aeromonas* dominated the vibrionaceae family. Gram-positive cocci were mainly represented by *Micrococcus*.

Higher ranges of the bacterial groups were recovered from the gastro-intestinal tract of freshwater fishes as also observed in the present study (Trust and Sparrow,1974; Sugita *et al.* 1983, 1986; Olayemi *et al.*, 1990).

Bacillus was the dominant genus isolated from the gut of carps in the present study. Singh (1994) isolated *Bacillus* as the dominant genus from water medium and sediments of catfish pond of CIFA fish farm. Okpokwasili and Alapiki (1990) observed *Bacillus* as the dominant genus in sediments, water,feed and skin of a Nigerian freshwater fish culture. Olayemi *et al.* (1990) isolated *Enterobacter, Proteus, Salmonella and Serratia* including other members of enterobacteriaceae such as *Escherichia, Shigella, Edwardsiella* and Klebsiella. Other gram-negative bacteria included *Aeromonas, Acinetobacter* and *Pseudomonas*. Gram-positive bacterial genera included *Staphylococcus* and *Micrococcus*. Sugita *et al.* (1983) isolated facultative anaerobes, enterobacteriaceae and *Vibrio-Aeromonas* group as predominant in the gastro-intestinal tract of freshwater fish in Tama River. In the present study, *Vibrio* was not isolated as it is generally prevalent in seawater fishes.

Sakata *et al.* (1980a) studying the variations in intestinal microflora of *Tilapia* reared in freshwater and seawater, reported that intestinal microflora of fresh or seawater fishes mainly comprised *Aeromonas* or *Vibrio* and *Aeromonas* respectively. Sakata *et al.* (1984) isolated *Vibrio, Aeromonas* and *Pseudomonas* as dominant genera from gastro-intestinal tract of tilapia (*Sarotherodon niloticus*) obtained from different locations.

Trust *et al.* (1979) isolated *Pseudomonas, Vibrio, Flavobacterium, Bacillus, Aeromonas, Micrococcus,* enterobacteriacea, *Alcaligenes* and *Acinetobacter-Moraxella* from gut of grass carp. Aerobic and facultative anaerobic bacteria isolated from intestine of gold fish (*Carassius auratus*) by Sugita *et al.* (1988) included genera like, *Pseudomonas, Flavobacterium, Aeromonas,* enterobacteriaceae and *Acinetobacter-Moraxella*. *Aeromonas, Pseudomonas, Flavobacterium* and members of enterobacteriaceae were isolated from the gut of carp, *Cyprinus carpio* obtained from rearing pond in Japan by Sugita *et al.* (1985c).

The above observations are similar to the findings in the present study where the gastro-intestinal tract bacterial flora largely comprised the facultative anaerobic and aerobic bacterial genera allocated with the natural fish food in the ambient waters.

5.2 ENZYME ASSAYS

5.2.1 Protease

Strong protease activity was observed in cases of catla, rohu and mrigal while the activity level was moderate in grass carp, in line with their feeding habits. Kawai and Ikeda (1972) and Shcherbina (1976) reported adaptive changes in the activity of proteolytic enzymes in *Cyprinus carpio* in relation to the type of diet. In case of catla and mrigal, high activities were observed in the fore-part of the intestine. The strong activity of protease in the fore-part of the gut of carps may be attributed to neutral range of pH of the stomachless carps comprising trypsin or chymotrypsin-type. Das and Tripathi (1991) recorded optimum proteolytic activity between pH 7.6 and 8.4 in both the fingerlings and adult grass crap. Bitterlich (1985) Observed sharp decrease of proteolytic activities from fore-gut to hind-gut of silver carp.

5.2.2 Amylase

Dhage (1968) and Phillips (1969) suggested that amylase activity in the intestine of herbivorous carp is more intense than in carnivorous fish. In the present study also, strong amylase activity was observed in all carp species except rohu, where it was comparatively lower. Higher amylase activity in catla was observed in anterior intestine. Kawai and Ikeda (1973) (as quoted by singh (1987)) showed strong amylase activity in mid-intestine of grass carp.

5.2.3 Lipase

Al-Hussaini (1949) observed the occurrence of lipase in cyprinids with higher activity in the anterior intestine than posterior intestine. In the present

study, moderate activities were observed in catla, rohu and mrigal while low activity was observed in grass carp. Lipase activity was concentrated in the anterior one-fifth of the intestine in mrigal and rohu but was found to be totally absent in the entire intestine of catla (Dhage, 1968). Ni *et al.* (1990) observed higher lipase activity in the mid-gut of intestine than in fore-gut and hind-gut of silver carp and big head carp while incase of common carp, it gradually reduced from fore-gut to hind-gut.

5.2.4 Invertase

Invertase activity was low in the carp species except grass carp, which showed relatively high activity. The levels gradually reduced towards posterior part of the gut. Ghosh and Saigal (1984) observed the presence of invertase in *Pangasius pangasius* with a greater activity in the anterior parts of the intestine . They also observed similar pattern of distribution for other enzymes viz, protease, amylase and lipase. Invertase was found to be moderate in the intestine of carnivorous cat fish *Heteropneustes fossilis* (Saigal *et al.*, 1974).

The present investigation was an attempt in correlating the gut microflora with the digestive enzyme profile. Among the four species of carps studied, highest gut bacterial load was observed in rohu and significantly lower gut load was observed in mrigal ($p < 0.10$). The comparison of the amylase activity between the two species showed a higher activity in mrigal than rohu. This inverse relationship between gut bacterial loads and amylase activities Indicated the possible role of the bacterial flora in the digestion process of carbohydrate substrates. Shiranee *et al.* (1993) observed that gut microflora of pearl spot (*Etroplus suratensis*) was predominated by amylolytic forms, corroborating the present observations.

In addition to digestion, intestinal microflora also play important role in other nutritional functions such as vitamin synthesis (Teshima and Kashiwada, 1967, 1969), essential amino acid synthesis (Lesauskiene *et al.*, 1974), etc.

Considering the possibilities of development of probiotics for better feed digestion and assimilation, further studies are suggested with regard to isolation and evaluation of gut bacterial flora with reference to digestive enzyme profile of carp species, ambient environmental regime, natural and supplementary feeds, etc. for formulating fish feed probiotics.

CHAPTER - 6

SUMMARY

6. SUMMARY

Heterotrophic bacteria play a major role in aquatic culture systems in maintenance of water quality, nutrient recycling as also nutrition of fish. While the role of heterotrophic bacterial communities in algae decomposition and nutrient liberation is well documented, their role in trophic sustenance and digestion of fish is yet to be studied in details. Considering the importance of this aspect, a comprehensive investigation of gut microflora of carps with reference to digestive enzyme profile was conducted. The studies were carried out in four species of carps, *viz.*, catla, *Catla catla*, rohu, *Labeo rohita*; mrigal, *Cirrhinus mrigala* and grass carp, *Ctenopharyngodon idella* reared in the fish farm at Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, Orissa, India (Lat. 20°11' 06"-20°11' 45"N; Long. 85°50'52"-85° 51' 35" E) during July to December, 1995.

The sources sampled during the study period comprised body surface, gills and gut of fishes. The ambient water and sediment were also sampled for bacteriological studies. The assessment of heterotrophic bacterial loads of different sources was done using nutrient glucose agar medium (Beef extract 3g, peptone 5g, sodium chloride 8g, Agar 15g, Distilled water 1000ml with pH 7.3 ± 0.2) by standard dilution plate count technique. The activities of digestive enzymes *viz.*, protease, lipase, amylase and invertase in different sections of gut of the four species of carps were assessed through qualitative assays. Gut enzymes were evaluated using provision of substrates *viz.*, gelatin for protease, olive oil for lipase, starch for amylase and sucrose for invertase.

The ranges of heterotrophic bacterial counts in different sources were: 1. water 1.25-2.55x 10^3/ml; 2. Sediment 1.23-2.57x10^5/g; 3. Body surface of catla 0.10-10.00x10^3/cm^2; 4. Gills of catla 0.71-21.00x$10^5$1g;5. Gut of catla 3.28-15.00x10^5/g;6. Body surface of rohu 4.96-28.10x10^3/cm^2; 7. Gills of rohu 1.36-7.00x10^6/g; 8. Gut of rohu 0.87-37.04x105/g; 9. Body surface of mrigal 2.22-6.67x103/cm^2;10. Gills of mrigal 1.50-29.55x10^5/g;11. Gut of mrigal 0.16-5.26x10^5/g; 12. Body surface of grass carp 0.34-11.67x10^3/cm^2;13. Gills of grass carp 0.86-7.75x10^5/gand 14. Gut of grass carp 0.46-38.00x10^5/g with respective mean viable counts of 1.76x10^3/m^2, 1.99x10^5/g, 2.67x10^3/cm^2; 1.34x10^5/g, 7.06x10^5/g, 18.33x10^3/cm^2, 40.97x10^5/g, 15.03x10^5/g, 4.09x10^3/cm^2, 19.80x10^5/g, 2.38x10^5/g, 3.52x10^3/cm^2, 3.34x10^5/g, and 9.76x10^5/g.

Among body surfaces of the four carp species, there were significant differences in bacterial loads between rohu and other three species ($P<0.05$). In case of gills, significant differences were found between rohu and catla as well as rohu and grass carp ($P<0.05$). The differences in the gut bacterial loads between catla and rohu were significant ($P<0.10$).

Among the bacterial isolates collected from different sources, 36.99% comprised gram-negative bacilli, followed by 25.71% gram-positive bacilli, 15.36% gram-positive coccobacilli, 14.42% gram-positive cocci and 7.52% gram-negative coccobacilli.

The gastro-intestinal tract flora showed high incidence of aerobic and facultatively anaerobic bacteria as derived from the environment and ingested feed, in line with the earlier observations with regard to other fish species. Bacterial isolates collected from the guts of four species of carps comprised 41.18% gram-negative bacilli, 17.65% gram-positive coccobacilli, 11.76% gram-positive coccus and 5.88% gram-negative coccobacilli. Gram-positive bacilli and coccobacilli were represented by genera like *Bacillus, Kurthia, Plesiomonas, Listeria, Sporosarcina* and

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Sporolactobacillus, gram-negative by *Enterobacter, Pseudomonas, Aeromonas, Serratia, Alcaligenes, Proteus, Acinetobacter, Flavobacterium* and *Salmonella* and gram-positive cocci by *Micrococcus* and *planococcus*. *Bacillus* was found to be dominant form of gram-positive bacilli and coccobacilli. Among gram-negative strains, important genera were *Enterobacter, Pseudomonas, Aeromonas* and *Serratia*. *Micrococcus* was the dominant genus among the gram-positive cocci.

The digestive enzyme assays included analyses for protease, amylase, lipase and invertase. As regards protease activity, it was strong in the fore-gut of catla and mrigal, that reduced towards the posterior part. The activities were strong and moderate throughout the gut in rohu and grass carp respectively. Gradual decrease of amylase activity from the anterior to posterior gut was observed in catla. Very strong and moderate levels of amylase activity were observed in grass carp, mrigal and rohu respectively. Lipase activity was moderate in all the species except grass carp where it was weak. Invertase activity was observed to be weak and discontinuous in all the species except grass carp where it was strong in fore-gut, moderate in mid-gut and weak in the hind-gut.

The present study made an attempt at characterising the gut heterotrophic bacterial flora with reference to prevalent digestive enzymatic activities. High bacterial population counts were observed in the gut of rohu where amylase activity was comparatively low. In case of mrigal, the amylase activity was strong with low gut heterotrophic load. these observations indicated the possible role of bacterial flora in the digestion process of carbohydrate substrate.

The gut bacterial flora of carps were found to be influenced by the generic composition of the water media. Being derived from the environment and associated with the feed ingested, further studies are suggested on the isolation of gut bacterial flora, assay of enzyme and possible incorporation of probiotics in fish feed formulations to enhance the feed digestibility and nutrient levels.

CHAPTER - 7

CONCLUSIONS

7. CONCLUSIONS

Investigations on the bacterial flora associated with carp species like catla, rohu, mrigal and grass carp with an emphasis on gut microflora with reference to their digestive enzyme profile carried out at the Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar during July to December 1995, led to the following conclusions:

1. The mean heterotrophic bacterial counts of different sources of four species of carps, *viz.*, catla, rohu, mrigal and grass carp were observed to be 2.67×10^3, 18.33×10^3, 4.09×10^3 and 3.52×10^3/cm^2 for body surface, 1.34×10^5, 40.97×10^5, 19.80×10^5 and 3.34×10^5/g for gills, and 7.06×10^5, 15.03×10^5, 2.38×10^5 and 9.76×10^5/g for gut, respectively.

2. The activities of digestive enzymes, *viz.*, protease, amylase, lipase and invertase along the gut from anterior to posterior region were observed to be strong to weak, strong to weak, moderate and low for catla; strong, moderate, moderate and low for rohu; strong to moderate, strong, moderate and weak for mrigal and moderate, strong, weak and strong to weak for grass carp, respectively.

3. Gram-positive bacteria were predominant in all the sources comprising 52.94%, 50.00%, 62.12%, 55.81% and 52.94% of the isolates collected from water media, sediment, body surface, gills and gut, respectively. In total, 55.48% of the isolates comprised gram-positive bacteria.

4. Generic representation of gut heterotrophic bacteria comprised *Bacillus* 30.15%, *Enterobacteria* 11.03%, *Pseudomonas* 11.03%, *Micrococcus* 9.56%, *Aeromonas* 5.88%, *Serratia* 5.88%, *Kurthia* 5.88%, *Alcaligenes* 4.41%, *Proteus* 3.68%, *Plesiomonas* 2.94% and others (*Planococcus, Listeria, Acinetobacter, Sporosarcina, Sporolactobacillus, Flavobacterium* and *Salmonella*) 9.56%.

5. The gut microflora of carps were found to be influenced by the generic composition in the ambient waters and had a role in the digestion process as evidenced from the high bacterial population in gut of rohu with comparatively low amylase activity. The study provided further lines of works with reference to isolation of gut bacterial flora, assay of enzymes and possible incorporation of probiotics in fish feed formulation.

BIBLIOGRAPHY

8. BIBLIOGRAPHY

*Adoni, A.D. 1975. *Studies on Microbiology of Sager Lake*. Ph. D. Thesis, Univ. Sagar, Sagar, M.P., 254 pp.

*Al-Hussaini, A.H. 1949. On the functional morphology of the alimentary tract of some fish in relation to differences in their feeding habits. Cytology physiology. *Quart.J. Micros. Sci.*, **90**: 323-354.

Alikunhi, K.H. 1952. On the food of young carp fry. *J. Zool. Soc. India*, **4**(1): 77-84.

Alikunhi, K.H. 1957. Fish culture in India. *Fm. Bull.* I.C.A.R. Delhi, **20**: 144 pp.

Alikunhi, K.H. and Sukumaran, K.K. 1962. Preliminary observations on Chinese carps in India. *Proc. Indian Sci. Congr.*, **49**(3): 387 p.

Allen, D.A., Austin, B., and Colwell, R.R. 1983. Numerical taxonomy of bacterial isolates associated with a freshwater fishery. *J. Gen. Microbiol.*, **129**: 2043-2062.

*Ananichev, A.V. 1959. Digestive enzymes of fish and seasonal changes in their activity. *Biokhimiya*, **24**: 1033-1040,(English transl: Biochemistry, **24**: 952-958, 1959).

Andersson, I.B. 1983. Bacterioplankton in the acidified lake Gardsjon. *Hydrobiologia*, **101**: 51-53.

Austin, B. 1983. Bacterial microflora associated with coastal, marine fish-rearing unit. *J.Mar. Biol. Ass.* UK, **63**: 585-592.

Austin, B. 1985. Antibiotic pollution from fish farms: Effects on aquatic microflora. *Microbiol. Sci.*, **2**: 113-117.

Avault, J.W. 1965. Preliminary studies with grass carp for aquatic weed control. *Prog. Fish-cult.*, **27**(4): 207-209.

Ayyappan, S. 1987. *Investigations on the limnology and microbial ecology of a lentic habitat*. Ph.D. Thesis, Bangalore University. Bangolore, 326 pp.

Ayyappan, S., Shakuntala, K. and Parameswaran, S. 1990a. Production efficiencies of a tropical freshwater lake. *Aqua. Hung.*, **6**: 65-75.

Ayyappan, S., Shakuntala, K. and Parameswaran, S. 1992. Microbial ecology of a tropical freshwater Lake. *J. Indian Inst. Sci.*, **72**: 1-13.

Ayyappan, S., Saha, P.K., Purushothaman, C.S. and Pani, K.C. 1987. Decomposition of water hyacinth, *Eichhornia crassipes* (mort.) Solms in response to nutrient enrichment. *In*: M. Mohan Joseph (ed.) *Proc. First Indian Fisheries Forum*, Asian Fisheries society. Indian Branch, Mangalore, 41-44.

Ayyappan, S., Pandey, B.K. and Tripathy, S.D. 1990b. Nutritive quality of microbial commouities as feed components in experimental carp culture. *Indian J. Exp. Biol.*, **28**: 977-980.

Ayyappan, S., Purushothaman, C.S., Saha, P.K. and Pani, K.C. 1991a. Heterotrophic bacterial populations and primary production in fish ponds under different management practices. *J.Aqua.*, **1**: 11-31.

Ayyappan, S., Pandey, B.K., Sarkar, S., Saha, C.D. and Tripathi, S.D. 1991b. Potential for spirulina as a feed supplement for carp fry. *In*: *Proc. Natl. Symp. On New Horizon In Freshwater Aquaculture*, Central Institute of Freshwater Aquaculture, Bhubaneswar, 23-25 Jan. Abstracts No. 86.

Barat, S. and Jana, B.B. 1990. Density difference of heterotrophic bacteria of water between carnivorous catfish farm and polyculture system of herbivorous carps. In: G.K. Manna and B.B. Jana (eds.) *Impact of Environment on Animal and Aquaculture*, University of Kalyani, Kalyani (India), 207-214.

Bardach, J.E., Ryther, J.H. and McLarney, W.O. 1972. *Aquaculture.* New York, John Wiley and Sons, Inc., 868 pp.

Barton, D.R. and Lock, M.A. 1979. Numerical abundance and biomass of bacteria, algae and macrobenthos of a large northern river, the Athabasca. *Int. Rev. ges. Hydrobiol.*, **64**(3): 345-350.

Bell, G.R., Hoskins, G.E. and Hodgkiss, W. 1971. Aspects of the characterisation, identification and ecology of the bacterial flora associated with the surface of stream incubating pacific salmon (*Oncorynchus*) eggs. *J. Fish. Res. Bd. Can.*, **28**: 1511-1525.

Bergey's Manual of Systematic Bactriology, 1984. N.R. Krieg (ed.) Vol. 1, The Williams and Wilkins Co. Baltimore, USA, 1268 pp.

Bergey's Manual of Systematic Bactriology, 1986. Peter, H.A. Sneath (ed.) Vol.2, The Williams and Wilkins Co. Baltimore, USA, 1599 pp.

*Bhat, K.C. 1986. *Studies on the formulation and relative merits of some artificial fish feeds.* M.F.Sc. Thesis, University of Agricultural Sciences Bangalore, India.

Bitterlich, G. 1985. Digestive enzyme pattern of two stomachless filter feeders silver carp, *Hypophthalmichthys molitrix* Val. and bighead *Aristichthys nobilis* Rich. *J. Fish Biol.*, **27**: 103-112.

Bondi, A. and Spandorf, A. 1953. The activity of digestive enzymes of the carp. (In Hebrew) *Bamidgeh*, **5**: 116-130.

Bondi, A. and Spandorf, A. 1954. The action of the digestive enzymes of the carp. *Br. J. Nutr.*, **8**: 240-246.

Chakroborty, R.D., Sen, P.R., Chatterjee, P.K. and Kowtal, G.V. 1973. Observations on the relative usefulness of different feed for carp spawn and fry. *J.Inland Fish. Soc. India,* **5**: 182-188.

Chang, Y.F. 1966. Culture of freshwater Fish in China *In*: E.O. Gangstad (ed.). Chinese fish culture, Report I. Aquatic plant control Research Program. *Tech. Rep*. U.S. Army Eng. Waterways Exp. Stn. Wash, (A-79), Draft transl. by T.S.Y. Koo, 1980.

Chiu, Y.N. and Benitez, L.V. 1981. Studies on the carbohydrate digestion in the digestive tract of the milk fish, *Chanos chanos. Mar. Biol.*, **61**: 247-254.

*Cohen, T. (1981). *Pancreatic proteolytic enzymes from carp cyprinus carpio* (In Hebrew with English) Ph.D. Thesis, Hebrew Univ of Jerusalem: 80-111 (mimeographed).

Collins, C.H. and Lyne, P.M. 1976. *Microbiological Methods*. Butterworth and Co. (Publ.) Ltd., London, 521 pp.

Colwell, R.R., 1962. The bacterial flora of Puget sound fish. *J. Appl. Bacteriol.*, **25**: 147-158.

Crissafi, E. and Maugaeri, T.L. 1988. Primary data on heterotrophic bacteria in deep sediments. *Mem. Biol. Mar. Oceanogr.*, **30**: 65-69.

Dabrowski, K. 1977. Protein requirements of grass carp fry (*Ctenopharygodon idella*). *Aquaculture*, **12**: 63-73.

Dabrowski, I.K. and Kazak, B. 1978. The use of fish meal and soybean meal as a protein source in the diet of grass carp fry. *Aquaculture*, **18**: 107-114.

Das, B.C. 1960. Effectiveness of yeast, Vit. B_{12} and alternative treatment on the survival of Indian carp during the early period of life. *Can. J. Biochem. Physiol.*, **28**: 453-458.

Das, M. and Singh, C.S. 1989. Economical supplementary feed for fish production. *In*: *Proc. Natl. Sem.* on Forty years of freshwater Aquaculture, Bhubaneswar, 7-9 Nov. 1989. Abstract SVN/17.

Das, K.M. and Tripathi, S.D. 1991. Studies on the digestive enzymes of grass carp, *Ctenopharyngodon idella* (Val.). *Aquaculture*, **92**: 21-32.

Dash, B. 1993. *Biological nitrogen fixation in freshwater fishpond ecosystems with reference to hydrobiological conditions.* Ph.D thesis, Orissa Univ. Agric. Tech., Bhubaneswar, 318 pp.

Devraj, K.V., Krishna Rao, D.S. and Keshavappa, G.Y. 1981. Utilization of duck weed and waste cabbage leaves in the formulation of fish feed. *Mysore J. Agric. Sci.*, **15**: 131-135.

Dhage, K.P. 1968. Studies of the digestive enzymes in the three species of the major carps of India. *J. Bio. Sci.*, **11**: 63-74.

Edwards, D.J. 1973. Aquarium studies on the consumption of small animals by O-group grass carp, *Ctenopharyngodon idella* (Val.). *J. Fish. Biol.*, **5**(5): 599-605.

Edwards, D.J. 1974. Weed preference and growth of young grass carp in New Zealand. *N.Z.J. Mar. Freshwat. Res.*, **8**(2): 341-350.

Edwards, D.J. 1975. Taking a bite at the water weed problem. *N.Z.J. Agric.*, **30**(1): 33, 35-36.

*Eichler, L.W. and Pope, D.H. 1984. The effect of lake acidity levels on microbial flora of selected fish species. *In*: J.S. Jacobson, L.S. Raymond (eds.) *Proc. New York state symp. on Atmospheric Deposition*. October 26-27, 1983. New York, Publ.: Cornell Univ., Cent. Environ. Res. Ithaca NY (USA), 199-208.

Esteve, C. and Garay, E. 1991. Heterotrophic bacterial flora associated with European eel, *Anguilla anguilla* reared in freshwater. *Bull. Jap. Soc. Sci. Fish.*, **57**(7): 1369-1375.

Falge, R., Schpanof, L. and Jurss, K. 1978. Amylase, esterase and protease activity in the intestine content of rainbow trout *Salmo gairdneri* Rich., after feeding with feed containing different amounts of starch and protein. *J. Ichthyol.*, **18**: 283-287.

Fang, X., Guo, X., Wang, T., Fang, Y. and Liu, Z. 1989. The preliminary study of heterotrophic bacteria in a high yielding fish pond. *J. Fish. China.*, **13**(2): 101-107.

Fischer, Z. 1968. Food selection in grass carp (*Ctenopharyngodon idella* Val.) under experimental conditions. *Polar Arch. Hydrobiol.*, **15**(1): 1-8.

Fish, G.R. 1960. The comparative activity of some digestive enzymes in the alimentary canal of tilapia and perch *Hydrobiol.*, **15**: 161-178.

Fisher, R.A. and Yates, F. 1982. *Statistical Tables*. 6th. edition, Hing Yip Printing Co., 146 pp.

Forester, T.S. and Avault, Jr. J.W. 1978. Effects of grass carp in freshwater red swamp crayfish in ponds. *Trans. Am. Fish. So.*, **107**(1): 157-160.

Gantar, M.F. 1985. The effect of heterotrophic bacteria on the growth of *Nostoc* sp. (Cyanobacterium). *Arch. Hydrobiol.*, **103**(4): 445-452.

Geldreich, E.E. and Clarke, N.A. 1966. Bacterial pollution indicators in the intestinal tract of freshwater fish. *Appl. Microbiol.*, **14**: 429-437.

Georgala, D.L. 1958. The bacterial flora of the skin of North Sea cod. *J. Gen. Microbiol.*, **18**: 84-91.

*Gerald, O. 1985. *Characteristics of bacterial flora in the digestive tracts of an African catfish, Clarias lazera.* Thesis Doct. Vet., ENV Toulouse (France), pp: 1-39.

Ghosh, A. and Saigal B.N. 1984. Observation on the digestive enzymes in the catfish *Pangasius pangasius* (Ham.) in relation its food habits. *J. Inland Fish. Soc. India*, **13**(1): 91-92.

Ghosh, A., Datta, A.K. and Saigal, B.N. 1977. Observations on the digestive enzymes of *Heteropneustes fossilis* (Bloch): Proteinase and Lipase. *J. Inland Fish. Soc. India,* **9**: 197-198.

Gillespie, N.C. and MacRae, I.C. 1975. The bacterial flora of some Queensland fish and its ability to cause spoilage. *J. Appl. Bacteriol.*, **39**: 91-100.

Gilmour, A., McCallum, M.F. and Allan, M.C. 1976. a study of the bacterial types occurring on the skin and in the intestines of farmed plaice, *Pleuronectes platessa. Aquaculture*, **7**: 161-172.

*Goel, N.R. 1975. Histochemical study of the activity of acid and alkaline phosphatases and lipase in the gastrointestinal tract of *Cirrhinus mrigala. Acta. Histochem.*, **54**: 48-55.

Gorbach, E.I. 1971. Condition and fatness of the grass carp (*Ctenophayngodon idella* Val.) in the Amur basin. *J.Ichthyol.*, **11**(6): 880-890.

Gorbach, E.I. 1972. Fecundity of the grass carp (*Ctenopharyngodon idella* Val.) in the Amur basin. *J.Ichthyol.*, **12**(4): 616-625.

Gupta, A.B. and Ahmad, M.A. 1966. Studies on the effect of feeding freshwater fishes with *Scendesmus obliquus* (Turpin) Kutz.*Hydrobiologia*, **28**: 42-48.

*Guthke, G. 1980. Quantitative mikrobiologiche untersuchngen in der Mittienen saale (Quantitative microbiological investigations of the middle part of river Saale, GDR). *Limnologica.*, **12**(2): 243-254.

Hamid, A., Sakata, T. and Kakimoto, D. 1979. Microflora in the alimentary tract of grey mullet 4. Estimation of the enzymatic activity in the intestinal bacteria. *Bull. Jap. Soc. Sci. Fish.*, **45**(1): 99-106.

Henebry, M.S., Gorden, R.W. and Buck, D.H.1988. Bacterial population in the gut of the silver carp (*Hypophthalmichthys molitrix*). *The Progr. Fish-cult.*, **50**: 86-92.

Henson, R.H. 1990. Spirulina algae improves Japanese fish feeds. *Aquacult. Mag.*, **16**(6): 38-43.

*Hickling, C.F. 1971. *Fish Culture,* 2nd. ed. Faber and Faber, London, 317 pp.

Hofer, R. 1979a. The adaptation of digestive enzymes to temperature, season and diet in roach, *Rutilus rutilus* and rudd, *Scardinius erythrophthalmus*: proteases. *J. Fish Biol.*, **15**: 373-379.

Hofer, R. 1979b. The adaptation of digestive enzymes to temperature, season and diet in roach, *Rutilus rutilus* L. and rudd. *Scardinius erythrophthalmus* L. 1. Amylase. *J. Fish Biol.*, **14**: 565-572.

Hora, S.L. and Pillay, T.V.R. 1962. Handbook on Fish culture in the Indo-Pacific Fisheries Region, *FAO Fish Biol. Tech. pap.*, (14): 203p.

Horsley, R.W. 1973. The bacterial flora of the Atlantic salmon (*Salmo salar L.*) in relation to its environment. *J. Appl. Bacteriol.*, **36**: 377-386.

Horsley, R.W. 1977. A review of the bacterial flora of teleosts and elasmobranchs including methods for its analysis. *J. Fish Biol.*, **10**: 529-553.

Hsu, Y.L. and Wu, J.L. 1979. The relationship between feeding habits and digestive proteases of some freshwater fishes. *Bull. Inst. Zool. Acad. Sinica*, **18**(1): 45-53.

Igarshi, M.A., Sugita, H. and Deguchi, Y. 1989. Microflora associated with eggs and nauplii of *Artemia salina. Bull. Jap. Soc. Sci. Fish.*, **55**(II) p. 2045.

Ingraham, J.L. 1962. Temperature relationship. *In*: J.C. Gunsalus and R.Y. Stanier (eds.). The bacteria. 4. The physiology of growth, Academic press, New York: 265-296.

Insam, H. and Domsch, K.H. 1988. Relationship between soil organic carbon and microbial biomass on chronosequences of reclamation sites. *Microb. Ecol.*, **15**: 177-188.

Jadhav, A.G. and Prasad Rao, P.D. 1991. Effect of supplementary feed such as abattoir and fish market waste on growth of the fingerlings of *Labeo rohita* (Ham). *In*: *Proc. Natl. Sem. on New Horizons in Freshwater Aquaculture*, Central Institute of Freshwater Aquaculture, Bhubaneswar, 23-25 Jan., 1991, 81-83.

Jana, B.B., Patel, G.N., Roy, S.K. and De, U.K. 1980. Growth characteristics of heterotrophic bacterial populations of water and bottom sediments in tanks under different trophic conditions. *Hydrobiologia*, **75**: 231-239.

Jana, B.B. and Roy, S.K. 1983. Estimates of microbial population involved in the N-cycle and their activity in water and sediments of fish farming ponds under mono and polyculture systems. *Int. Rev.ges. Hydrobiol.*, **68**: 581-590.

Jana, B.B. and Barat, S. 1992. Management induced changes in water quality in fish farming systems: Pattern and magnitude. *J. Agua. Trop.*, **7**: 53-92.

Jana, B.B. and Patel, G.N. 1984. Measurements of some groups of microbial populations with special reference to denitrification in water and sediments of fish farming ponds under mono and polyculture systems. *Int. Rev. ges. Hydrobiol.*, **64**(2): 231-240.

Jana, B.B. and Patel, G.N. 1986. Seasonal and spatial distribution of iron precipitating heterotrophs in water and sediments of fish ponds of differing farming managements. *Int. Rev. ges. Hydrobiol.*, **2**: 259-270.

Jhingran, V.G. and Pullin, R.S.V. 1985. *A Hatchery Manual for the Chinese and Indian major carps*. Asian Development Bank, ICLARM, Manila, Phillipines, 199 pp.

Jhingran, V.G. 1991. *Fish and Fisheries of India*. Rev. Eighth edition, Hindustan Publ. Co. (India), Delhi, 727 pp.

Jonas, E., Ragyanszki, M., Olha, J. and Boross, L. 1983. Proteolytic digestive enzymes of carnivorous (*Silurus glanis* L.),herbivorous (*Hypophthalmichthys motilrix*.Val.)and omnivorous (*Cyprinus carpio* L.)fishes. *Aquaculture*, **30**: 145-154.

Jones, J.G. 1971. Studies on freshwater bacteria: Factors which influence the populations and its activity. *J. Ecol.*, **59**: 593-613.

Jones, J.G. and Godini-Orlandi, M.J.L. 1981. The distribution of some genera of filamentous bacteria in littoral and profoudal lake sediments. *J. Gen. Microbiol.*, **123**: 99-101.

Kapoor, B.G., Smith, H. and Verijhina, I.A. 1975. The alimentary canal and digestion in teleosts. *Adv. Mar. Biol.*, **13**: 109-239.

Kawai, S.I and Ikeda, S. 1971. Studies on digestive enzymes of fishes. I. Carbohydrases in digestive organs of several fishes. *Bull. Jap. Soc. Sci. Fish.*, **37**(4): 333-337.

Kawai, S.I. and Ikeda, S. 1972. Studies on digestive enzymes of fishes. II. Effect of dietary change on the activities of digestive enzymes in carp intestine. *Bull. Jap. Soc. Sci. Fish.*, **38**: 265-270.

*Kawai, A. Takatori, N. and Sugiyama, M. 1975. Aquaculture environments and microbes. *In*: *The Japanese Society of Scientific Fisheries* (Editor), Marine Ecosystem and Microbes. Koseisha-Koseikaku, Tokyo: 112-125.

Kawamoto, N.Y. 1957. Production during intensive carp culture in Japan. *Progr. Fish-Cult.*, **19**(1): 26-31.

Khan, H.A. 1971. Artificial feeding in composite fish culture. *Proc. 1st. all India co-ordinated research project on composite culture of Indian and exotic fishes,* 19 p., CIFRI, Cuttack.

Kilgen,R.H. and Smitherman, R.O. 1971. Food habits of the white amur stocked in ponds alone and in combination with other species. *Prog. Fish-cult.*, 33(3): 123-127.

Kirchman, D.L.,Keil, R.G. and Wheeler, P.A. 1990. Carbon limitation of ammonium uptake by heterotrophic bacteria in the subarctic pacific. *Limnol. Oceanogr.*, **35**(6):1258-1266.

Kitamikado, M. and Tachino, S. 1960a. Studies on the digestive enzymes of rainbow trout. I. Carbohydrases. *Bull. Jap. Soc. Sci. Fish.*, **26**(7): 679-684. Transl. Ser., Fish. Res. Bd Can. (2193).

Kitamikado, M. and Tachino, S. 1960b. Studies on digestive enzymes of rainbow trout.II. Proteases. *Bull.Jap. Soc. Sci. Fish.*, **26**: 685-690. Transl. Ser., Fish. Res. Bd. Can. (2195).

Kitamikado, M.,Morishita, T. and Tachino, S. 1964. Digestibility of dietary protein in rainbow trout II. Effect of starch and oil contents in diets, and size of fish. *Bull. Jap. Soc. Sci. Fish.*, **30**: 50-54.

Kuzmina, V.V. 1990. Temperature influence on the total level of proteolytic activity in the digestive tract of some species of freshwater fishes. *J. Ichthyol.*, **30**(7): 97-109.

*Kuznetsov, S.I.1952. *Role of microorganisms in the material turnover of lakes.* Nauka press, Moscow, 300 pp. (in Russian).

*Lesauskiene, L., Jankevicius, K. and Syvokiene, J. 1974. The role of the digestive tract microorganisms in the nutrition of pond fishes, 6. Content of free amino acids in the body of second-year fish and their synthetization by the digestive tract microorganisms (In Russian with English Summary.) Tr. An. Lit. SSR (Biol.), (2), 127-135.

*Lesel. R. 1981. Bacterial microflora of the digestive tract of fish presented at Nutrition des poissons. Actes coll. CNERNA, Paris (France) May 1979. Publ. by: CNRS, parts (France), 1981, pp: 89-100.

*Lesel, R. and Rouel, M. 1987. Relations entre temperature et flore bacterienne dans let tube digestif de la truite. Arch-en-ciel, Une nouvelle analyse, *Bull. Cent. Etud. Rech. Sci. Biarritz,* **15**(1-2): 13-21.

Lesel, R. and Peringer, P. 1981. Influence of temperature on the bacterial microflora in *Salmo gairdneri* Richaradson. *Arch. Hydrobiol.*, **93**(1): 109-120.

Lindsay, G.J.H. 1986. The significance of chitinolytic enzymes and lysozyme in rainbow trout (*Salmo gairdneri*) defence. *Aquaculture*, **51**: 169-173.

*Ling, S.W. 1967. Feed and feeding of warm water fishes in ponds in Asia and far east. *FAO Fish. Rep.*, (44) Vol **3**: 291-309.

*Liston, J. 1956. Quantitative variations in the bacterial flora of flatfish. *J. Gen. Microbiol.*, **15**: 205-216.

*Liston, J. 1957. The occurrence and distribution of bacterial types on flatfish. *J. Gen. Microbiol.*, **16**: 304-314.

Lovell, R.T. 1981. Intestinal synthesis of nutrients in fish. *Aquacult. Mag.*, **7**(3): 34-35.

Lovell, R.T., Prather, E.E., Tres-Dick, J. and Chhorn, L. 1974. Effect of addition of fish meal to all-plant feeds on the dietary protein needs of channel catfish in ponds. *In*: *Proc. 28th. Ann. Conf. of the Sourth-Eastern Association of Game and fish Commissioners*, 1974.

MacCormack, W.P. and Fraile, E.R. 1989-90. Bacterial flora of newly caught Antarctic fish *Notothenia neglacta. Polar Biol.*, **10**: 413-417.

MacFarlane, R.D., McLaughlin, J.J. and Bullock,G.L. 1986. Quantitative and qualitative studies of gut flora in striped bass from estuarine and coastal marine environments. *J. Wild L. Dis.*, **22**(3):344-348.

Margolis, L. 1953. The effect of fasting on the bacterial flora of the intestine of fish. *J. Fish. Res. Bd. Canada.*, **10**: 62-63.

*Mary, P.P. 1977. Studies on the gastrointestinal microflora of the mullet *Liza dussumieri* (Val.). Ph.D. Thesis, CAS in Mar. Biol., Annamalai Univ. 122 pp.

Mazumdar, A. and Dickman, M.D. 1989. Factors affecting the spatial and temporal distribution of phototrophic sulphur bacteria. *Arch. Hydrobiol.*, **106**(2): 209-226.

Minami, Z., Kajinta, M. and Hibino, S. 1972. Studies on the utilization of petro-yeast as diet of freshwater fish. 1. The distribution of chitin-decomposing bacteria in digestive tracts. *Bull. Jap. Soc. Sci. Fish.*, **38**: 1013-1019.

Mitra, B., Chaudhury, A. and Mukherjee, K. 1977. Variation of muscle lipid in fishes. *Proc. Zool. Soc. Calcutta*. **30**: 115-119.

Mohanty, S.N. and Swamy, D.N. 1986. Enriched conventional feed for Indian major carps. *In*: L. Maclean, J.L., Dizon, L.B. and Hosillos, L.V. (eds.), *The First Asian Fisheries Forum*, Asian Fisheries Society, Manila, Phillippines.

Mohanty, S.N., Swamy, D.N. and Tripathi, S.D. 1990. Growth, nutritional indices and carcass composition of the Indian major carp fry *Catla catla, Labeo rohita* and *Cirrhinus mrigala* fed with four different dietary protein levels. *Aqua. Hung.*, 211-217.

Moriarty, D.J.W. 1976. Quantitative studies on the bacteria and algae in the food of mullet *Mugil cephalus* L., and the prawn *Metapenaeus* bennettae (Tack and Dall). *J. Exp. Mar. Biol. Ecol.*, **22**: 131-143.

Moriarty, D.J.W. 1989. Ingestion and digestion of microorganisms by aquatic animals and the function of gut flora. p. 49 *Int. Colloquium Microbiol. Poecilotherms*. Paris, July 1-12, 1989.

Morishita, T., Noda, H., Kitamikado, M., Takahashi, T. and Tachino, S. 1964. The activities of the digestive enzymes in fish. *J. Fac. Fish*, Univ of Mie (Japan), 239-246.

Mudarris, M. and Austin, B. 1988. Quantitative and qualitative studies of the bacterial microflora of turbot, *Schophthalmus maximus* L., gills. *J. Fish Biol.*, **32**: 223-229.

Mukhopadhay, P.K., Dehadrai, P.V. and Banerjee, S.K. 1978. Studies on intestinal protease: Isolation purification and effect of dietary proteins on alkaline protease activity of the air-breathing fish, *Clarias batrachus* (Linn.). *Hydrobiologia*, **57**(1): 11-15.

Muroga, K., Higarhi, M.and Keitoku, H. 1987. The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegeli*) at larval and juvenile stages. *Aquaculture*, **65**: 79-88.

Murray, R.E. and Hudson, R.E. 1984. Microbial biomass and utilization of dissolved organic matter in the *Okefenokee* swamp ecosystem. *App. Env. Microbiol.*, **47**(4): 685-692.

Nagayama, F. and Saito, Y. 1968. Distribution of amylase glucosidase and galactosidase in fish. *Bull. Jap. Soc. Sci. Fish.*, **34**: 944-949.

Nandeesha, M.C., Devraj, K.V. and Sudhakar N.S. 1986. Studies on the growth response of four species of carps to different protein sources in the pelleted feeds. *In*:J.L. Maclean, L.B. Dizon and L.V. Hosillos (eds.), *The First Asian Fisheries Forum*, Asian Fisheries Society, Manila, Phillippines, 25-31.

Nandeesha, M.C., Das, S.K., Keshavanath, P. and Verghese, T.J. 1989a. Do fish require same level of protein everyday. *In*: *Proc. Natl. Sem. on Forty Years Freshwater Aquaculture*, Central Institute of Freshwater Aquaculture, Bhubaneswar, 7-9 Nov., 1989. Abstract SN/2.

Nandeesha, M.C., Srikant, G.K., Basavaraja, N., Keshavanath, P. Verghese, T.J., Banot, K., Roy, A.K. and Kale, R.D. 1988. Influence of earthworm meal on the growth and flesh quality of common carp. *Biological wastes* , **26**: 189-198.

Nandeesha, M.C., Basavaraja, N., Keshavanath, P., Verghese, T.J., Sudhakara, N.S., Srikant, G.K. and Ray, A.K.1989b. Formulation of pellets with sericulture wastes and their evaluation in carp culture. *Indian J. Anim. Sci.*, **59**: 1198-1205.

Natarajan, A.V. and Jhingran, A.G. 1961. Index of preponderance- a method for grading the food elements in the stomach analysis of fishes. *Indian J. Fish.*, 8(1): 54-59.

Newman, J.T., Jr, Cosenza, B.J. and Buck,J.D. 1972. Aerobic microflora of the blue fish (*Pomatomus saltatrix*) intestine, *J. Fish. Res. Bd. Canada*, **29**: 333-336.

Ni, Shouwen, Gui, Mingyuan, Liu, Huanliang, 1990. A comparative study on activity of lipase in grass carp, common carp, silver carp, bighead carp and Tilapia. *J. Dalian Fish. Coll.*, **5**(3-4):19-24.

Niamat, R. and Jafri, A.K. 1984. Preliminary observations on the use of water hyacinth leaf real as protein source in fish feed. *Curr. Sci.*, **50**(6): 338-340.

Nikolsky, G.V., 1963. (L. Birkett, Transl.) *The ecology of fishes,*. London, Academic Press: 352 p.

Noaillac-Depeyre, T. and Gas, N. 1974. Fat absorption by the enterocystes of the carp (*Cyprinus carpio* L.). *Cell. Tissue Res.*, **155** : 353-365.

Norris, J.R. and Ribbons, D.W. 1970. *Methods in Microbiology.* Volume 3A, Academic Press Inc., London, 505 pp.

Odum, W.E. 1968. The ecological significance of fine particle sclection by the striped mullet, *Mugil cephalus. Limnol. Oceanogr.,* **13**:92-98.

Ogbondeminu, F.S. 1993. The occurrence and distribution of enteric bacteria in fish and water of tropical aquaculture ponds in Nigeria. *J. Aqua. Trop.,* **8**: 61-66.

Ogbondeminu, F.S. 1994. Commensal bacterial microflora associated with incubating eggs of *Clarias anguillaris* in a tropical hatchery. *J. Aqua. Trop.,* **9**: 151-156.

Ogbondeminu, F.S. and Okoye, F.C. 1992. Microbiological evaluation of an untreated domestic wastewater aquaculture system. *J. Aqua. Trop.*, **7**: 27-34.

Ogbondeminu, F.S., Ovie, S.I. and Adeniji, H.A. 1991a. Development of heterotrophic bacterial community in outdoor ponds treated with fermented animal wastes for natural fish food production studies. *Ann. Rep. Natl. Inst. Freshwat. Fish. Res* .(Nigeria), 148-151.

Ogbondeminu, F.S., Omorinkoba, W.S., Madu, C.T. and Ibikunle, G.F. 1991b. Bacterial microflora associated with fish production in outdoor concrete ponds and their feed. *J. Aqua. Trop.*, **6**: 55-62.

Ohwada, K., Tabor, P.S. and Colwell, R.R. 1980. Species composition and barotolerance of gut microflora of deep sea benthic microfauna collected at various depth in Atlantic ocean. *Appl. Environ. Microbiol.*,**40**(4): 746-755.

Okafor, N. and Nzeakor, B.C. 1985. Microbial flora of fresh and smoked fish from Nigerian freshwater. *Food Microbiol.,* **2**: 71-75.

Okpokwasili, G.C. and Alapiki, A. M. 1990. Bacterial flora associated with a Nigerian freshwater fish culture. *J. Aqua. Trop.*, **5**: 87-90.

Olah, J. and Vasarhelyi, R. 1970. Comparative nutrient agar studies on the qualitative survey of Saprophytic water microorganisms. *Annl. Biol.*, Tihany, **37**: 223-234.

Olayemi, A.B., Adedayo, O. and Ojo, A.O. 1990. Microbiological studies on freshwater fishes from the Asa river, Ilorin, Nigeria. *J. Aqua. Trop.*, **5**: 135-139.

Onishi, T., Murayama, S. and Takeuchi, M. 1976. Sequence of digestive enzyme levels on carp after feeding. III.Response of protease and amylase to twice-a-day feeding. *Bull. Jap. Soc. Sci.Fish.,* **42**: 921-929.

Opuszynski, K. 1972. Use of phytophagous fish to control aquatic plants. *Aquaculture*. **1**(1): 61-74.

Opuszynski, K. 1979. Weed control and fish production. Proc. of the grass carp conference . In J.V. Shireman (ed.) *Proc. of the grass crap conference,* Univ. of Florida, Institute of food and Agricultural Sciences, 103-138.

Pandian, T.J. 1987. Fish Energetics. *In*: T.J.Pandian and F.J. Vemberg (eds.), *Animal energetics,* Vol.2, Academic Press, New York, 357-465.

Patankar, V.B. 1973. Esterase in the stomach of fishes with different food habits. *Folia Histochem. Cytochem.*, **11**: 253-258.

Patra, B.C. and Ray, A.K. 1988. A preliminary study on the utilization of the aquatic weed, *Hydrilla verticiallata* (I.F.) royle feed by the carp, *Labeo rohita* (Ham.): Growth and certain biochemical composition of flesh. *Indian Biologist*. Vol XX (1).

Patralekh, L.N. 1991. Bacterial population of three freshwater ecosystems. *Eviron. Ecol.,* **9**(1): 218-220.

Pattanaik, S. and Das, K.M. 1979. Utilisation of some aquatic weeds as food for rearing carp spawn and fry. *In*: *Symp on Inland Aquaculture,* (Abstracts), February 12-14, 1979, CIFRI, Barrackpore, p. 116.

Pattanaik, S., Swamy, D.N., Rout, M. and Das, K.M. 1989. On water hyacinth leaf meal as a protein source in pelleted feed for Indian major carps. *Proc. Natl. Sem. on Forty Years Aquaculture.,* 1989.

Peleteiro, M.C. and Richards, R.H. 1985. Identification of lymphocytes in the epidermis of the rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Dis.*, **8**: 161-172.

Phillips, A.M. 1969. Nutrition, digestion and energy utilization. *In*: W.S. Hoar and D.J. Randall (eds.), *Fish physiology,* I. *Excretion ionic regulation and metabolism,* Academic Press, New York, 391-432.

Prabhavathy, G. and Sreenivasan, A. 1977. Cultural prospects of Chinese carps in Tamilnadu. *Proc. IPFC,* **17**(3): 354-362.

Ragynszki, M. 1980. Preliminary investigations on the proteolytic digestive enzymes of carp fry. *Aqua. Hung.* (Szarvas), **2**: 27-30.

Rai, H. and Hill, G. 1980. Classification of Central Amazon Lakes on the basis of their microbiological and physico-chemical characteristics. *Hydrobiologia*, **72**: 85-99.

Rai, H. and Hill, G. 1982. Establishing the pattern of heterotrophic bacterial activity in three Central Amazonian lakes. *Hydrobiologia*, **86**: 121-126.

Ralizaona, C., Wambeke, F. Van, Bianchi, A. 1983. Isolation of the bacterial microflora in the digestive tract of abyssal fishes, *Rev. Int. Oceanogr.* Med., **70-71**: 67-78.

Ram, N.M., Ulitzer, S. and Avnimelech, Y. 1980. Microbial and chemical changes occurring at the mud-water interface in an experimental fish pond. *Technion-Israel, Inst. Tech. Israel* Publ., **317**: 19.

Ram, N.M., Zur, O. and Avnimelech, Y. 1982. Microbiological changes occurring in sediment-water interface in an intensively stocked and fed fish pond. *Aquaculture*, **27**: 63-72.

Rao, S.S. and Burnison, B.K. 1976. Bacterial distribution in lake Erie (1967,1970). *J. Fish. Res. Bd. Can.*, **33**: 574-580.

Ray, A.K. 1988. On digestive enzymes in three Indian freshwater perches in relation to food and feeding habits. *J. Inland. Fish. Soc. India*, 20(1): 1-5.

Reimer, G. 1986. The relationship between the digestive enzymes in Arctic char, *Salvelinus alpinus* (Salmonidae, Osteichthyes) and its ability to survive extreme environments *Hydrobiologia*, **133**(1): 65-72.

Renukaradhya, K.M. and Verghese, T.J. 1986. Protein requirement of the carps *Catla catla* (Ham.) and *Labeo rohita* (Ham.). *Proc. Indian Acad. Sci.*, **95**: 103-107.

Sabapathy, U. and Teo, L.H., 1993. A quantitative study of some digestive enzymes in the rabbitfish, *Siganus canaliculatus* and the sea bass, *Lates calcarifer. J. Fish Biol.* **42**: 595-602.

Saigal, B.N., Ghosh, A. and Datta, A.K. 1974. Observations on the carbohydrate digestive enzymes in carnivarious catfish *Heteropneustes fossilis* (Bloch). *J. Inland Fish. Soc. India,* **6**: 83-84.

Sakata, T., Okabayashi, J. and Kakimoto, D. 1980b. Variations in intestinal microflora of *Tilapia* reared in fresh and seawater. *Bull. Jap. Soc. Sci. Fish.*, **46**: 313-317.

Sakata, T., Sugita, H., Mitsuka, T., Kakimoto, D. and Kadota, H. 1980b. Isolation of obligate anaerobes from the intestinal tract of freshwater fish. *Bull. Jap. Soc. Sci. Fish.*, **46**(4): 871.

Sakata, T., Uno, K. and Kakimoto, D. 1984. Dominant bacteria of the aerobic microflora in Tilapia intestine. *Bull. Jap. Soc. Sci. Fish.*, **50**(3): 489-493.

Sarbahi, D.S. 1951. Studies of the digestive tract and the digestive enzymes of the goldfish *Carassius auratus* (Lin.) and the largemouth blackbass, *Micropterus salmoides* (Lacepede). *Biol. Bull.,* **100**: 244-257.

Scaperclaus, W. 1933. Textbook of pond culture: Rearing and keeping of carp, trout and allied fishes (Tr. F Hund). *Fish Leaflet Wash.* (311): 260 p.

Schmidt, D.W. 1970. Numbers of bacteria and algae and their interrelations in some Amazonian waters. *Amazonia*, **2**(4): 393-400

*Schornik, J. and Ram, N. 1978. Nitrification in four acidic streams in Southern New Jersey. *Wat. Res. Invest,* US Geol. Survey, 22-121.

Sen, P.R., Rao, N.G.S., Gosh, S.R.and Rout, M. 1978. Observation on the protein and carbohydrate requirements of carps. *Aquaculture*, **13**: 245-255.

Seneriches, M.L.M. and Chiu, Y.N. 1988. Effect of fish meal on the growth, survival and feed efficiency of milk fish (*Chanos chanos*) fry. *Aquaculture*, **71**: 61-69.

Sepers, A.B.J., Overback, J., Hoefle, M.G., Krambeck, C. and Witzel, K.P. 1984. The influence of varying environmental conditions on the activity of heterotrophic bacteria. *Adv. Limnol.*, No 13.

Sera, H., Ishida, Y. and Kadota, H. 1972. Bacterial flora in the digestive tracts of marine fish. IV. Effects of H^+ concentration and gastric juices on survival of the indigenous bacteria. *Bull. Jap. Soc. Sci. Fish.*, **38**: 859-863.

Shantithirumani, S. and Chandrika, V. 1992. Seasonal variation of heterotrophic bacteria in aqucultural ponds. *Proc. 32nd. Ann. Conf. Assoc. Microbiologist of India*, Madurai, Jan. 10-12, 1992. Abstract EMP 2-20.

Shcherbina, M.A., Trofimova, L.N. and Kazlaskene, O.P. 1976. The activity of protease and the intensity of protein absorption with the introduction of different quantities of fat into the food of the carp, *Cyprinus carpio. J. Ichthyol.*, **16**: 632-636.

Shell, E.W. 1967. Feed and feeding of warmwater fish in North America. *FAO Fish. Rep.,* **44**(3): 310-325.

Shewan, J.M., 1961. The microbiology of seawater fish. *In*: G. Borgstrom (ed.) *Fish as food*, Vol I. Academic Press, London. 487-560.

Shiranee, P., Natarajan, P. and Dhevendran, P. 1993. The role of gut and sediment bacterial flora in the nutrition of cultured pearl spot (*Etroplus suratensis*, Bloch). *The Israel J. Aqua. Bamidgeh*, **45**(2): 45-58.

Shivokene, Ya.S. and Tryapshene, O.P. 1985. Bacteria numbers and biomass in the digestive tract of pond fish as a function of their diet. *J. Ichthyol.*, **25**(5): 95-102.

Singh, S.B., Dey, R.K. and Reddy, P.V.G.K. 1976. Some additional notes on the piscivorous habits of the grass carps (*Ctenopharyngodon idella*). *Aquaculture.*, **9**(2): 195-198.

Singh, B.N. and Bhanot, K.K. 1988. Protein requirement of fry *Catla catla* (Ham.). *In*: M. Mohan Joseph (ed.). *The First Indian Fisheries Forum Proceedings*, Asian Fisheries Society, Indian Branch, Mangalore pp. 77-88.

Singh, S.B., Banerjee, S.C. and Chakrabarthi, P.C. 1967. Preliminary observations on response of young Chinese carps to various physico-chemical factors of water. *Proc. Natl. Acad. Sci. India*, **37B**(3): 320-324.

Singh, B.N. 1987. Digestive physiology of carps and freshwater catfishes. In: USAID Training Programme in Freshwater Aquaculture, 14th.July-13th.Aug 1987, Central Institute of freshwater Aquaculture, Bhubaneswar, India.

Singh, B.N. and Bhanot, K.K. 1970. Nutrient value of algal feeds of carp. *J. Ireland fish. Soc. India*, 121-127.

Singh, B.N., Sinha, V.R.P. and Kumar, K. 1987. Protein requirements of Indian major carp, *Cirrhinus mrigala* (Ham.). *Intl. J. Acad.*, Ichthyol., **8**(1,2).

Singh, S.K. 1994. *Studies on the bacterial flora of freshwater catfish and prawn hatchery systems.* M.F.Sc. Thesis, Orissa Univ. Agril. Tech., Bhubaneswar, 146 pp.

Smith, L.S. 1980. Digestion in teleost fishes. *In*: *Aquaculture development and coordination programme*-Lectures presented at the FAO/UNDP Training Course in Fish Feed Technology, ADCP/REP/80/11, p. 3-17.

Sobolev, Y.A. 1970. Food interrelationships of young grass carp, silver carp, and carp reared jointly in ponds in Belorussia. *J.Ichthyol.*, **10**(4): 528-533.

Spannhof, L. and Plantikow, H. 1983. Studies on carbohydrate digestion in rainbow trout. *Aquaculture*, **30**: 95-108.

Srivastava, K.P. and Floodgata, G.D. 1986. Studies on the intestinal micoroflora of Dab *Limanda limanda* L. *J. Mar. Biol. Assoc. India.*, **8**: 1-7.

Stickney, R.R. and Shumway, S.E. 1974. Occurrence of cellulase activity in the stomachs of fishes. *J. Fish Biol.*, **6**: 779-790.

*Streichsbier, F. 1982. Oekologische untersuchungen zum mikrobiellen chitinabbu in Stadtnahen Fliessgewassern. Wasser Abwasser, **25**: 53-70.

Sugita, H., Takuyama, K. and Deguchi, Y. 1985a. The intestinal microflora of carp *Cyprinus carpio*, grass carp *Ctenopharyngodon idella* and tilapia *Sarotherodon niloticus. Bull. Jap. Soc. Sci. Fish.*, **51**(8): 1325-1329.

Sugita, H., Tamura, M. and Deguchi, Y. 1985b. Enterobacteria present in the gastrointestinal tract of freshwater fish in the Tama River. *Bull. Coll. Agric. Vet. Med. Nihon Univ.* no. 42, 211-215.

Sugita, H., Tsunohara, M., Ohkoshi, T. and Deguchi, Y. 1988. The establishment of an intestinal microflora in developing gold fish (*Carassius auratus*) of culture ponds. *Microb. Ecol.*, **15**: 333-344.

Sugita, H., Oshima, K., Fushino, J. and Deguchi, Y. 1987. Substrate specificity of heterotrophic bacteria in the water and sediment of a carp culture pond. *Aquaculture*, **64**(1): 39-46.

Sugita, H., Oshima, K., Tamura, M. and Deguchi, Y. 1983. Bacterial flora in the gastrointestine of freshwater fishes in the river. *Bull. Jap. Soc. Sci. Fish.*, **49**(9): 1387-1395.

Sugita, H., Takayama, M. Oshima, T. and Deguchi, Y. 1992. Occurrence of microaerophilic bacteria in the water and sediment of a grass carp culture pond. *Aquaculture*, **103**: 135-140.

Sugita, H., Ushioka, S., Kihara, D. and Deguchi, Y., 1985 Changes in the bacterial composition of water in a carp rearing tank. *Aquaculture*, **44**: 243-247.

Sukhatme , P.V. and Amble, V. G. 1989. *Statistical Method for Agricultural Workers*. Indian Counc. Agric. Res., New Delhi, 359 pp.

Sutton, D.L. 1977. Grass carp (*Ctenopharyngodon idella* Val.) in North America. *Aquat. Bot.*, **3**: 157-164.

Swamy, D.N., Mohanty, S.N. and Tripathi, S.D. 1988. Growth of mrigal (*Cirrhinus mrigala* Ham.) fingerlings fed on fish meal based formulated diets. *In*: M. Mohan Joseph (ed.),*Proc.of the Indian Fisheries Forum*, Asian Fisheries Society, Indian branch, Mangalore, 81-83.

Tacon, A.G. and Jackson, A.J. 1985. Utilization of conventional and unconventional protein sources in practical fish feeds. *In*: C.B. Cowey, A.M. Mackie and J.G. Bell (eds.). *Nutrition and Feeding in Fish*. Academic Press, London, 119-145.

*Tanaka, N. and Kadota, H. 1980. Bacterial densities and their growth rates during *Uroglena* blooms in lake Biwa. *Bull. Natl. Rev. Inst. Aquacult.* (Japan), **1**: 87-93.

*Teshima, S. and Kashiwada, K. 1967. Studies on the production of B vitamins by intestinal bacteria of fish. III. Isolation of vitamin B_{12} synthesizing bacteria and their bacteriological properties. *Bull. Jap. Soc. Sci. Fish.*, **33**: 979-983.

*Teshima, S. and Kashiwada, K. 1969. Studies on the production of B vitamins by intestinal bacteria of fish. IV. Production of nicotinic acid by intestinal bacteria of carp. *Mem. Fac. Fish. Kagoshima Univ.*, **18**: 87-91.

Trust, T.J. 1971. Bacterial counts of commercial fish diet. *J. Fish. Res. Bd. Can.*, **28**: 1185-1189.

Trust, T.J. 1975. Bacteria associated with the gills of salmonid fishes in freshwater. *J.Appl. Bacteriol.*, **38**: 225-233.

Trust, T.J. and Sparrow, R.A.H. 1974. The bacterial flora in the alimentary tract of freshwater salmonid fishes. *Can. J. Microbiol.*, 20(9): 1219-1228.

Trust, T.J., Bull, L.M. Currie, B.R. and Buckley, J.T. 1979. Obligate anaerobic bacteria in the gastrointestinal microflora of the grass carp (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*) and rainbow trout (*Salmo gairdneri*). *J.Fish. Res. Bd. Canada,* **36**: 1174-1179.

*Ushiyama, H., Fujimori, T., Shibata, T. and Yoshimura, K. 1965. Studies on carbohydrates in pyloric caeca of the salmon, *Oncorhynchus keta*. *Bull. Hokkaido Univ. Fish.*, **16**(3): 183-188. *Transl.Ser. Fish. Res. Bd. Can.*(2183), 12 pp.

Varadraj, P. and Ayyappan, S. 1989. Population and distribution of heterotrophic bacterial flora in freshwater bodies of Bangalore. *Indian J. Environ. Hlth.*, **31**(2):162-170.

Verghese, T.J., Devraj, K.V., Shantaram, B. and Shetty, H.P.C. 1976. Growth response of common carp, *Cyprinus carpio* var. *communis* to protein rich pelleted feed. *Proc. Sem. on the development and utilization of Indian Fishery resources*, Colombo, p. 1-9.

Venugopal, M.N. and Keshavanath, P. 1987. Digestion coefficient conversion rates of three pelleted feeds fed to common carp, *Cyprinus carpio. Mysore J. Agril. Sci.*, **21**: 342-347.

*Verigin, B.V., Viet, N. and Dung, N. 1963. Data on the food selectivity and daily ration of White Amur. *In*: (Problems of the fisheries exploitation of plant-eating fishes in the water bodies of the USSR).*Ashkhabad*, Akademic Nauk, Turkmenistan, SSSR, 192-195.

Willey, R.G., Diskocil, M.J. and Lembi, C.A. 1974. Potential of the white amur (*Ctenopharyngodon idella* Val.) as a biological control for aquatic weeds in Indiana. *Proc. Indian Acad. Sci.*, **83**:173-178.

Wilson, G.S. and Miles, A. 1975. *In*: Arngld, E. (ed.) Topley and Wilson's principles of bacteriology and immunity, London, 129-130.

Wood, E.J.F. 1967. Microbiology of oceans and estuaries. 1-319, Elsevier Publishing Company, Amsterdam.

Yoshimizu, M., Kimura, T. and Sakai, M.1980. Microflora of the embryo and fry of salmonids. *Bull. Jap. Soc. Sci. Fish.*, **46**(8): 967-975.

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* Not consulted in originals.