Microflora in the Alimentary Tract of Gray Mullet III : Study on the Characteristics of the Intestinal Microflora in Different Conditions

Mem. Fac. Fish., Kagoshima Univ.
Vol. 26 pp. 79~87 (1977)

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Study on the Characteristics of the Intestinal Microflora in Different Conditions

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Summary

In consideration of the possibility that some bacteria might play an important role as symbionts or commensals which may aid fish in nutrition and in growth, the current study was intended to provide more information on the characteristics of the intestinal microflora. It was observed that the microflora isolated from the intestine of gray mullet living in fresh water and sea water possessed optimum temperatures for growth from 25°C–37°C and 0–2% optimum sodium chloride concentrations at optimum pH 7 or 8, whereas microflora isolated from environmental water grew at optimum temperature from 20°C–30°C and 0.5%–3% sodium chloride concentration at pH 7. It was also found that the intestinal *Vibrio* and *Enterobacter* were resistant to SLS (Sodium Lauryl Sulphate), and they were neither affected by antibiotics nor were able to produce antibiotics.

Many of the activities and the importance of the intestinal microflora in the nutrition and well being of their hosts have been established (FLOCH et al, 1970).

In contrast to the large body of information about the activities of the intestinal microflora, little is available concerning the inter-relationship of the bacterial population.

In the previous paper of this series the authors have carried out the isolation and identification of the intestinal bacteria of gray mullet, *Mugil cephalus*, an amphidromous fish cultivated in fresh and sea water. It was found that certain specific bacteria, members of the genus *Enterobacter* were the only survivor in the intestine, when the fish were transferred back to the original fresh water. The finding concluded that gray mullet possessed an ability in concert with the environment, to select microorganisms in their intestinal tract. The present study was undertaken to detect the characteristics of the microflora which is supposed to possess the ability to contribute in nutrition and growth of gray mullet.

Materials and Methods

The authors have studied the intestinal microflora of gray mullet living in fresh water, and sea water. Effects of temperature, salinity, pH, SLS susceptibility, sta-

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bility of growth in poor nutrients, antibiotic sensitivity and production of antibiotics were observed in media as described below;

Medium I was used for bacteria isolated from the intestine of fish living in fresh water and from environmental fresh water. In this medium polypeptone 10 g, beef extract 3 g, yeast extract 3 g, KCl 2 g, $MgSO_4 \cdot 7H_2O$, 6 g, $CaCl_2 \cdot 2H_2O$ 3 g, were dissolved in 1 liter distilled water and NaCl was supplimented on the basis of requirement. The stock cultures were maintained on 1.5% agar and 0.6% NaCl containing medium at 30°C.

Medium II (ZoBell 2216 E modified) was used for bacteria isolated from the intestine of fish living in sea water and from the environmental sea water. The original NaCl concentration was replaced according to the requirement. Stock cultures were maintained on 1.5% agar and 2.5% NaCl containing medium at 25°C.

Medium III (fresh water medium) was used for bacteria isolated from the fish living in half fresh and half sea water mixture. In this medium the original concentration of artificial sea water (ASW) was diluted to one sixth strength. No effective growth was in this medium. The pH for stock cultures was adjusted in all the cases to 7.5.

Estimation of bacterial growth

7 ml volume of culture media I and II in which the concentration of NaCl varies from 0%–9% were kept for 24 hours at 20°C–37°C and the bacterial growth at different temperatures and salinities were estimated by following the change in optical density. Some culture media were made of pH 5, 5.5, 6, 6.5, 7, 7.5, and 8 which were determined by pH meter (Hitachi Horiba Model no F7–7DE). The bacterial growth in marine culture media were estimated by measuring the turbidity after 24 hours incubation at 25°C and 30°C.

All the isolates were tested for lytic susceptibility to SLS (Sodium Lauryl Sulphate). SLS lysis of the test organisms was examined by measuring the turbidity decrease due to the addition of 0.05 ml of 0.5 M SLS aqueous solution to 7 ml of the culture grown on both the media after 24 hours incubation. In all these cases the controls consisting of uninoculated media which were treated identically. The degree of lysis was calculated by the following equation:

$$\% \text{ SLS lysis} = \frac{A-B}{A} \times 100$$

Where A=optical density of bacterial suspension after 24 hours incubation.

B=Residual turbidity after lysing the suspension with SLS for 30 minutes.

In all of the above cases growth was estimated by following the change in optical density (OD) at 540 mμ as measured by spectrophotometer, (Model Tokyo photoelectric Co, Ltd). Tween 80 was used to avoid the thick pellicle formed especially by *Bacillus*..

In order to identify the inhibitory effect of some antibiotics, such as penicillins, leucomycin, erythromycin, streptomycin, and tetracyclines, growth of the intestinal

bacteria was carried out on solidified medium. The growth inhibition was determined by the antibiotic discs (Eiken Kagaku Co, Ltd), after incubated at 25°C and 30°C for 24 hours. The clear areas were observed around the discs on the plate and were measured in centimeters.

In consideration of the possibility of mutual relationships among the intestinal bacteria and the assessment of the capacity to produce antibiotics in the culture media, growth was observed after 24 hours incubation of both the test and the standard organisms. Two lines were drawn horizontally and vertically on the agar plate with inoculating needle. Each line held of test organism and standard organisms or two test organisms, which were allowed to diffuse overnight into the surrounding agar plate at 30°C.

To examine their contributary effect on nutritional requirements, growth was carried out in a basal medium containing 3 g glycerol as medium A, 3 g sodium citrate as medium B, 3 g glucose as medium C and 3 g glycine as medium D, as sole carbon source. Basal medium was composed of following components;
K_2HPO_4 7 g, KH_2PO_4 2 g, $(NH_4)_2SO_4$ 1.5 g, $MgSO_4$ 1 g, $CaCl_2$. 0.01 g and $FeSO_4$ $7H_2O$ .0005 g in 1 liter distilled water and pH was adjusted to 7.1.

Results

Microflora isolated from the intestine of gray mullet living in fresh water, sea water and from environmental water were identified as *Bacillus, Enterobacter, Micrococcus, Acinetobacter, Pseudomonas, Vibrio, Aeromonas, Achromobacter, Corynebacterium and Staphylococcus* as described in the previous papers. All the identified bacteria were studied and the characteristic of the type of representative isolates were shown in Table 1. Optimum temperature for all *Bacillus* and *Micrococcus* was 37°C under the optimum pH 8.0 without any supplement of NaCl. *Enterobacter* from the intestine of the fish living in fresh water grew well at their optimum temperature of 30°C at optimum pH 7.0 and 0.5% supplement of NaCl. On the other hand, *Enterobacter* isolated from the fish living in sea water show somewhat different characteristics as shown in Fig. 1, where they need 2.0% NaCl at 25°C. Thus, according to Fig. 2(a) and 2(b) intestinal *Vibrio* and the environmental *Vibrio* also show different temperature and salinity requirements and growth through a wide pH range. The differences in the characteristics of bacteria isolated from the intestine and the environmental water were supposed to possess specific properties which are favourable for their adaptation with the environment.

In the course of the stability study against SLS (Sodium Lauryl Sulphate) by the intestinal and the environmental bacteria, the average lytic ratio of each representative genus was presented in Table 2. The ratios were observed indicated greater lytic sensitivity of the intestinal isolates than the environmental isolates. *Bacillus,* and *Micrococcus* were found to be more strongly stable to SLS than the other intestinal bacteria. Slight lysis was observed with some intestinal *Aeromonas* and *Pseudomonas.*

However, *Enterobacter* and *Vibrio* were found strongly resistant to SLS. On the other hand, bacteria in the environmental water, especilly in sea water isolates, were completely lysed by SLS. From the above result it is concluded that the environmental

Table 1. Effect of temperature, salinity and pH on bacterial growth.

Name of bacteria isolated	Optimum temperature °C				Optimum salinity (%)				Optimum pH			
	A	B	C	D	A	B	C	D	A	B	C	D
Bacillus	37	—	—	—	0	—	—	—	—	—	—	—
Entero-bacter	30	25	30	—	0.5	2.0	0.5	—	8.0	7.0	8.0	—
Micrococcus	37	—	30	—	0	—	0.5	—	8.0	—	8.0	—
Acineto-bacter	—	25	30	20	—	2.0	0.5	3.0	—	7.0	7.0	7.0
Pseudomonas	—	25	30	20	—	2.0	0.5	3.0	7.0	7.0	7.0	7.0
Vibrio	—	25	—	20	—	2.0	—	3.0	—	7.0	—	7.0
Aeromonas	—	25	—	20	—	2.0	—	3.0	—	7.0	—	7.0
Achromo-bacter	—	—	30	20	—	—	0.5	3.0	—	—	7.0	7.0
Coryne-bacterium	—	—	—	20	—	—	0.5	3.0	—	—	7.0	7.0
Staphylo-coccus	—	—	30	—	—	—	0.5	—	—	—	8.0	—

A=Bacteria isolated from the intestine of fish living in fresh water, B=bacteria isolated from the intestine of fish living in sea water, C=bacteria from environmental fresh water and D=bacteria from environmental sea water.

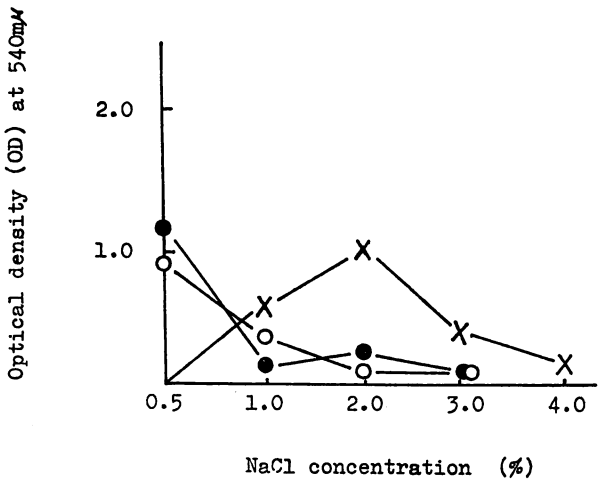

Fig. 1. Characteristic curves of intestinal and environmental bacteria. (*Enterobacter*).
—●— Fresh water intestinal bacteria at 30°C.
—○— Fresh water bacteria at 30°C.
—×— Sea water intestinal bacteria at 25°C.

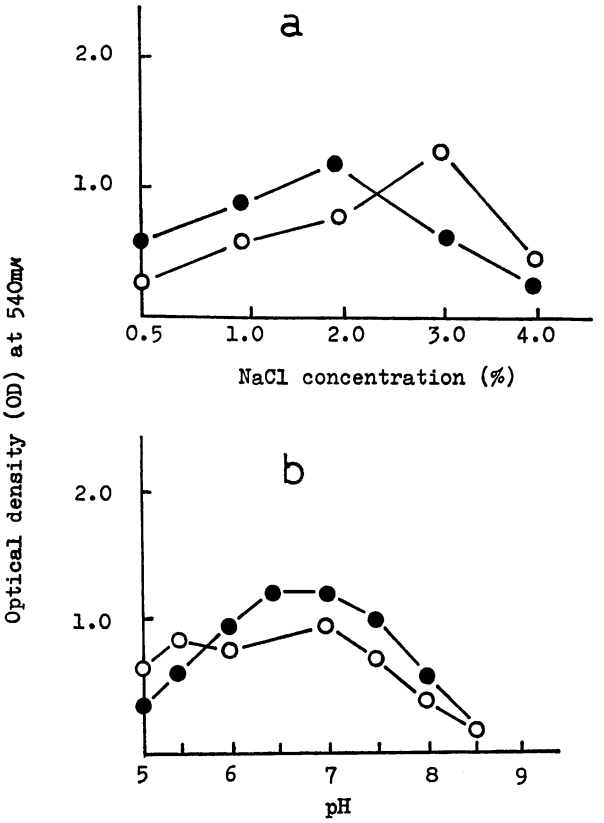

Fig. 2. Effect of (a) temperature and salinity, (b) pH on the growth of *Vibrio*.
—●— Sea water intestinal bacteria at 25°C.
—○— Sea water bacteria at 20°C.

microflora were rather unstable compared with the intestinal microflora against SLS detergent.

Table 3 indicated the ability of intestinal microflora to grow with glycerol, citrate, glucose and glycine as a sole carbon source. Almost all the bacteria grew in glycerol. None of the isolates except *Vibrio* was able to grow in citrate as sole carbon source. Glucose and glycine supported the growth of very few bacteria. The extent of growth of bacteria for 24 hours was higher when glycerol was the carbon source than it was when glycine or glucose were used. The inability of bacteria to grow in presence of citrate, glucose or glycine may indicate an increased nutritional requirement or inability to use the substrate. On the other hand, *Vibrio* and *Enterobacter* utilized all the carbon sources indicating an important nutritional versatility for the fish. Considering the possibilities of producing vitamins or amino acids by the bacteria, further studies are in progress.

The effect of antibiotics at different conditions on the growth of the bacteria was

Table 2. SLS lytic susceptibility of isolates.

Name of bacterial isolates	Intestinal isolates		Environmental isolates*	
	Intact cells OD at 540 mμ	Lytic response (%)	Intact cells OD at 540 mμ	Lytic response (%)
Bacillus	1.00～1.50	16	—	—
Enterobacter	1.20～1.21	0	0.09～1.00	9
Micrococcus	0.07～0.09	12	—	—
Acinetobacter	1.20	16	1.00	50
Vibrio	1.10	0	1.00～1.50	16
Aeromonas	1.40～1.50	21	1.20～1.40	130
Pseudomonas	1.50～1.60	26	1.50～1.60	88
Achromobacter	—	—	0.08～1.00	100
Corynebacterium	—	—	0.09～1.20	100
Staphylococcus	—	—	0.07～0.09	100

* Lysis (%)$=\frac{A-B}{A}$ 100. A=Intact cell=optical density of growth suspension after incubation for 24 hours. B=Residual turbidity after lysing the suspension with SLS for 30 minutes.

Table 3. Utilization of carbon sources by the intestinal bacteria.

Name of the isolates	Medium A glycerol		Medium B Citrate		Medium C Glucose		Medium D Glycine	
Bacillus	0.03～0.04	±	0.01	−	0.01	−	0.01	−
Enterobacter	0.29～0.30	++	0.04～0.06	±	0.14～0.10	++	0.09～0.12	+
Micrococcus	0.01	−	0.01	−	0.01	−	0.01	−
Acinetobacter	0.09～0.10	++	0.01～0.03	−	0.01～0.02	−	0.01	−
Vibrio	0.40～0.45	++	0.04～0.08	+	0.15～0.20	++	0.06～0.09	+
Aeromonas	0.05～0.06	+	0.01	−	0.02～0.04	±	0.03～0.05	+
Pseudomonas	0.19～0.20	++	0.01	−	0.03～0.05	±	0.03～0.04	−

++: Heavy growth, +: considerable growth, ±: negative growth, −: no growth.

shown in Table 4. However increasing the level of antibiotic concentration, increased the sensitivity. Among five antibiotics tested (penicillin, leucomycin, streptomycin, erythromycin and tetracyclines) penicilline was found most effetive and it inhibited the growth of almost all bacteria except *Vibrio* and *Enterobacter*. They were found sensitive to chlortetracycline and streptomycin. The specific characteristics of these bacteria should not be overlooked and the problem should be studied further.

A study was also carried out on the possibility of producing antibiotics in the surrounding media by the intestinal isolates. *Escherichia coli, Bacillus subtilis* and *Staphylococcus citreus,* were used as reference organisms. No trace of antibiotic production was observed. The inability to produce antibiotics in the media is considered to be benificial to the fish.

Table 4. Composition of antibiotic sensitivities of the intestinal bacteria.

Isolated bacteria	Name of the antibiotics used				
	Pc	Lm	Sm	Em	cTc
Bacillus	+++	—	—	+	+
Enterobacter	—	—	+++	—	+
Micrococcus	+++	+++	++	++	+++
Acinetobacter	+++	++	—	+++	+++
Pseudomonas	+++	+++	+++	+++	++
Aeromonas	+++	—	+++	+++	+
Vibrio	—	—	++	—	+

Abbreviations used: Pc: Penicillin, Lm: Leucomycin, Sm: Streptomycin, Em: Erythromycin, cTc: Chlortetracycline, (−): Resistant to drug, (+): Sensitive to high concentration, (++): Sesitive to medium concentration and (+++): Sensitive to low concentration.

Table 5. Inhibitory effect among the intestinal bacteria.

Name of the isolates	B	E	M	Aci	P	Aer	V
Bacillus		—	—	—	±	±	—
Enterobacter	—		—	—	—	—	—
Micrococcus	—	—		—	—	—	—
Acinetobacter	—	—	—		—	—	—
Pseudomonas	—	—	—	—		—	—
Aeromonas	—	—	—	—	—		—
Vibrio	—	—	—	—	—	—	

Abbreviations used; ±; very weak inhibition, −, no inhibition, B=*Bacillus*, E=*Enterobacter*, M=*Micrococcus*, Aci=*Acinetobacter*, Aer=*Aeromonas*, and V=*Vibrio*.

Mutual relationship among the intestinal bacteria were shown in Table 5, where no remarkable inhibitory effect was observed. Although *Pseudomonas* and *Aeromonas* were found weakly inhibited by *Bacillus* strain, it may be ignored. Considering the fact that if *Bacillus* possesses the ability of producing an antibiotic in the medium, the reference organisms should also have responded to the antibiotic produced by *Bacillus*. Thus it is concluded that the bacteria isolated from the intestine of gray mullet may not produce harmful effects among themselves.

Discussion

The experimental results indicated that the optimum temperature, salt concentration and pH were different for intestinal bacteria and bacteria living in the environ-

mental water. It was concluded in the previous paper that gray mullet have an ability with the environment to select microorganisms in their intestinal tract and according to the present study it was found that bacteria also possesses capacity for adaptation with the environment in which they live. The earlier observation of ZOBELL and MITCHNER (1938) that marine isolates on growing in laboratory media became adapted to a Na free medium should not be overlooked and the problem should be studied further. The requirement for Na is found also in halophilic bacteria isolated from non marine environments, (LARSON; 1962) and has been reported in two instances among non halophilic non marine bacteria (SISTRAM 1960; BRYANT et al; 1959). According to the experiment intestinal microflora had high susceptibility to SLS and the intestinal *Vibrio* and *Enterobacter* were uneffected by SLS. On the other hand most of the bacteria isolated from the environmental water were lysed by SLS. This result agrees with the study of KAKIMOTO et al (1972) who concluded that marine isolates are lysed more easily by SLS than terrestrial organisms.

In the present findings some of the intestinal microflora grow in the presence of glycerol or glycine. *Vibrio* and *Enterobacter* grew well with all the compounds supplied as sole carbon sources. This seems to indicate that most of intestinal microflora especially *Vibrio* in marine environment and *Enterobacter* in fresh water environment are capable of production of nutrients in the intestine of gray mullet. The microflora may even be essential in free living fish feeding on materials lacking vitamins which the microflora can synthesize (TRUST and SPARROW; 1974). SEKI 1972 has suggested that the bacteria may even represent a source of nutrients for the fish. But not all the effects of the intestinal microflora may be benificial to the fish. Considering the need of more information on this point further studies are in progress.

The result of the effect of different antibiotics show that the organisms have different responses to the drugs. *Vibrio* and *Enterobacter* were found sensitive to streptomycin and chlortetracycline, but were more or less resistant to the other drugs.

The specific characteristics of *Vibrio* and *Enterobacter* indicate that these organisms have developed an adaptive capacity to protect against antibiotic invasion in the alimentary tract, which is may be produced in the intestine by other bacteria. However the member of other intestinal bacteria had no capacity to produce antibiotics. This capacity is supposed to have a benificial rather than a harmful effect for the fish.

Finally, the result indicated that the intestinal microflora had mutual inter-relationships. There was no remarkable inhibitory effect observed which may be proposed to possess contributary effects on fish nutrition.

From the findings above it may be speculated that the microflora which were identified from the intestine of gray mullet may be used in feeds as growth factors or sources of growth factors. There is some evidence that microorganisms as feed supplements contribute to the efficient utilization of nutrients, increased growth rates and generally more economical production of animals and animal products. Microorganisms have been studied as sources of specific amino acids that may be liberated as free amino acid into the growth media (CASIDA 1956). Microorganisms synthesize pro-

tein and some common amino acids from carbohydrates and simple sources of nitrogen (ANDERSON and JACKSON; 1958). Microorganisms including bacteria are sources of vitamins, antibiotics, proteins and amino acids, enzymes and related factors used in animal rations to improve feed efficiency and increase the rate of growth, (HALL; 1962).

It is apparent that greater attention must be given to the intestinal microflora of fish. The importance of further studies is indicated by the fact that once the nutritional requirements of those fish have been determined, large numbers may be cultivated under controlled condition.

Acknowdgements

The authors are grateful to Dr. SADAHIKO IMAI, Professor of Kagoshima University for his helpful discussions and suggestions during the progress of this work. Thanks are also due to Mr. WILLIAM P. SHARP of the Department of Botany and Microbiology, Arizona State University, who stayed in this Laboratory under exchange programme, for useful discussions and going through the manuscript.

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