THE BACTERIAL FLORA AND THEIR POSSIBLE ASSOCIATION WITH SPOILAGE IN A VARIETY OF FRESH-WATER FISH: *CYPRINUS CARPIO. VAR. COMMUNIS*

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The bacteria from a variety of fresh – water fish, *Cyprinus carpio. var. communis*, showed the presence of micrococci, Gram positive and Gram negative rods. These have been characterised as far as was possible. Of thirty-eight strains of bacteria used, only six strains were considered as causing spoilage of fish flesh in experiments where flesh was incubated with individual cultures of the bacteria. These six strains had been found on the surface and/or intestine of the fish and supports the suggestions that, after death, invasion of flesh by bacteria from the surface and intestine could be the cause of bacterial spoilage of fish.

INTRODUCTION

The flora of fish vary due to the geographical area, the environment,feeding habits and to some extent on the species. The former factors are considered to play much greater role than say species (Shewan, 1961). The spoilage occurring in fish has been thought to be due to the combined effect of enzymatic autolysis of fish muscle and of microbial action (Bramstedt & Auerbach, 1961). A "special spoilage microflora" has been thought to be active with regard to fresh water fishes, but the micro-organisms active in this respect are considered to originate largely from contamination subsequent to capture (Bramstedt & Auerbach, 1961).

A study was conducted jointly by the Central Inland Fisheries Research Institute, Barrackpore and the Department of Microbiology, All India Institute of Hygiene and Public Health, Calcutta to determine (a) the microflora in a variety of fish, *Cyprinus carpio. var. communis* and (b) to consider the possibilities of these bacteria being associated with the spoilage of this variety of fish after death.

Materials and Methods

(a) Isolation and characterisation of bacteria

The fishes used were caught from the ponds of the Central Inland Fisheries Research Institute, Barrackpore and brought alive to the laboratory. Five fishes were used in two batches of three on one day and two on another day. Along with the "catch", sample of the pond water was also included on each day.

Bacteria were isolated from the pond water, slime from the surface of live fishes, intestinal content, after killing the fishes in the laboratory and from the fluid exuding from the gills and vents after allowing the dead ungutted fishes to remain overnight in the laboratory.

The media used for each "specimen" were nutrient agar, MacConkey's agar, blood agar, potassium tellurite agar and desoxycholate-citrate agar plates. In addition to these, primary inoculation into selenite F and tetrathionate broths with subsequent subcultures onto desoxycholate-citrate agar plates were also undertaken. Anaerobic cultures consisted of inoculation into Robertson's cooked meat medium with subsequent subculture onto blood agar plates which were incubated anaerobically in "MacIntosh and Fildes' jars."

The temperature of incubation throughout the study was 37°C.

Growth on the "plates" were examined after overnight incubation (appr: 18 hours) and as many different types of colonies as could be ascertained were obtained in pure culture.

Anaerobic incubation did not yield "strict anaerobes" and it was found that all the strains isolated would grow on nutrient agar. "Pure cultures" were therefore maintained on nutrient agar slopes and when not in use, kept at 4°C with regular subculturing.

The isolates were examined for morphology by Gram's method. For motility and biochemical investigations as and when required, methods described by Ewing & Edwards (1960), Cruickshank (1960) and Wilson & Miles (1964) were used. In considering many of the Gram negative aerobic rods, the schema given by Shewan *et al* (1960 a, b) and Scholes & Shewan (1964) were used. The media were prepared according to methods described the Handbook of Bacteriology (1960)

(b) Experiments to associate bacteria with "spoilage" of fish flesh:

For these studies, flesh from freshly killed fish was incubated for varying periods in nutrient broth inoculated with the strains of bacteria. Freshly killed fish was washed thoroughly with sterile distilled water and with sterile instruments the flesh, without the skin, was cut into pieces of each approximately 0.5 g. Each piece of flesh was transferred to 10.0 ml nutrient broth and the tube inoculated with a loopful of overnight growth of the bacteria in nutrient broth. Two tubes of media containing "fish flesh" were used for each strain of bacteria and with each day's experiment, two tubes of media with "fish flesh" but not inoculated with bacteria were included to serve as "control".

The tubes were incubated aerobically at 37°C and changes in the flesh noted at intervals of "overnight", 48 hours, 72 hours and 96 hours incubation. The flesh first appeared swollen and subsequent changes were one of the following: slight pink colouration; pink colour; flesh being broken down; flesh completely disintegrated. If either of the last two changes were

found, this was taken to mean "rotting" as used in this communication.

The "control tubes" did not show any of these changes and if in any day's experiment the "control" tube showed growth of bacteria, the experiment with fish and inoculated bacteria for that day was repeated.

Results

(a) Bacterial flora from *Cyprinus capio (var. communis)*

Forty colonies examined from the media used showed species of micrococci, both yellow and white, enterococci, yellow-pigmented and non-pigmented aerobic asporogenous Gram positive rods, *Bacillus* spp., "Corynebacterium" spp., *Klebsiella* spp., non-lactose fermenting Enterobacteriaceae, *Pseudomonas* spp., *Achromobacter* spp., *Vibrio* spp., *Escherichia coli type I, Proteus* spp. and Gram negative asporogenous rods whose identity could not be established. The identity of the Enterobacteriaceae was not attempted. The distribution of these bacteria from the four types of "specimens", pond water, surface of fish, intestine and exudate are shown in Table I.

(b) Experiments to associate bacteria with "rotting" of fish flesh

Of the bacteria isolated, experiments to associate "rotting" of flesh with the isolates were undertaken with 4 strains of yellow micrococci, 3 strains of white micrococci and 31 strains of other bacteria. Two strains, the *Vibrio* spp. from pond water and a non-lactose fermenting strain of Enterobacteriaceae from the intestine, could not be used as the culture tubes unfortunately broke during storage.

The results of the degree of "spoilage" with the 38 strains tested are shown in Table II.

The "identity" and the source of isolation of these strains are given in Table III.

As shown in Table II, of the 38 strains examined, only six strains were considered as causing "rotting" of the fish flesh. These strains, the *Achromobacter* spp. from pond water and intestine, two strains of *Klebsiella* spp. from the surface of fish, the *Bacillus* spp. and a chromogenic, asporogenous Gram positive rod from the intestine, were used in another series of experiments where overnight growth of each strain of bacteria were diluted with nutrient broth in dilutions of "undiluted"; 1:2; 1:10; 1:50 and 1:100 and with each dilution a piece of fish flesh was incubated as in the experiments for "rotting". The changes in the flesh on overnight incubation at 37°C at different dilutions are shown in Table IV.

Discussion

The bacterial flora obtained from various sites in the variety of fresh-water fish, *Cyprinus carpio. var. communis*, consisted mainly of Gram negative aerobic rods and eleven broad groups were noted. Micrococci obtained were of three varieties and the Gram positive rods could be "classified" into four groups. The absence of strict anaerobes was of some interest. These "groupings" are shown in Table V.

It will be noted from Table V that the bacteria from the surface and from the intestine of the fish were more or less similar to those obtained from the water of the pond from which the "catch" was made. Such findings are in keeping with the suggestions made by Shewan (1962).

An attempt had been made to determine whether it was possible to arrive at a "spoilage microflora". Sterility of freshly caught healthy fish in respect to the bacterial species obtained in this study

TABLE I BACTERIA ISOLATED FROM SEVERAL SOURCES IN EXAMINING THE MICROFLORA OF *CYPRINUS CARPIO (VAR. COMMUNIS)*

Specimen examined	Bacterial species
Pond water of "catch"	Micrococci (yellow), *Proteus* spp., "Corynebacterium" spp., *Klebsiella* spp., **Vibrio* spp., *Achromobacter* spp., Gram negative rod (c)
Surface of fish	Micrococci (white & yellow), *Klebsiella* spp., Enterobacteriaceae, non-pigmented aerobic Gram positive rod, Gram negative rod (a), Pseudomonas Group III.
Intestine of fish	*Bacillus* spp., *Achromobacter* spp., Micrococci (yellow & white), pigmented Gram positive rod, Gram negative rod (c), Enterobacteriaceae, micrococci (? enterococci).
Exudate from dead fish	Micrococci (yellow & white), *Klebsiella* spp., pigmented Gram positive rod, Gram negative rod, (b), Enterobacteriaceae, *Esch. coli* I, Pseudomonas Group IV.

(a) Gram negative rod, motile, oxidase & Hugh–Leifson both negative, non-lactose fermenter.

(b) as above but non-motile

(c) Gram negative rod, motile, oxidase negative, alkaline in Hugh–Leifson, non-lactose fermenter.

* *Vibrio* spp. was non-agglutinable vibrio belonging to Heiberg I.

TABLE II SPOILAGE OF FISH FLESH WITH INDIVIDUAL BACTERIA AFTER INCUBATION AT 37°C IN NUTRIENT BROTH.

Results after incubation for				Obtained with strain no. of bacteria *
Overnight	48 hrs.	72 hrs.	96 hrs.	
—	—	—	—	6, 7, 12, 20, 22, 23, 24, 25, 29, 30, 31, and white micrococci from surface & exudate.
—	+	+	+	2, 4, 5, 14, 27 and yellow micrococci from surface & intestine and white micrococci from intestine.
+	+	+	+	1, 9, 10, 11, 15, 16, 26 and yellow micrococci from pond water.
+	++	++	++	3, 17, and yellow micrococci from exudate.
	or			
++	++	++	++	
+	+	+++	+++	8, 19, 21
+	++	+++	+++	13
++	++	+++	+++	18
—	++	+++	+++	19

— = flesh swollen only

\+ = slight pink colouration of flesh

++ = Pink colouration of flesh

+++ = Flesh broken / flesh disintegrated.

* Key to the strain numbers are given in Table III.

TABLE III IDENTITIY OF THE BACTERIA WITH THE SOURCE OF ISOLATION.

Strain no. as refferred to.	Source of isolation.	Identity of the strain
1	Pond water	Klebsiella spp.
2	Surface	Gram positive, asporogenous, aerobic rod.
3	Exudate	Gram positive, yellow pigmented, asporogenous rod.
4	Intestine	-do-
5	Pond water	*Proteus* spp.
6	Surface	Pseudomonas Group III
7	Exudate	Pseudomonas Group IV
8	Intestine	As in strain no. 3
9	Pond water	*Corynebacterium* spp.
10	Surface	Gram negative rod (a), see Table I
11	Exudate	Non-lactose fermenting Enterobacteriaceae.
12	Intestine	Cocci (? enterococci)
13	Surface	*Klebsiella* spp.
14	Exudate	Gram negative rod (b), see Table I
15	Intestine	Non-lactose fermenting Enterobacteriaceae.
16	Pond water	*Klebsiella* spp.
17	Surface	-do-
18	Pond water	Achromobacter spp.
19	Surface	*Klebsiella* spp.
20	Exudate	-do-
21	Intestine	*Achromobacter* spp.
22	Pond water	*Klebsiella* spp.
23	Surface	-do-
24	Exudate	*Esch.* coli I
25	Intestine	Gram negative rod (c), see Table I
26	Pond water	-do-
27	Exudate	Non-lactose-fermenting Enterobacteriaceae.
28	Intestine	*Bacillus* spp.
29	Surface	Non-lactose fermenting Enterobacteriaceae
30	Exudate	As in stain no. 3
31	Intestine	-do-

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9	Pond water	*Corynebacterium* spp.
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13	Surface	*Klebsiella* spp.
14	Exudate	Gram negative rod (b), see Table I
15	Intestine	Non-lactose fermenting Enterobacteriaceae.
16	Pond water	*Klebsiella* spp.
17	Surface	-do-
18	Pond water	Achromobacter spp.
19	Surface	*Klebsiella* spp.
20	Exudate	-do-
21	Intestine	*Achromobacter* spp.
22	Pond water	*Klebsiella* spp.
23	Surface	-do-
24	Exudate	*Esch.* coli I
25	Intestine	Gram negative rod (c), see Table I
26	Pond water	-do-
27	Exudate	Non-lactose-fermenting Enterobacteriaceae.
28	Intestine	*Bacillus* spp.
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15	Intestine	Non-lactose fermenting Enterobacteriaceae.
16	Pond water	*Klebsiella* spp.
17	Surface	-do-
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19	Surface	*Klebsiella* spp.
20	Exudate	-do-
21	Intestine	*Achromobacter* spp.
22	Pond water	*Klebsiella* spp.
23	Surface	-do-
24	Exudate	*Esch.* coli I
25	Intestine	Gram negative rod (c), see Table I
26	Pond water	-do-
27	Exudate	Non-lactose-fermenting Enterobacteriaceae.
28	Intestine	*Bacillus* spp.
29	Surface	Non-lactose fermenting Enterobacteriaceae
30	Exudate	As in stain no. 3
31	Intestine	-do-

TABLE IV

CHANGES IN FISH FLESH WITH SIX STRAINS OF BACTERIA AT DIFFERENT DILUTIONS. THE RESULT WERE RECORDED AFTER OVERNIGHT INCUBATION AT 37°C TAKING THE AVERAGE OF TWO READINGS FOR EACH STRAIN

* Strain No.	Dilutions					* * Viable count per ml. at dil. 1/2.
	Undil.	1/2	1/10	1/50	1/100	
8	+	+	+	+	+	5×10^7
13	+	+	—	—	—	1×10^{10}
18	++	+	—	—	—	2×10^9
19	++	+	—	—	—	5×10^8
21	++	+	—	—	—	5×10^8
28	++	+	—	—	—	5×10^9

"+" and "++" denote comparative degree of "rotting" and "–" indicates no. "rotting".

* Strain numbers of bacteria as in Table III

* * Viable count by surface method adopted from that of Miles and Misra (1938)

TABLE V BROAD "CLASSIFICATION" OF THE STRAINS AND THEIR SOURCE OF ISOLATION.

Classification of the strains.	Isolated from :			
	Water	Surface	Intestine	Exudate
I. Gram positive cocci:				
Micrococci, yellow	+	+	+	+
,, , white		+	+	+
,, , (enterococci)			+	
II. Gram positive rods:				
Bacilus spp.			+	
Corynebacterium spp.	+			
Pigmented, asporogenous rods.			+	+
Non-pigmented, asporogenous rods	+			
III Gram negative rods:				
Klebsiella spp.	+	+	+	+
Esch. coli I				
Vibrio spp.	+			+
Non-lactose fermenting Enterobacteriaceae.		+	+	+
Proteus spp.	+			
Pseudomonas spp.		+		+
Achromobacter spp.	+		+	
Rods (a) as in Table I		+		
Rods (b) as in Tadle I				+
Rods (c) as in Table I	+		+	

+ indicates the Isolation of the strain.

was evident from the "control tubes" where fish flesh was incubated in nutrient broth. The suggestions made by Shewan (1961) that in spoilage of fish, invasion of flesh by bacteria occurring after death through skin and peritoneal lining could be supported from the findings that the six strains implicated in spoilage in this study belonged to four types of bacteria which were present on the surface and / or in the intestine of the fish.

Scholes & Shewan (1964) had noted that the bacterial content of the intestine of newly caught fish was 10^3 to 10^8 per ml. of intestinal fluid. It was therefore of interest to find that "rotting" of flesh as considered in this study was noted with the presence of bacteria in the range 10^7 to 10^{10} per ml. It had also been observed by Hobbs (1953) that in decomposition detectable to human senses, bacterial counts of 1 to 10 million organisms per g were present.

The genesis of spoilage of fish is thought to occur due to the dual action of autolysis and bacteria. Kreuzer (cited by Bramstedt & Auerbach, 1961) has considered a "special spoilage microflora" to be active in this respect originating largely from contamination subsequent to capture of fish. Our studies have shown that some of the intrinsic microflora associated with the fish cannot be overlooked in causing spoilage.

While only 6 strains out of 38 bacterial strains used were considered by us to be associated with spoilage of this variety of fish, further studies need be made for definitive conclusions with regards to the question of the presence or absence of a "spoilage microflora" and and their source.

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