MICRO-ORGANISMS ASSOCIATED WITH THE "BOMBAY DUCK" OR *BOMBIL* (*HARPODON NEHEREUS* BUCH.)

By J. V. Bhat* and M. J. Albuquerque
(*St. Xavier's College, Bombay*)

Received November 28, 1952
(Communicated by Dr. T. S. Mahabale, F.A.SC.)

Introduction

With incredibly extensive and innumerable shoals, and occurrence practically all the year round, the 'Bombay Duck' or *bombil* (*Harpodon nehereus* Buch.) occupies a conspicuous position among the economically important fishes off the Indian coast. The fish, both fresh and dehydrated, has found an important place in the Indian dietary as such and connoisseurs always appreciate its distinctly fine flavour. Even Europeans had heard about the celebrity of the 'Bombay Duck' through Boswell (1926) the Biographer of Samuel Johnson as far back as the 18th century. Though it is true that the Bombay and Kathiawar shoals are the heaviest—hence the name Bombay Duck—the fish is also plentifully obtained in the estuaries of Bengal and other parts of India. Neither is the distribution of this fish limited to the Indian coast line alone, for it extends from Burma to Zanzibar and even China. However, the mass movements of this fish are fitful, and especially during the monsoons, Bombay is flooded with the heaviest shoals. This brings with it the problem of preservation, and inasmuch as all methods of preservation are primarily aimed at the control of microbial spoilage, a study of the micro-organisms commonly associated with the fish becomes not only apparent but imperative. Added to this, there appears to be no work reported on the microbial flora of this fish, though several workers since Hamilton Buchanan made his first scientific observation to it (1822) have studied its taxonomic, morphological and anatomical details and have even made attempts to study its food and feeding habits as well as the zoological parasites present in the fish (Sethna, 1932; Hora, 1934; Pinto, 1935; Moses, 1946; and Pillay, 1951). The results obtained on studying the micro-flora of this fish are presented in this paper.

Collection and treatment of fish.—The fish utilised for examination was of unquestionable freshness, complying with the tests for freshness recommended by Cox (1946). The samples were collected in a large sterile petri

* Present address: Indian Institute of Science, Bangalore.

dish, which in its turn was placed in another large dish packed with ice, so as to prevent to a considerable extent, any increase in bacterial count during transport. Moreover, the use of a closed sterile petri dish permitted no further contamination by handling, flies, etc. The ice, since it is not allowed to come in contact with the fish, does not further contaminate the same with its own flora. The fresh fish immediately on transportation to the laboratory was thoroughly washed 6 times serially in fresh quantities of sterile water kept in 6 different petri dishes. Other methods employed were (1) the use of 1 : 500 mercuric chloride for 5 minutes as a surface disinfectant, subsequently washing the fish free of the chemical in sterile water, (2) rubbing the exterior of the fish with cotton wool soaked in alcohol and then washing the fish to free it from alcohol prior to dissection and/or analysis. Except for securing the superficial body flora of the fish, where the above treatment was not undertaken, these methods of pretreatment were adopted in all cases and found satisfactory.

Materials for the study of the normal flora.—The methods employed for isolating the flora from various parts of the fish varied according to the nature and location of the part, and the possibilities of its contamination by aerial or other microbes. It must be emphasized here that all dissections undertaken in the process of obtaining the flora from the internal viscera of the fish, were carried out under the strictest aseptic precautions. The contents of the stomach was obtained by directly inserting a blunt probe attached to a sterilized tuberculin syringe, and withdrawing some of the stomach contents, which had been previously flushed with about 0·5 ml. of sterile water. The first drop of the withdrawn suspension was discarded and subsequent drops used for plating procedures on appropriate media. Other methods examined, like suspending incised stomach in sterile water, or boring a hole through the fish with a hot platinum loop to withdraw the stomach contents, were found to be either too laborious or to produce inconsistent results. The flora of the intestine could neither be obtained directly nor as easily as the flora of the stomach because of its position away from the mouth as well as its delicate structure. The walls of the intestine are thin and appear to be turgid and full of fluid when the fish is fresh, whereas in the partially decayed samples this region is usually ruptured and the contents are spilt in the alimentary cavity. Great care is therefore required both in selecting fresh samples as also in the procedure adopted in obtaining the flora from this region. The intestinal region is slightly levered by means of an arrow-head needle and a sterile platinum loop is pierced into the intestinal layer when hot. The heat is sufficient to rupture the wall. The contents are allowed to leak out for some time and then the sterilized platinum wire is inserted a few centimetres

into the intestinal cavity. The contents thus collected, are plated on appropriate media. The flora of the rectum was obtained as in the case of the intestines, and not directly *via* the anus, which present possibilities of contamination. The microflora of the skin and gills were easily examined by preparing suspensions of skin scrapings and incised gills. By adhering to the methods outlined, we have succeeded in getting consistent results and secured a flora which may be said to be normally associated with the fish.

Methods employed for the isolation and identification of micro-organisms.— The selection of various media for the detection of the flora associated with the fish was made very carefully and with due regard to the natural environments of these micro-organisms. At the same time, it was ensured that a nutritionally exacting organism did not escape being isolated. The media usually employed were the following:—

1. Nutrient agar, pH 7 to 7·2 without NaCl.
2. Nutrient agar, pH 7 to 7·2 with 3% NaCl.
3. Fish infusion agar, pH 7 to 7·2 with 3% NaCl.
4. Czapek-Dox-Thom medium of pH 6·6.
5. 5% Blood agar, pH 7·6.
6. MacConkey's agar, pH 7·4.
7. Enrichment media without NaCl.

Inasmuch as some of the media selected are routinely employed in most laboratories, their composition is too well known to require any mention. The composition of other media which are not so well known is given below.

Fish infusion agar

Fish infusion*	1000 ml.
Bacto-peptone	10 g.
Sodium chloride	30 g.
Agar	30 g.
pH	7·0–7·2

Enrichment media.—The basal solution for the various enrichment media was made up as follows:—

Distilled water	1000 ml.
Ammonium sulphate	0·5 g.
Di-potassium hydrogen phosphate	0·5 g.

* For every gram of minced fish, 2 ml. of distilled water were used for extraction in the refrigerator (overnight) and thereafter in the autoclave.

Magnesium sulphate	0·1 g.
Ferrous sulphate	0·02 g.
Calcium sulphate	traces†

The composition of the various enrichment media was as follows:—

Yeast autolysate medium

Basal solution	1000 ml.
Lactose	10 g.
Yeast autolysate	1 g.

Yeast autolysate was prepared as follows and 0·1 g. represents roughly 1 ml. of the liquid preparation. One pound of pressed yeast was suspended in small amounts at a time in 500 ml. of distilled water. The mixture was kept in a flask and incubated at 50° C. for 24 hours, after which it was boiled and alkali added to adjust the pH to 6·4. It was then filtered and sterilized after standardisation.

Bile salt medium

Basal solution	1000 ml.
Lactose	10 g.
Bile salts (Difco)	2 g.
pH	7·0

Sodium desoxycholate medium

Basal solution	1000 ml.
Lactose	10 g.
Sodium desoxycholate	5 g.
pH	7·0

The principal methods employed for estimating the abundance of micro-organisms in the fish, both fresh and dehydrated, were direct microscopic counts and plating procedures. Each of the various media enumerated succeeded in eliciting only a part of the microflora of the fish, though collectively they appear to satisfy the growth requirements of a wide range of micro-organisms. The incorporation of 3% salt to various media produced a noticeable increase in count. As an alternative to the addition of salt, it has been suggested that the particular medium be prepared in sea water. Though such a medium is recommended (Berkeley, 1919; Lipman, 1926), in our experience the incorporation of 3% NaCl serves equally well the purpose. A 3% salt agar was selected to represent the marine environment and the

† 1 ml. of a saturated solution of $CaSO_4$ was the amount used.

yeast autolysate enrichment medium was primarily chosen to detect the presence of moulds and yeasts. Fish infusion agar and 5% blood agar were chosen to correspond more closely to the nature of fish and animal tissue. MacConkey's agar and the other enrichment media facilitated the growth of intestinal organisms, and as will be noted later was to a great extent beneficial in the detection and study of the colony characters of micro-organisms found in the ventriculus, intestines and rectum of the fish, as well as in certain epidemiological studies on the fish conducted in this laboratory.

Results

A microbiological examination of 42 *bombils*, both fresh and dehydrated, according to the methods already described, revealed a flora which was consistent enough to be regarded as normal. These bacteria, being marine in origin, differed in certain respects from their terrestrial counterparts, notably in cell morphology, certain cultural and physiological characteristics, and salinity requirements.

Cell morphology.—A large percentage of the bacteria found on the fresh *bombil* were gram negative rods, capsulated in the animal body, but losing this property on artificial cultivation. Collectively, this group is not morphologically differentiable, and pleomorphism is quite pronounced in most of them. A comparatively smaller proportion of the bacterial flora was made up of cocci, gram positive rods, and sporeforming bacilli. From the ventriculus of the commercially dehydrated fish, the flora appeared to be more characteristic.

Cultural and physiological characteristics.—In general, bacteria from the *bombil* grow more slowly, producing smaller colonies than their terrestrial counterparts from soil, sewage, milk, etc. The colonies on agar are often depressed and those from the alimentary canal are mostly non-pigmented and translucent while surface organisms appear both pigmented as well as non-pigmented. Opacity of the colonies was a variable character. Inasmuch as the habitat of *Harpodon nehereus* is tropical waters, which according to ZoBell (1946) have surface temperatures as high as 28% C. to 30° C., or 38 to 40° C. in localized regions near shore, it is not surprising that with regard to the microflora of the *bombil*, moderately good growth was observed at room temperature (28 to 30° C.). Although ZoBell and Grant (1943) present evidence which suggests that all heterotrophic marine bacteria are able to assimilate glucose, only 46 of the 60 cultures studied by ZoBell and Upham (1944) fermented glucose with the formation of acid and none of them produced gas from glucose. The normal micro-flora of the intestine, ventriculus and rectum of fresh *bombil* present evidence to the contrary. These

micro-organisms isolated are as strongly saccharolytic as they are proteolytic. Practically all of them fermented with 'acid and gas', besides fermenting in a like manner, maltose, saccharose, starch and dextrin. Formation of hydrogen sulphide from 2% peptone salt water in 24 hours was a property common to nearly all these cultures. Likewise almost all isolates reduced nitrates to nitrites. However, these bacteria were conspicuous by their inability to produce indol from tryptone salt water and to ferment lactose. A frequently occurring gram negative coccobacterium which is remarkably heat resistant and persists even in the dehydrated fish, was observed to be physiologically inactive. Besides those mentioned, there occur other bacteria whose occurrence appear fitful and hence may be classified as a group of adventitious flora of the alimentary canal. Briefly speaking, the following genera have been encountered in the various regions.

Fresh fish

Alimentary canal.—Bacterium, Escherichia, Achromobacter, Pseudomonas and *Flavobacterium.*

Skin, Gills and Flesh.—Micrococcus (also *Gaffkya* and *Sarcina* species), *Bacillus, Escherichia, Aerobacter, Bacterium, Achromobacter.*

Dehydrated fish

Ventriculus or Stomach.—Micrococcus, Sarcina, Bacillus.

We have encountered considerable difficulties in placing these organisms isolated from the *bombil*, in Bergey's (1948) classification. Similar difficulties have been met with by Thjotta and Somme (1943), ZoBell and Upham (1944) and other workers who have been familiar with marine bacteria. We have accordingly recognized slight differences in character of the bacteria isolated only as strain differences and have preferred to refer them to the nearest species in Bergey's (1948) Manual.

Bacterial flora of the fresh bombil.—Though the bacterial flora of the *bombil* varies with the region of the fish examined, certain types of organisms are common to most, if not all, of the regions examined, and this more than justify their claims to be regarded as the normal flora of the *bombil.* Among these, a species of *Sarcina, viz., Sarcina littoralis* and a near-luminiscent non-spore-forming organism which closely answers to the description of *Bacterium phosphoreum* deserve special mention, as they or their variants have been isolated in large numbers in all regions of the alimentary tract of the fish. In addition to these normally occurring species, an adventitious flora made up mostly of *Achromobacter* and *Bacterium* species also occur in the ventriculus and the rectum, but not in the intestines which appear to be free from an

adventitious flora. *Escherichia* (*E. freundi*) and *Aerobacter* species have also been encountered, but only in fish taken near shore. Other isolates obtained from the fresh fish include those of *Micrococcus* species, *Bacillus* species (*B. cereus* and others), *Pseudomonas ovalis* and *Vibrio percolans*.

Though there has been seldom any consistency about the microflora of the surface of the fish, species of *Aerobacter*, *Escherichia* (*E. intermedium*), *Achromobacter* (*A. delicatulum*, *A. superficiale*), *Flavobacterium* (*F. lutescens*), *Bacterium* (*B. mutabile* and others), *Micrococcus* (*M. caseolyticus*, *M. epidermidis*), *Sarcina*, *Gaffkya*, and *Bacillus* have been isolated and identified from the gills, skin and flesh of fresh samples of fish.

Bacterial flora of the dehydrated bombil.—Unlike the micro-organisms present in the ventriculus of the fresh fish, those present in the same region in the dehydrated form have a characteristic flora of their own. Species of *Micrococcus* seem to be very well represented and among the more common micrococci isolated and identified were *M. candidus*, *M. freudenreichii*, *M. epidermidis*, *M. aurantiacus*, *M. flavus*, *M. ureæ*, and *M. albus*. *Sarcina littoralis*, *Achromobacter delicatulum* and two species of unidentified bacilli were also isolated from the dehydrated *bombil*.

Discussion

On the basis of morphological and anatomical details, the *bombil* (*Harpodon nehereus*) is classified among the deep sea fishes, though its shoals are found in abundance along the coast. It has been shown that the skin and alimentary canal of the *bombil* have a characteristic flora of their own and that this flora is not only stenohaline in nature but possess powerful enzymes which apparently are responsible for the spoilage of the fish, the moment it dies. The micro-flora of the live fish and the dead one of course, vary to an extent depending upon the environments, conditions under which they are transported and handled, as also upon the death-examination interval. The occurrence of the colon group of organisms in the ventriculus of fresh fish collected from harbour waters, is significant from an epidemiological standpoint. But for the organism resembling *Sarcina litto alis*, the flora of the dehydrated fish is characterised by the complete absence of bacteria associated with the fresh *bombil*. The presence of *Sarcina littoralis*, even in dehydrated fish, suggests the resistant nature of this organism to adverse conditions like heat treatment and ultra-violet radiation. The frequent occurrence of species of *Micrococcus* (many of which secrete enterotoxins) in the dehydrated form of the fish, stresses the possibilities of this fish being a potential source of food poisoning.

SUMMARY

The fresh 'Bombay Duck' (*Harpodon nehereus*) has a normal bacterial flora of its own, among which *Bacterium phosphoreum* and *Sarcina littoralis* or their variants predominate. The latter species also persists in the dehydrated variety of the fish, which otherwise presents a microbial picture, totally different from that of fresh fish. Besides the species mentioned, the fresh fish is also associated with an adventitious flora, in which the following genera are represented: *Escherichia*, *Aerobacter*, *Micrococcus*, *Gaffkya*, *Achromobacter*, *Flavobacterium*, *Bacillus* and *Pseudomonas*. The occurrence of a large number and variety of micrococci in the dehydrated fish has a probable epidemiological significance.

REFERENCES

Berkeley, C. .. "A study of marine bacteria, Straits of Georgia, B. C.," *Trans. Roy. Soc.*, Canada, 1919, **13**, 15-43.

Boswell, J. .. *Tour to Hebrides with Samuel Johnson*, 1926, J. M. Dent & Sons, Ltd., London.

Bergey, D. H., Murray, E. G. D. Breed, R. S. and Hitchens, A. F. *Manual of Determinative Bacteriology*, 1939, The Williams & Wilkins Co., Baltimore.

Breed, R. S., Murray, E. G. D., and Hitchens, A. P. .. *Ibid.*, 1948, The Williams & Wilkins Co., Baltimore.

Buchanan, H. .. *Gangetic Fishes*, 1822, Edinburgh 209.

Cohn, F. .. "Zwei neue Beggiatoen," *Hedwigia*, 1865, **4**, 81-84.

Cox, M. E. L. .. *Practical Cookery*, Bombay, 1946.

Ehrenberg, C. G. .. *Die Infusionsthierchen als vollkommene Organismen. Ein Blick in das tiefere organische Laben der Natur.*, 1838, Folio, Leipzig.

Hora, S. L. .. "Wanderings of Bombay Duck in Indian Waters," *J. Bomb. Nat. Hist. Soc.*, 1934, **37**, 640-54.

Lipman, C. B. .. "The concentration of sea water as affecting bacterial population," *J. Bact.*, 1926, **12**, 311-13.

Moses, S. T. .. *Baroda Fisheries Bulletin*, **8**, 1946.

Pillay, T. V. R. .. "A preliminary note on the food and feeding habits of *Hanpodon nehereus* in the river Mattol," *Sci. & Culture*, 1951, **17**, 261-62.

Pinto, M. .. "Anatomy of the Bombay Duck," 1935, *Thesis*, University of Bombay.

Sethna, S. B. .. "Fishing for Bombay Duck," *J. Bomb. Nat. Hist. Soc.*, 1932, **35**, 867-72.

Thjotta, Th. and Somme, O. M. .. "Skrift Norske Udenske," *Akad. Oslo Matem-Naturvidenskap*, 1942, **4**, 1-86.

Warming, E. .. "Om nogle ved Denmarks Kyster levende Bakterien," *Medd. Naturhist for Kjöbenhavn.*, 1875, 7, 356-76.

———— .. *Ibid.*, 1876, **8**, 21-42.

Zobell, C. E. and Grant, C. W. .. "Bacterial utilization of low concentration of organic matter," *J. Bact.*, 1943, **45**, 555-64.

————, and Upham, H. C. "A list of marine bacteria including descriptions of sixty new species," *Bulletin Scripps. Inst. Oceangr.*, 1944, **5**, 239-92.

———— .. *Marine Microbiology*, 1946, Chro. Bot. Co.. Waltham, Mass.