QUANTITATIVE AND QUALITATIVE STUDIES ON THE BACTERIAL FLORA OF FRESH SARDINES.

T. C. KARTHIAYANI AND K. MAHADEVA IYER
Central Institute of Fisheries Technology, Ernakulam, Cochin-11

Plate counts at RT and 8 C on the skin with muscle and the gut contents of absolutely fresh sardines *(Sardinella longiceps)* caught off Cochin showed a seasonal variation when sampling was done over a period of 12 months. The counts of the gut contents ran parallel with those of the skin with muscle, but at a higher level of magnitude. Qualitatively, the analysis of 360 strains of bacteria isolated from the skin with muscle and 100 strains from the guts during an year's study revealed a very high preponderance of Gram negative rods, mainly of *Achromobacter, Vibrio,* and *Pseudomonas* groups. The percentage of Gram positive organism was very low or nil at times in the ocean-fresh sardines.

INTRODUCTION

Though a considerable amount of information is available on the quantitative and qualitative aspects of the bacterial flora of fish in the North Sea (Stewart, 1932; Reay and Shewan, 1949; Liston, 1956, 1957; Georgala, 1958), the North Atlantic (Reed and Spence, 1929; Gibbons, 1934a, 1934b; Dyer, 1947), the North Pacific (Hunter, 1920; Fellers, 1926; Snow and Beard, 1939; Kiser, 1944; Liston, 1959) and also the South Pacific (Liston and Colwell, 1963), data with respect to fishes in the tropical seas are scanty. The work of Wood in Australia in this field is worthy of mention (Wood, 1940, 1950, 1952, 1953). He studied the number and types of bacteria found in marine fish caught in the warmer coasts of Australia and reported the predominance of Gram positive organisms, especially *Micrococci,* in these fishes. This was, probably, the first indication that geographical locations influenced the qualitative nature of the flora as some of his results were at variance with those reported by workers in northern temperate zones. In India, Venkataraman and Sreenivasan (1952, 1954) studied the bacterial flora of Mackerel caught off the west coast of India. The present study was undertaken with the object of examining

systematically the microflora of fish caught along the west coast of India, especially those caught in Cochin waters. The investigations reported in this paper were designed (1) to study the quantitative variations in the bacterial load at different parts of the fish, viz., the skin with muscle and the guts with season (2) to get an idea of the qualitative composition of the bacterial flora associated with fresh uncontaminated sardines.

Materials and Methods

Fish: Fresh specimens of sardines *(Sardinella longiceps)* were examined each month for a period of one year. The fish, caught off the coast of Cochin, were brought to the landing places in country crafts. The specimens, still in rigor, were transferred aseptically into wide-mouthed sterile bottle and brought to the laboratory where they arrived within 4 hrs of being caught.

Sampling and enumeration of the bacterial flora: On arrival, 10–12g of muscle sample along with the skin were cut aseptically from 6 to 8 specimens. The entire guts from 4 to 5 specimens were also removed for sampling. Duplicate viable counts were carried out on the suitably diluted samples of both skin with muscle and guts using sea water agar (SWA) and distilled water agar (DWA) by the usual pour plate techniques.

Sea water based medium consisted of 1% peptone (Difco), a trace of ferric phosphate and 1.5% agar dissolved in sea water. The sea water used was aged by storing it in glass carboys in the dark for at least a month. The distilled water agar had similar composition except that it contained, in addition, 0.5% NaCl and made up in distilled water.

Duplicate series of plates were incubated at room temperature (28–30 C) and at 8 C (refrigerator temperature) and the colonies developed counted after 2 days and 21 days respectively.

Collection and classification of bacterial isolates: Random colonies were picked from SWA plates poured out from both skin with muscle and guts. The colonies appearing after 5 days at RT were picked and inoculated into sea water peptone. Each isolate was restreaked three times to ensure purity before their morphological and biochemical characteristics were studied. The pure cultures were maintained on sea water agar slants for preservation and subsequent studies. A total of 360 isolates from skin with muscle samplings and 100 isolates from the gut samplings was examined in detail. This represented the sum total of cultures isolated, at an average of 40 cultures a month, from both skin with muscle and guts over a period of one year.

Morphology and Gram stain were observed of 24 hr cultures grown on sea water agar slants. Sensitivity to Penicillin, CTC and OTC (Shewan, Hodgkiss and and Liston, 1954) was determined on the cultures growing on the agar medium (Peptone, 1%, NaCl 1%, Agar 1.5% in distilled water) in petri plates by observing the zone of inhibition around filter paper discs dipped in the respective antibiotic solution. The antibiotic plates were read after 24 hrs incubation at RT. The mode of attack of glucose by the cultures was determind by the Hugh and Leifson test (Hugh and Leifson, 1953). The presence of oxidase systems in the cultures was tested by means of Kovacs test (Kovacs, 1956). The production of pigment was observed on the sea water agar slants after 5–6 days of incubation at RT. The generic classification of the bacterial isolates was done according to a modified scheme of Usio Simidu and Kazuzoshi Aiso (1962) (Table I).

Results

Quantitative: The average of two monthly counts of skin with muscle and guts on SWA are shown logarithmically in Fig 1. The counts vary between 4.4×10^3 and 1.1×10^7 organisms/g for skin with muscle and between 6.0×10^4 and 8.5×10^8 organisms/g for the guts. The corresponding counts obtained by incubation of duplicate sets of plates at 8 C run parallel to those at RT.

Table II gives the counts on DWA as compared to those on SWA. In general, the counts on the former medium are lower and in 50% of the gut samplings there is no growth on DWA. Further, the ratios of counts in SWA to those in DWA in gut samplings are comparatively higher than the corresponding ratios for the skin with muscle samples.

Qualitative: Fig. 2 represents, graphically, the percentage of different morphological forms found on skin with muscle and guts for each quarter. The percentage was the average of the values for the three months of a quarter.

Table III shows the generic distribution of bacteria isolated during the year from skin with muscle and guts.

Discussion

Quantitative aspects: The log of counts, in general, varies from a minimum of 3.7 to a maximum of 7 in the case of skin with muscle and from 4.8 to 8.9 in the case of guts. This means that the "bacterial density" is higher in the guts than on the skin with muscle. This is in agreement with the results reported by Liston (1956) who records a count of 10^3 to 10^5 organisms/g on the skin and 10^3 to 10^7 organisms/g in the gut samples. He had also reported (Liston, 1956) that during most months, the counts obtained by incubation at 0 C was somewhat lower than that obtained at 20 C, but the difference was not excessively great. In the present study also a similar trend is observed; the counts at 8 C run parallel to those at RT at a slightly lesser magnitude. Further Fig. 1 suggests a seasonal variation, the skin counts recording a high peak in the months of June and September and the gut contents, in June and October. Georgala (1958) had reported such a high peak in June and October for the skin counts of the North Sea Cod. It is interesting that both the skin with muscle counts and the gut counts show peaks at almost the same periods of the year. This indicates that the conditions obtaining in the environs from which the fish is caught affect equally the bacteria present on the surface and the intestinal contents of the fish. However, if the phenomenon is due, simply, to the general increase in the ambient temperature of incubation for the RT-kept plates during the hot months, then the corresponding peaks obtained by incubation at 8 C become difficult to be explained unless there occurs a general increase in the bacterial load in fish during these months.

Regarding the capability of the organisms to grow in DWA it was observed that the counts were always slightly lower than the corresponding values obtained with SWA. This difference was more perceptible in the case of gut bacteria where in 50% of the platings there was no growth in the DWA at RT, though there was good growth in the sea water based medium (See Table I). This observation and also the higher ratio of SWA counts to DWA counts for the gut samplings indicate the true marine nature of the gut bacteria in as much as they seem to be nutritionally exacting as regards the requirement of sea water for growth.

Qualitative aspects: In all the quarters of the year the Gram negative rods predo-

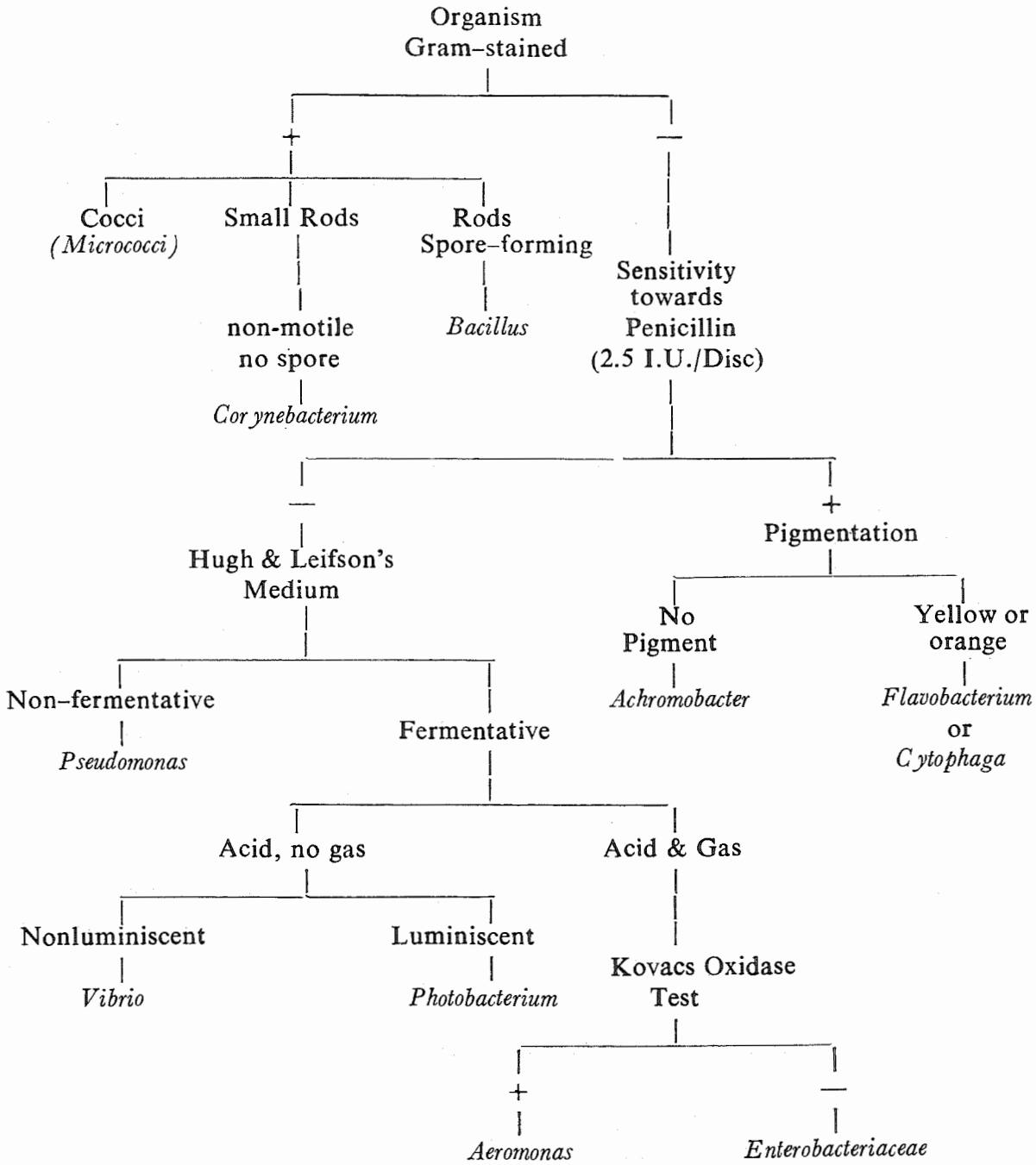
minated. It was never less than 79% for the skin with muscle isolates and 90% for the gut isolates. In the quarter from October to December, the gut flora consisted entirely of Gram negative rods. It is interesting to note that during these months, the corresponding counts of the guts in DWA were nil which indicates that these organisms are essentially marine types. Similar preponderance of Gram negative rods has been reported by Shewan (1966), by Georgala (1958) in the investigations on the bacterial flora of North Sea Cod, by Liston (1957) on the bacterial types of Flat fish and by Colwell and Liston (1962a. 1962b) in their investigations on the bacterial flora of Marine Invertebrates of the Pacific Ocean and of fishes of Central Pacific. In this respect, the present study seems to corroborate more with the reports for fish in Northern waters than those reported by Wood (1940) and Venkataraman and Sreenivasan (1952) who had found large numbers of *Micrococcus* and *Bacillus* species respectively in their specimens. The present authors agree with the explanation advanced by Colwell and Liston (1962b) that "such factors as fresh water run-off or low sea water-fresh water interchange in the inshore areas where the fish were captured alter the composition of the bacterial commensal floras". Hence the presence of Bacillus, essentially a terrestrial type, is not surprising in the fish examined by Venkataraman and Sreenivasan.

From Table III, it can be seen that the bulk of the flora of skin with muscle and guts consisted of Gram negative, asporogenous rods of the *Achromobacter, Vibrio, Pseudomonas* and *Flavobacterium* and *Cytophaga* groups. The relative percentages of these genera are at variance with those reported for fishes of Northern waters where *Pseudomonas* predominated followed by *Achromobacter* (Georgala, 1958; Liston, 1957). But then geographical location is known to cause such variations in the generic distribution. Thus Liston and Colwell (1963) report greater abundance of *Achromobacter* species on the fish caught in the warm water areas (Eniwetok Atoll, in Southern Pacific Ocean) than on the fish from Northern Pacific areas (Puget Sound, off Washington coast). Our results, too, show such a greater prevalence of *Achromobacter* and also *Vibrio* groups in freshly caught sardines. Such occurrence is possibly not due to the phenomenon of host specificity as prawn, a crustacean, caught in the same area also harbours a greater proportion of *Achromobacter* in the fresh state (unpublished data).

The incidence of luminous strains, classified as *Photobacterium* was observed in the guts (Table III) and noted in every sampling of the guts with infrequent occurrence at a lesser proportion on the skin. This corroborates with the reported occurrence of a large number of luminous strains in the guts of Flat fish (Liston, 1957).

Our experiments (unpublished work) have further shown that at least a part of the bacterial isolates from marine fish fails to form colonies, during initial isolation, in DWA. It is now well established that truly marine types of bacteria are more or less exacting, nutritionally, for sea water or some of the constituents of sea water (MacLeod, Onofrey and Norris, 1954; Tomio Hidaka, 1965). This may partly explain the total absence of colonies in DWA in some of the samplings of the guts or the high ratios of counts on SWA to those on DWA reported here. Hence choice was made of SWA plates, rather than DWA plates, for the isolation of individual microorganisms. But the use of SWA for the isolation of *Bacillus* or the *Micrococci* – essentially terrestrial types – when present on fish leaves us at no disadvantage. It has been found that such

TABLE I THE OUTLINE OF PROCEDURE FOR SCREENING OF CULTURES



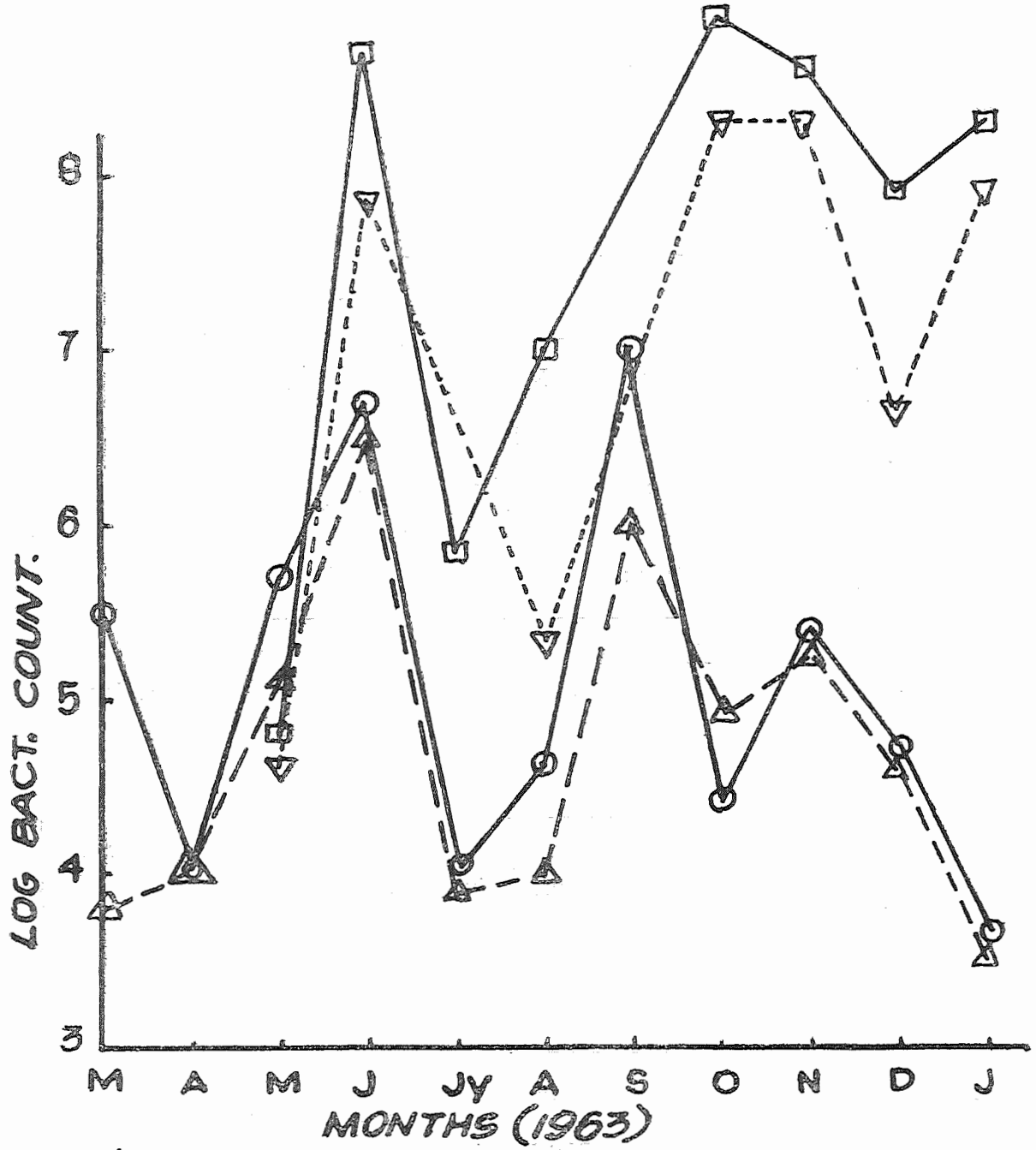


Fig.1.

○—○ Skin with Muscle counts at RT.
△---△ " " " 8c.
□—□ Gut counts at RT.
▽----▽ " " 8c.

TABLE II COUNTS OF SKIN WITH MUSCLE AND GUTS OBTAINED WITH SWA & DWA

Month	Counts at RT of					
	Skin with muscle			Guts		
	SWA	DWA	SWA counts/ DWA counts	SWA	DWA	SWA counts/ DWA counts
Mar	3.2×10^5	3.0×10^3	106.70	—	—	—
Apr	1.0×10^4	2.0×10^3	5.00	—	—	—
May	5.0×10^5	1.0×10^4	50.00	6.0×10^4	1.0×10^3	60.00
Jun	4.7×10^6	3.7×10^6	1.27	5.0×10^8	2.0×10^4	25,000.00
Jul	9.0×10^3	4.0×10^3	2.25	7.0×10^5	No growth	—
Aug	4.0×10^4	1.0×10^4	4.00	9.0×10^6	6.0×10^4	150.00
Sep	1.1×10^7	7.7×10^4	142.90	—	—	—
Oct	3.0×10^4	No growth	—	8.5×10^8	No growth	—
Nov	2.5×10^5	4.7×10^4	5.32	3.6×10^8	No growth	—
Dec	5.4×10^4	5.0×10^3	10.80	7.5×10^7	No growth	—
Jan	4.4×10^3	1.7×10^3	2.60	2.0×10^8	1.2×10^5	1,666.00

TABLE III DISTRIBUTION OF BACTERIA ON SKIN WITH MUSCLE AND GUTS

Bacterial Genus	Percentage in	
	Skin with muscle	Guts
Achromobacter	32	30
Vibrio	27	15
Pseudomonas	14	30
Flavobacterium & Cytophaga	11	2
Corynebacterium	7	1
Micrococci	6	1
Bacillus	1	0
Aeromonas	1	3
Photobacterium	1	18

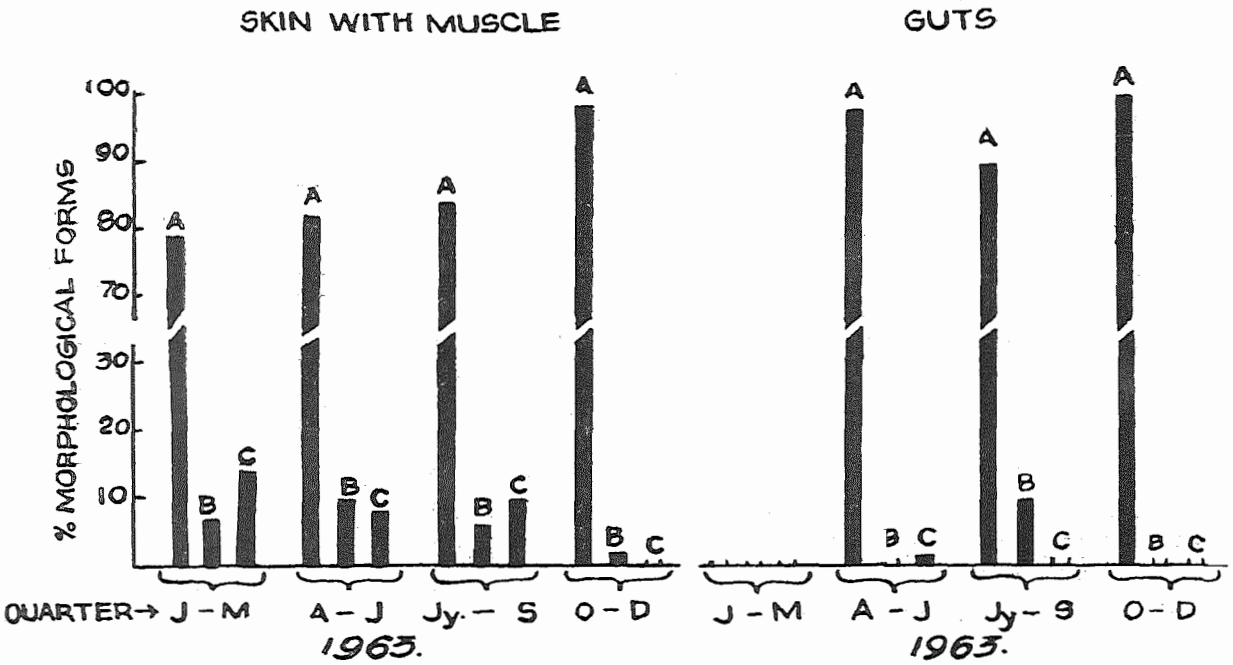


Fig. 2 Percentage of different morphological forms.

A = GRAM NEGATIVE RODS
B = GRAM POSITIVE COCCI
C = GRAM POSITIVE RODS

N. B. No sampling done for guts during the 1st quarter, 1963.

organisms, in pure cultures as well as in mixed cultures with marine types, grow within the time allotted for incubation, viz. 5 days, at RT (unpublished work). Consequently the nature of the flora isolated from SWA would probably present a truer picture of the flora of the fresh fish than the use of DWA warrants.

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