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STUDIES ON THE INTESTINAL MICROFLORA OF THE DAB, *LIMANDA LIMANDA* L.

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INTRODUCTION

ALTHOUGH the bacterial flora of marine fish has been extensively studied from the point of view of fish spoilage (for references see Shewan, 1961), comparatively little work has been done on the intestinal flora of fish either with regard to its variation or its composition. Liston (1956) found that the microflora of the skin and gills of North Sea skate (*Raja batis*) and lemon sole (*Pleuronectes microcephalus*) varied seasonally in a manner which suggested that the variation was associated with the plankton outbursts. Similarly, Georgala (1958) found a relation between the increase in the skin bacterial flora of North Sea Cod (*Gadus callarias*) and plankton growth. Presumably the increase in plankton led to an increase in the number of bacteria in the water as the plankton decayed. Liston (1956) also suggested that the protected environment of the gut of lemon sole made its flora somewhat insensitive to seasonal changes. However, a critical examination of his data indicates an irregular but definite tendency for the count of bacteria in the gut of this species to increase to a maximum around September-October, and to fall away to a minimum in December-January.

Several workers (Obst, 1919 ; Hunter, 1920 ; Blake, 1935 ; Margolis, 1953 ; Shewan, 1961) have reported that the number of intestinal bacteria is closely associated with the feeding habits of the fish ; starving fish having very few bacteria present in the gut. In this connection, it is interesting to note that Rae (1965) reported that the lemon sole is a seasonal feeder with maximum stomach contents in June, and a minimum in February ; a situation which parallels Liston's (1956) data for the gut flora. Moreover, the close similarity between the bacterial flora of the fish and that of the environment found by Shewan (1961) strongly suggests that the intestinal flora had originated from the ingested food.

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The dab (*Limanda limanda* L.) was chosen as the subject of this study because it can usually be caught by line close to these laboratories, and examined with the minimum of delay and handling. Furthermore, little is known of the feeding habits of this fish.

Materials and Methods

(A) Treatment of Fish.

(1) *Method of catching :* During the period from January-September 1965, 2-5 freshly caught specimens of dab, *L. limanda* L., were examined each month except March in which not even a single fish could be caught in spite of many attempts during the month. Each specimen was caught between 11 a.m. and 4 p.m. in the Menai Straits close to the laboratory ; the fish were placed, with the minimum of handling, in separate clean polythene bags which were then carefully put in a clean aluminium bucket. The time between catching and the beginning of the laboratory examination was kept to a minimum and was never more than 1 hour. The length and weight of each fish were recorded before taking the gut content sample.

(2) *Collecting the intestinal contents :* Live fishes were killed by a blow on the head before collecting the samples. The intestinal contents were collected in tared, sterile, corked test tubes containing 10 ml. of sterile, aged sea water. The area around the anus was sterilised by wiping with ethanol. The region of the body containing the intestine was carefully pressed to cause the extrusion of the faecal matter through the anal opening. In certain cases it was found that the amount of intestinal material obtained by this method was very small. In these cases the intestine was removed from the fish and the intestinal contents sampled aseptically.

(3) *Examination of food :* After sampling, the whole digestive tract of each fish was dissected out and preserved in formalin for the qualitative examination of the food. Subsequently, the dry weight of undigested food from each gut was determined by drying to constant weight in an oven at 110°C.

(B) Bacteriological Methods.

(1) *Enumeration of bacteria :* The bacteria were counted by the standard viable count method. After thoroughly shaking the sample, serial 1 in 10 dilutions were prepared by adding 1 ml. of inoculum to 9 ml. of sterile, aged sea water diluent until it was judged that the bacterial numbers per ml. were down to a countable value. Duplicate petri dishes were inoculated with 1 ml. of the appropriate dilutions using the usual pour plate technique. The medium used was 0.3% sea water peptone agar which Floodgate (1964) found optimum for the bacteria of the Menai Straits. This medium consisted of agar (Oxoid) 12 grams, peptone (Oxoid) 3 grams, and 1,000 ml. of aged sea water. The medium was sterilised by autoclaving at 15 lbs. per square inch for 15 minutes, the pH being adjusted, if necessary, to give a value of 7.6 after autoclaving.

The plates were incubated aerobically at 20°C. and counted after 6 days. This incubation temperature was used because both Georgala (1958) and Liston (1956) found that this was the optimum for the growth of the majority of bacteria from fish. Furthermore, Floodgate (1964) found that organisms from the Menai Straits also grew very well at this temperature.

From June onwards the luminous colonies were also counted in a dark room after 36 and 40 hours of incubation at 20°C.

(2) *Examination of bacterial isolates :* Since it was impossible to examine in detail all the colonies which grew on the count plates, a limited number were isolated for further morphological and biochemical investigation. In order to ensure that the colonies were taken at random, each plate was divided into 1 cm. squares. Each square was numbered and a number was taken from a table of random numbers. All the colonies growing in the square with this number were then cultured in 0.3% sea water broth. In this way pure cultures were obtained from 720 colonies and each of these was examined for colony morphology, cell morphology, Gram-reaction and motility. On the basis of this examination, a further 185 strains were selected to give as wide a range of colony and cell morphology as possible. These strains were then tested for their ability to produce acid and gas from glucose, galactose, lactose, maltose, mannitol, sucrose and xylose, and to liquefy gelatin. The media used were 0.3% peptone sea water broth enriched with 0.5% of the carbohydrate with bromocresol purple as indicator. Ability to liquefy gelatin was determined with Oxoid charcoal gelatin discs in 0.3% sea water broth.

Results and Discussion

(a) *Variation in bacterial counts*

The date of catching, the length, weight and the concentration of bacteria per gram faecal matter in the intestines of each of the fish is shown in Table I together with the dry weight of food and the feeding index (F.I.). One of the two fishes caught in January (fish No. 1) was diseased having a lesion on the ventral surface of the body, and the fishes in February were caught on different dates. In spite of repeated attempts, no fish could be caught at all during the month of March. This lack of fish during the early part of the year suggests that the dab either migrates offshore during the winter, or stops feeding.

Table I

Details of fish examined

Fish No.	Date of catching	Length (cm.)	Weight (gm.)	Dry wt. of Food (mg.)	F.I.	No. bacterial gm. Intestinal contents
1	Jan. 6th. 1965	28.2	209.4	100.0	0.48	4.51×10^{6}
2	Jan. 6th. ,,	20.6	90.2	0.1	0.011	7.06×10^{4}
3	Feb. 15th ,,	19.8	77.6	0.1	0.013	1.01×10^{5}
4	Feb. 25th. ,,	18.3	55.6	40.0	0.71	4.87×10^{4}
5	April 12th. ,,	15.8	36.8	170.0	4.61	1.15×10^{5}
6	April 12th. ,,	20.6	93.2	20.0	0.21	1.38×10^{6}
7	May 10th. ,,	21.4	126.4	450.0	3.56	7.91×10^{8}
8	May 10th. ,,	20.5	103.1	70.0	0.68	1.19×10^{5}
9	June 28th. ,,	21.3	109.8	50.0	0.46	6.93×10^{7}
10	June 28th. ,,	19.8	89.1	798.0	8.95	2.50×10^{8}
11	June 28th. ,,	17.0	63.0	0.1	0.015	6.09×10^{7}
12	June 28th. ,,	15.5	45.6	210.0	4.6	1.20×10^{7}
13	June 28th. ,,	12.4	24.2	80.0	3.31	2.34×10^{8}
14	July 16th. ,,	19.8	90.4	140.0	1.54	6.60×10^{9}
15	July 16th. ,,	18.3	75.2	50.0	0.66	8.12×10^{4}
16	July 16th. ,,	16.8	58.6	100.0	1.70	1.28×10^{5}
17	Aug. 12th. ,,	25.4	210.8	1400.0	6.64	2.10×10^{7}
18	Aug. 12th. ,,	19.0	84.4	100.0	1.18	2.40×10^{7}
19	Sept. 3rd. ,,	23.6	170.7	1050.0	6.15	2.22×10^{6}
20	Sept. 3rd. ,,	16.5	50.2	120.0	2.39	1.00×10^{6}

Gastropods	*Buccinum* sp.
Bivalves	*Mytilus edulis.*
Echinoderms	*Ophiothrix* sp. ; Holothurians.
Compound ascidians	*Diplosoma* sp.

Details of the kinds of food found in each fish are shown in Table II. No relationship was established between the species of the animals found in the gut and the number of bacteria per gram of intestinal contents, or between the dry weight of the gut contents and the concentration of bacteria. However, the monthly mean weight of food (Table I) showed a seasonal variation reaching a maximum in August and a minimum in February. In this respect the feeding activity of the dab is similar to that of the lemon sole (Rae, 1965).

(c) *Description of intestinal bacterial flora*

On the basis of cell and colony morphology and the biochemical tests, the bacterial flora of the dab may be divided into several groups as follows.

Group 1 organisms are motile, Gram-negative, non-spore forming rods ; producing acid from glucose, galactose, maltose, mannitol and sucrose ; may or may not liquefy gelatin ; forming a non-pigmented colony. These organisms comprised 70% of the total of the isolates and may belong to the family, *Pseudomonadaceae.*

Group 2 organisms are motile, Gram-negative, non-spore forming rods ; they do not produce acid from any of the sugar solutions used, nor do they liquefy gelatin ; they form a non-pigmented colony. These organisms, which comprised 12% of the total, resemble the *Achromobacter* as described by Shewan, Hobbs and Hodgkiss (1960).

Group 3 organisms are motile, Gram-positive, spore forming rods ; they do not produce acid from any of the sugar solutions used, nor do they liquefy gelatin ; they form a non-pigmented colony. These organisms, which comprised 6% of the total, probably belong to the genus, *Bacillus.*

Group 4 organisms are motile, Gram-negative, non-spore forming rods ; they occasionally produce acid late from glucose and maltose, but not from any of the other sugar solutions used, nor do they liquefy gelatin ; they form a yellow or orange coloured colony. These organisms, which comprised only 2% of the total, probably belong to the genus, *Flavobacterium.*

From June onwards, luminous organisms were observed on the count plates. On one occasion (fish No. 14) 40% of the total organisms isolated were luminous. Their numbers could not be correlated with the food in the gut. Most of the luminous organisms isolated were Gram-negative, motile rods which produce acid from carbohydrates and liquefied gelatin. They probably belong to the genus, *Vibrio.*

SUMMARY

The number of intestinal bacteria of the dab, *Limanda limanda* L., from the Menai Straits, was found to vary seasonally. The numbers of viable intestinal bacteria appeared to remain more or less constant from January to April, increased

in May and reached a maximum in June. The bacterial counts again decreased in July and increased in August. The difference between the counts carried out in August and September was not very great. A statistical analysis suggests that the feeding rate of the fish is an important factor in determining the bacterial intestinal load.

An examination of the stomach and intestinal contents suggests that the dab, *Limanda limanda* L., feeds on common coastal benthic invertebrates.

The possible taxonomic position of the majority of the bacterial isolates is discussed.

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