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A BACTERIOLOGICAL STUDY OF FROZEN FILLETS
PRELIMINARY REPORT

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N.E. GIBBONS

HALIFAX, 1930.

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Title

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PRELIMINARY REPORT

Author

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A Bacteriological Study of Frozen Fillets.
Preliminary Report

By N.E. Gibbons

During the development of the quick freezing method of preserving fish it has been found necessary to store the fillets at temperatures of about -20°C. Fillets stored at -5°C. and also at -10°C. became unfit for use after a few months. The cause of this was thought to be bacteriological.

A number of fillets were examined which had been in storage at different temperatures for periods of from one to two years. A series of haddock fillets were used for preliminary experiments. These had been stored for 13 months at -18°C. Some cod fillets, which had been kept at -10°C. for two years, were also examined. In addition to these, two series of haddock fillets were put up in the regular way but examined bacteriologically at intervals. One of these series was stored at -5°C, the other at -18°C.

A short outline of the method used in preparing frozen fillets is given in order that later points may be more easily understood.

The gutted fish are received as fresh as possible. These are kept in holding tanks at a temperature of one to two degrees above freezing until used. The fish are washed and the fillets removed, the operator cutting from the head down along the backbone. The whole fillet is then turned over, skin side down, while the latter is removed by running a knife through the fascia. This procedure, of course, covers the table with a rich flora from the slime, and although everything is washed frequently, it is impossible to prevent considerable contamination. The fillet is laid on this slime-coated table and cut into pieces 4 to 5 inches long. These pieces are again washed and taken to the cutting machine. Here the

pieces are forced into the machine which cuts them into blocks about 5"x3"x1". The block may be made up of one piece or of several. These are wrapped in wax paper, placed in a frame which in turn is slid into a water-tight container and the whole immersed in brine at -20°C. or lower. Freezing is accomplished in 20 to 30 minutes. The fillets are then packed and stored.

Methods

Our first problem was to find the most suitable medium. Ordinary nutrient agar, ordinary agar +1.5% sea salt(Turk's Island), fish infusion agar and a synthetic agar (Drew, 1911) were tried. The highest counts were obtained with the 1.5% salt agar, while the counts with the fish infusion agar were slightly lower. As a result a fish infusion-salt-agar was tried. This gave about the same results as the 1.5% salt-nutrient agar. As the latter is much easier to prepare and has less precipitate to cloud the plates, it was used for most of the work.

In sampling the fillets the technic of Weizirl and Newton (1914) was adopted. The wax paper was removed from the frozen fillet and the surface flamed. A one or two gram sample was then removed with sterile knives and forceps. The superficial layer was always avoided. The sample was weighed on sterile paper, transferred to a mortar previously rinsed with alcohol and flamed, and the tissue then triturated with sterile sand and water. Appropriate dilutions were made and plates poured. The plates were incubated at 25, 10 and 3°C. Counts were made after 8, 14 and 21 days for the respective temperatures, using a 2.5X hand lens. The most representative types on the plates were isolated, isolation being made at each temperature. In this way there is necessarily considerable duplication.

Stock strains have been carried on 1.5% salt-beef extract agar and stored in the ice-box. For purposes of identification the following characteristics have been determined: morphology, motility gram reaction, growth on agar, potato and in nutrient broth, growth in gelatin, reduction of nitrates and action in dextrose. In cases where there was no growth on potato the organism was set up on the salt potato described by Sanborn (1930). The potato slants are soaked in a 3% solution of NaCl for a few hours before sterilization. All cultures were incubated at room temperature (20 to 22°C.) These reactions are generally sufficient to identify the cocci, Flavobacteria and Achromobacters.

Where special colours and shades are mentioned, these have been arrived at by comparison with those given in Winsor and Newton's Specimens of Artists' Oil and Water colours.

I Quantitative Examination.

Quantitative bacteriological examinations of such products as fish and meats are never very satisfactory, due to the uneven distribution of the organisms through the specimen. This is particularly true of the fillets in question as some may be in one piece in which case there may be a few organisms in the interior; others may be made up of several pieces and the samples removed for examination may include a portion from the exterior surface of the original fillet which had been heavily laden with bacteria. This must be kept in mind when examining the following tables.

In Table I are given the number of organisms per gram in a number of haddock fillets which had been frozen on May 7, 1929, and kept at a temperature of -18°C. The examinations were made between June 11 and June 19, 1930. The fillets showed no signs of spoilage or bacterial growth. However, on thawing, they were dull and soft, while a fresh fillet on thawing is firm and glistening. There was no objectionable and only a slight "fishy" odour. The counts show a wide variation, not only in different fillets but in different samples from the same fillet. The highest counts were obtained at 25°C., the lowest at 3°C. There is a greater drop in numbers between the 3° and the 10° counts than between those at 25° and 10°.

Some cod fillets which had been put up on July 19, 1928 and stored at -10°C. were also available. These were examined between July 10 and July 29, 1930. The results of the examination are given in Table II. These fillets were very dry in appearance, yellowish and dull with numerous ice crystals in the spaces. But there were no signs of bacterial or fungus growth. The counts on these fillets are much lower than on the haddock fillets stored for a year at -18°C., those on the cod never being above 4,000 per gram. The counts at 3°C. were also higher in proportion. Many more molds were found in this series. However, no definite conclusions are warranted from these two experiments, as we know nothing of the original condition of the fish, nor their subsequent treatment.

In order to obtain some idea of the early changes in the bacterial flora, two series of twelve fillets were taken from the regular run and examined. Haddock was used in both cases.

TABLE I

Frozen Fillets, Haddock. Serial No. 2068. Put up May 7, 1929. Stored at -18°C.

Date Sampled 1930	Fillet No.	Count per gram at 25°C.	10°C.	3°C.
June 11	1 A	195,000	124,000	39,500
	B	19,000	17,300	9,000
June 12	2 C	141,000	63,000	22,700
	D	21,000	33,500	3,600
June 13	3 E	600,000	660,000	300,000
	F	40,000	25,200	5,800
June 16	4 G	20,000	3,000	1,000
	H	12,000	3,000	1,100
	5 J	7,000	2,000	500
	K	52,000	25,000	21,500
June 19	6 L	15,000	22,000	2.400
	M	45,000	39,000	24,700
	7 N	13,000	7,000	1,800
	O	6,000	3,000	100

TABLE II

Frozen Fillets, Cod. Put up July 19, 1928. Stored at -10°C.

Date Sampled 1930	Fillet No.	Count per gram 25°C.	10°C.	3°C.
July 10	V13	4,000	-----	1,000
	V18	4,000	4,000	1,000
July 17	V12	2,000	-----	3,500
	V14	1,500	1,000	500
July 29	V11	3,500	1,500	---

The first series was put up on June 26, 1930. Two haddock were examined in the holding tank. The skin was seared by flaming with alcohol and the sample taken from the inner portions of the muscle. In both cases no bacterial growth was obtained on plating out, although one mold colony appeared on one plate and two on another. However, as a high dilution (1-10,000) was used, the organisms may have been lost. In any case the count must have been rather low.

The twelve fillets were taken after going through the regular procedure and sampled just before freezing. After freezing they were stored at -18°C. One of them was examined the next day and the others at intervals. Up to the present, six have been examined with the results tabulated in Table III.

There is a great increase in the number of organisms in the fillets before freezing, due to the handling during the preparation. Shortly after freezing (24 hours) the bacterial count is much lower; and up to six weeks there seems to be very little drop after that due to the initial freezing. The sample taken at seven months shows a slight drop. However, this sample was shipped from Halifax to New York by way of a ship's refrigerator kept near the freezing point and brought from New York to New Haven packed in "Dry Ice". Consequently too much cannot be deduced from this sample unless substantiated by subsequent examination of the other fillets in the series.

TABLE III

Frozen fillets, Haddock. Put up June 26, 1930. Stored at -18°C.

Date Sampled	Fillet No.	Count per gram at 25°C.	10°C.	5°C.
6-26-30	1	600,000	250,000	100,000
6-27-30	1a	100,000	68,000	35,000
		35,000	- -	5,000
2-26-30	2	100,000	30,000	20,000
7- 3-30	2a	74,000	35,000	8,000
		46,000	54,000	10,000
6-26-30	3	320,000	130,000	70,000
7-10-30	3a	64,000	74,000	16,000
		68,000	68,000	18,000
6-26-30	4	150,000	- -	10,000
7-24-30	4a	105,000	90,000	22,000
		106,000	38,000	68,000
6-26-30	5	710,000	90,000	10,000
8- 7-30	5a	99,000	- -	- -
		88,000	- -	- -
6-26-30	10	60,000	30,000	- -
2- 9-31	10a	9,500(20°)	25,000(8°)	
		750	1,000	

TABLE IV

Frozen Fillets, Haddock. Put up July 9, 1930. Stored at -5°C.				
Date Sampled	Fillet No.	Count per gram at 25°C.	10°C.	3°C.
7--9-30	21	245,000	295,000	70,000
7-10-30	21a	130,000	154,000	50,000
		254,000	174,000	43,000
7- 9-30	22	50,000	65,000	15,000
7-23-30	22a	100,000	103,000	46,000
		116,000	88,000	65,000
7- 9-30	23	115,000	125,000	15,000
8- 6-30	23a	48,000	- -	- -
7- 9-30	24	123,000	70,000	5,000
2- 9 31	24a	99,000(20°)	168,000(8°)	- -

A second series was treated in exactly the same manner except that it was stored at -5°C. A lower dilution was used in sampling the fish in the holding tank, and growth was obtained on the 25° plates but not on those at lower temperatures. In one case the count was 90 per gram, in the other 4,500. The results of the examination after freezing are tabulated in Table IV. The remarks regarding Table III are also applicable here.

2. Qualitative Examination

I. Coccaceae.

About one third of the strains isolated were cocci. Of these, one hundred and forty-six have been grouped in the species which they most closely resemble. Seventeen species are represented.

Micrococcus nitrificans Bergey et al. is by far the most prevalent species, having been isolated 59 times. These strains do not liquefy gelatin, reduce nitrates to nitrites and have little or no action on milk. According to Hucker's classification (1924), these would be grouped in the species M. epidermididis. There are minor differences in their growth on potato and action on dextrose. Thirty-four strains grow on ordinary potato, and of these six produce acid in dextrose; seven strains do not grow on potato and of these three produce acid in dextrose; sixteen do not grow on ordinary potato but grow on salt potato; two strains give a scanty growth on plain potato, but none on salt potato.

Micrococcus candicans Flugge was isolated nine times. These strains show great variability in their ability to grow on potato; most of them give abundant growth on plain potato, but, if the growth is scanty, there is generally good growth on salt potato.

An organism resembling Staphylococcus albus Rosenbach was isolated seven times.

Of the yellow cocci, Micrococcus varians Migula was the most common. Eleven strains were isolated. Their growth on potato varies from moderate to none, and the colour on agar ranges from a cream, through yellow to Naples yellow. There were also five strains which come under this species, except that they do not produce acid from dextrose. This is probably a variety of the same species.

Eleven strains have been classified as Micrococcus conglomeratus Migula, although all of these differ from the regular description in their ability to grow on potato. However, nitrates are reduced and we believe this is a more constant character than growth on potato.

This same characteristic has been taken into consideration when eight strains are classified as Micrococcus perflavus Bergey et al. These organisms give only a scanty growth on potato, nitrates are reduced and acid is produced in milk.

Five cultures, which had a cadmium orange pigment when isolated, had lost most of their colour when finally examined. These liquefy gelatin slowly, give little or no growth on potato, do not reduce nitrates and do not act on dextrose or milk. Although Micrococcus subgranulatus Freund is yellow rather than orange, these five strains have been grouped under this heading. According to Hucker they may be considered as M. aurantiacus.

Organisms resembling Micrococcus Flavescens Henrici (Bergey et al.) or M. flavus (Hucker) and Micrococcus subflava Bunn were each isolated twice.

Organisms resembling Staphylococcus aureus Rosenbach were also isolated twice. These strains liquefy gelatin slowly, give an abundant orange growth on potato and slight acidity in dextrose. One strain reduces nitrates; the other does not. Both give an alkaline reaction in milk, with peptonization. Although the latter characteristic differs from Bergey's description, on the basis of Hucker's work this organism resembles Staph. or M. aureus more than any other species described.

Two strains are grouped as Micrococcus aurantiacus (Schroeter) Cohn, according to Hucker's classification. These organisms do not liquefy gelatin, do not grow on potato, reduce nitrates to nitrites, produce acid from dextrose and do not change milk. Two more strains are classified as Hucker's species, Micrococcus luteus (Schroeter) Cohn.

Micrococcus subcitreus Migula and M. ochraceus Rosenthal were each isolated once. Several strains of red cocci have been isolated, but owing to different pigment production on different media, only two have been classified, and we have again resorted to Hucker's classification (Hucker, 1928). Both strains are non-motile, reduce nitrates, give only a scant growth on potato and do not liquefy gelatin. One gives a rose doree pigment on agar and has been classified as Micrococcus roseofulvus Lehmann and Neumann. The other gives a deeper pigment, rose madder, and has been assigned to the species Micrococcus rhodochrous Zopf.

In Table V the distribution of the various species is given according to temperature. The figures refer to the number of samples from which the species was isolated at the various temperatures. Of the samples examined, cocci were isolated from twenty-six. It is interesting to note that only M. nitrificans and M. conglomeratus were isolated at 5°. At 10° M.nitrificans was isolated more often that at either of the other temperatures. It would seem then that generally a temperature of 25°C. is more favourable for the growth of this genus of organisms.

In many cases the same species was isolated several times from the same sample, probably due to picking from duplicate plates. In the above table only one isolation at each temperature has been considered.

TABLE V

Distribution of the species of cocci according to temperature.

Species	No. of strains isolated at		
	25°C.	10°C.	5 °C.
M. nitrificans	8	13	7
M. conglomeratus	5	-	2
M. varians	9	2	-
M. perflavus	5	-	-
Staph. albus	5	1	-
M. candicans	6	3	-
M. subgranulatus	2	1	-
M. flavescens	1	-	-
M. luteus	2	-	-
M. aurantiacus	1	1	-
Staph. aureus	2	-	-
M. ochraceus	1	-	-
M. subflava	2	-	-
M. subcitreus	1	-	-
M. roseofulvus	1	-	-
M. rhodochrous	1	-	-

II. Bacteriaceae.

Genus Flavobacterium.

Thirty-seven rods were isolated which produce a yellow pigment on agar. Thirteen have been placed in the species which they most resemble. None of these form indol. Only one is motile. This strain produces a cadmium yellow pigment on agar, liquefies gelatin slowly, does not reduce nitrates or change milk. In these respects it resembles Flav. aurescens (Ravenel) Bergey et al.

The remaining twelve strains are non-motile. Three of these liquefy gelatin, peptonize milk, reduce nitrates and give an abundant yellow growth on potato. Two are short rods, giving a pale yellow growth on agar and are probably Flav. lutescens (Lutig) Bergey et al. The other strain is a larger thicker rod and gives a deeper yellow growth on agar. In this it corresponds to Flav. fucatum isolated by Harrison (1930). Two other strains resemble these very closely except that the growth on agar is a lemon yellow and they produce a slight alkalinity in milk. These have been classed as Flav. fuscum (Zimmermann) Bergey et al.

The remaining four strains. of those which have been classified, do not liquefy gelatin and do not change milk. Two do not reduce nitrates and grow well on potato. In spite of their deeper pigmentation they are placed in the species Flav. solare (Lehmann and Neumann) Bergey et al. Another strain which resembles this closely, except that it reduces nitrates, is probably Flav. ovails (Wright) Bergey et al. A strain which does not reduce nitrates and gives only a scant growth on potato resembles Flav. brevis (Frankland) Bergey et al.

Up to the present we have been unable to place nine strains by Bergey's classification.

Genus Pseudomonas.

Twenty one strains of this genus have been recognised. Sixteen strains are of the Ps. fluorescens (Flugge) Migula type. Seven cultures are undoubtedly of this species. Two grow on potato as a white spreading growth but are otherwise typical. In one the potato becomes almost black, later changing to a dirty greenish black. Six other strains are not as active in the reduction of nitrates attacking them more slowly.

Five strains resemble the above but do not reduce nitrates. Although these show no indol production in 7 days we have classified them as Ps. schuylkilliensis (Wright) Chester.

None of the cultures produce the characteristic soluble pigment on the 1.5% salt-extract agar, nor is any pigment noticeable, even on infusion agar, when grown at 30°C.

Six strains have been isolated which produce a brown soluble pigment on agar. These have the general characteristics of the Pseudomonas genus but at the present time we cannot definitely assign them to any particular genus.

Genus Achromobacter

Representatives from this genus make up the largest group of organisms isolated. However, only a few have been classified. Further cultural characteristics are necessary to identify the remaining strains.

Achromobacter ambiguum (Wright), Ach. multistriatum (Wright), Ach. refractum (Wright), Ach. venosum (Vaughan), Ach. album (Copeland), Ach. superficialis (Jordan) Bergey et al. and Ach. sulfureum Bergey et al. have been isolated.

An organism which resembles Ach. solitarium (Ravenel) Bergey et al. but is non-motile has been isolated several times. Some of these strains give a white growth on potato, others a spreading metallic grey growth. Harrison (1926) mentions an organism similar to this isolated from frozen fish.

We have also isolated a number of strains which cannot be classed in any group given by Bergey. These are short, Gram negative rods, non-motile which do not liquefy gelatin and do not change milk. Harrison (1926) found that a number of his isolations came under such a sub-group. Besides the above characteristics this organisms grew on potato and reduced nitrates. We have also found this type. Some strains, however, do not grow on potato, two produce acid in dextrose and one gives a slight amount of indol. Still others which we believe should come under this sub-group, do not reduce nitrates.

Other Genera

Twelve strains belong to the Tribe Bacteriae but as yet we have not classified them according to genus and species.

Five rods have been isolated which give a very faint pink pigment on agar. These, too, we have not been able to place. The same is true of three rods producing a red pigment.

Besides the true bacteria, eight yeasts and one Actinomyces were found. No attempt has been made to identify these.

SUMMARY

A rather extensive survey of the bacterial flora of frozen fillets has been attempted. The most representative types have been isolated and many identified according to accepted classifications. This phase has not yet been completed.

However, a few general conclusions are obvious.

There is a rich bacterial flora in frozen fish stored over long periods at temperatures of -18°C. The initial freezing seems to be more destructive than subsequent storage at the low temperature. The bulk of the flora is due to gross contamination during the handling of the fish, although unavoidable to a great extent.

The large number of cocci is striking. We believe this represents an outside contamination in a great many cases. While Achromobacters are also present in large numbers this is more to be expected as this genus is largely made up of water forms. The scarcity of spore-bearing rods is noticeable, only one or two strains having been isolated.

While many strains have been assigned to known species, at the present time we wish this grouping to be considered as tentative. We make this reservation because of several observations which we hope further study may clear up. It was noticed, that of the strains isolated from fillets frozen and stored for a long time, none produced gas from dextrose or gave indol. These characteristics were noticed in some strains isolated from the series put up by the author and examined after a few weeks at the low temperature. This may have been a coincidence; it may mean that this type of organism is killed off after a time. Still we believe it merits further study and experiments are now in progress which we hope may shed some light on the matter.

We hope soon to be able to determine the role played by these various types of organisms in fish spoilage, particularly at low temperatures.

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