Bacterial Flora of EDTA Treated Oil Sardine (*Sardinella longiceps*), Indian Mackerel (*Rastrelliger kanagurta*) and Prawn (*Metapenaeus dobsoni*) in Ice Storage*

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The native flora of fresh oil sardine and mackerel consisted mainly of *Pseudomonas* spp; *Moraxella* spp; *Acinetobacter* spp. and *Vibrio* spp. During spoilage in ice, nearly 75% of their bacterial flora belonged to *Pseudomonas* spp. alone. But Na_2 EDTA treatment reduced the proportion of *Pseudomonas* spp. considerably and the major bacterial groups at the time of spoilage were *Moraxella* spp. and *Acinetobacter* spp. In the case of fresh prawn, the native flora were constituted by *Pseudomonas* spp; *Moraxella* spp; *Acinetobacter* spp. and *Vibrio* spp. At the time of spoilage of prawn in ice, *Moraxella* spp. and *Acinetobacter* spp. predominated, together constituting 74% of the total population. Na_2 EDTA treatment did not alter significantly the spoilage flora of prawns. *Moraxella* spp. and *Acinetobacter* spp. accounted for 86% of the spoilage flora in ice storage of Na_2 EDTA treated prawns.

The most important cause of spoilage of fresh fish and prawn held in ice, is the growth of micro-organisms. The growth of micro-organisms results in development of off odours and off flavours, which are the indications of spoilage. Application of chemical preservative to slow the rate of growth of micro-organisms results in extended ice storage life for fish and prawn. Chemical preservative like ethylene diamine tetra acetic acid (EDTA) salts were reported to be effective in extending the storage life of raw fish (Levin, 1967). Boyd & Southcott (1968) found that disodium salt of EDTA (Na_2 EDTA) retarded the growth of bacteria. Pelroy & Seman (1969) found that EDTA treatment caused a shift in the predominant spoilage flora. Surendran (1980) investigated various aspects of the preservation of oil sardine, Indian mackerel and prawn with Na_2 EDTA dips and subsequent storage in ice. The effect of the Na_2 EDTA treatment on the selection of bacterial population during ice-storage of these fish and prawn is reported in this paper.

Materials and Methods

Fresh oil sardine (*Sardinella longiceps*), mackerel (*Rastrelliger kanagurta*) and prawn (*Metapenaeus dobsoni*) were procured from fishing crafts operating off Cochin and brought to the laboratory within 2 to 4 h after catch.

Disodium ethylene diamine tetra-acetate (Na_2 EDTA) solutions of 1000 p.p.m. (0.1% w/v) and 10,000 p.p.m. (1% w/v) strength were used as dip solutions. The prawn/fish were dipped in the Na_2 EDTA solutions for 10 min, drained well and packed in ordinary crushed ice, in the prawn/fish to ice ratio of 1 : 1 and stored in thermocole insulated ice boxes. In all cases, untreated prawn/fish stored in ordinary crushed ice served as the control. The samples were stored for 20–30 days and ice loss was made up by addition of crushed ice, usually on alternate days.

The samples were analysed immediately on the begining of the study and thence, after intervals of 3 to 5 days during storage. Total plate count (TPC) was determined using sea water agar (SWA). The plates were incubated at 28 ± 2°C for 3 days and counts taken. Bacterial cultures were isolated from TPC plates of Na_2 EDTA treated samples and the control, during each sampling. The cultures were biochemically and morphologically characterised as described by Surendran & Gopakumar (1981). They were classified by the scheme of Surendran (1980) and Surendran & Gopakumar (1981).

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The sensitivity of cultures to various levels of Na_2 EDTA was determined by the agar plate technique (Surendran, 1980).

Results and Discussion

1. *Flora of oil sardine*

Typical results on the pattern of change in the bacterial flora of both control and EDTA treated oil sardine during ice storage are presented in Table 1. The succession of genera during ice storage of untreated oil sardine, was almost the same as reported earlier, (Surendran & Gopakumar, 1981) in connection with the CTC storage of oil sardine. The initial flora of fresh oil sardine comprised mainly of *Pseudomonas* spp. (20%), *Moraxella* spp. (12%), *Acinetobacter* spp. (18%) and *Vibrio* spp. (28%). As the storage in ice progressed, the flora changed significantly and by the time the fish spoiled, 75% of the bacteria belonged to *Pseudomonas* spp., the rest constituted by *Moraxella* (6%), *Acinetobacter* (9%), *Vibrio* (4%), *Flavobacter/Cytophaga* (2%) and *Micrococcus* (2%). The qualitative change in the flora of EDTA treated oil sardine during ice storage was entirely different. By the beginning of the ice-storage itself, the proportion of *Pseudomonas* spp. was found to be considerably reduced. Thus after 4 days in ice, the flora of 0.1% Na_2 EDTA treated fish consisted of *Pseudomonas* (7%), *Moraxella* (21%), *Acinetobacter* (30%) and *Vibrio* (22%), while the corresponding values for 1% Na_2 EDTA treated fish were, *Pseudomonas* (6%), *Moraxella* (18%), *Acinetobacter* (31%) and *Vibrio* (26%). However, as the number of days of storage in ice increased, the *Pseudomonas* spp. were found to recover from the initial fall. On the 24th day in ice, 20% of the flora of 0.1% Na_2 EDTA treated oil sardine and 18% of the flora of 1% Na_2 EDTA treated oil sardine, were composed of *Pseudomonas* spp. The other prominant groups were *Moraxella* and *Acinetobacter* spp., which together constituted 63% of the flora of the 0.1% Na_2 EDTA treated sample and 72% of the 1% Na_2 EDTA treated sample. *Vibrio* spp. diminished to 6% of the total.

The pattern of change in the percentage of gelatin liquefiers in the control and treated samples were similar; however, at the time of spoilage, 78% of the flora of the control

Table 1. *Pattern of change in the bacterial flora of untreated and EDTA treated oil sardine during ice storage*

	Percentage of micro-organisms at different intervals									
	Ordinary ice stored				Dip in 0.1% Na_2 EDTA and ordinary ice stored			Dip in 1% Na_2 EDTA and ordinary ice stored		
Days	0	4	10	24	4	10	24	4	10	24
Pseudomonas	20	21	34	75	7	12	20	6	12	18
Moraxella	12	17	14	6	21	20	26	18	24	30
Acinetobacter	18	24	22	9	30	34	37	31	38	42
Vibrio	28	18	10	4	22	18	8	26	16	6
Flavobacter/Cytophaga	4	5	4	2	5	4	3	6	3	2
Micrococcus	7	6	4	2	7	6	4	4	5	0
Others	11	9	12	2	8	6	2	9	2	2
Gelatin liquefiers	81	22	30	78	21	26	65	30	37	70
Putrefiers (fish media)	14	17	24	61	22	20	46	12	18	38
Capable of growth at 0°C	22	69	80	93	54	78	86	46	68	90
Sensitivity to 1% Na_2 EDTA	30	27	42	62	24	22	19	26	18	12
No. of cultures identified	65	62	51	56	40	51	63	70	59	52

sample were gelatin liquefiers, while only 65 to 70% of the flora of the treated samples were capable of gelatin hydrolysis. The succession of the putrefiers during ice storage seemed to be significantly different between the control and the EDTA treated fish. While 61% of the untreated oil sardine at the time of spoilage in ice, were putrefiers, only 46% and 38% of the flora of 0.1% Na_2 EDTA treated and 1% Na_2 EDTA treated fish were respectively putrefiers. This marked difference in the proportion of putrefying bacteria was evidently a reflection of the qualitative difference in the spoilage flora of the control and the treated samples.

It can also be noted from Table 1 that EDTA sensitivity of the flora of the treated fish decreased with increase in storage period. Thus only 19% of the flora of the 0.1% Na_2 EDTA treated sample and 12% of the flora of 1% Na_2 EDTA treated sample after 24 days in ice, were sensitive to 1% Na_2 EDTA, while 62% of the flora of the untreated oil sardine, was sensitive to 1% Na_2 EDTA.

2. *Flora of mackerel*

Table 2 gives typical results on the qualitative changes in the flora during ice storage of untreated and EDTA treated Indian mackerel. A comparison of this data with those presented in Table 1 shows that, the pattern of change in the flora of mackerel was almost similar to that of the flora of oil sardine, similarly treated and stored. Physiological group-wise also, similar patterns were observed.

In the case of both oil sardine and mackerel, EDTA treatment has brought about a qualitative change in the spoilage flora during ice storage of the fish. While, the dominant flora, of the untreated control samples at the time of spoilage in ice, were *Pseudomonas* spp., constituting 75 to 80% of the total flora, the major flora of the spoiling EDTA treated fish were the *Moraxella-Acinetobacter* group, forming 62% to 86% of the total, the *Pseudomonas* spp. accounting for only 8 to 17%. This significant difference in the flora is reflected in the trimethyl amine nitrogen (TMAN) values of the control and the EDTA treated samples, as well as in the percentage of putrefiers in the flora at the time of spoilage of the fish.

Pelroy & Seman (1969) have made similar observations in the qualitative changes of the bacterial flora of untreated and EDTA treated petrale sole and ocean

Table 2. *Pattern of change in the bacterial flora of untreated and EDTA treated mackerel during ice storage*

	Percentage of micro-organisms at different intervals									
	Ordinary ice stored				Dip in 0.1% Na_2 EDTA and ordinary ice stored			Dip in 1% Na_2 EDTA and ordinary ice stored		
Days	0	4	12	24	4	12	24	4	12	24
Pseudomonas	10	14	38	81	4	16	21	6	12	17
Moraxella	18	21	11	7	24	20	32	22	26	29
Acinetobacter	26	24	18	6	27	31	38	24	30	33
Vibrio	32	21	16	4	20	12	6	28	14	5
Flavobacter/Cytophaga	5	6	4	0	3	4	1	5	5	2
Micrococcus	5	7	6	1	4	3	0	4	3	2
Others	4	7	7	1	8	14	2	11	10	12
Gelatin liquefiers	82	20	31	90	16	37	68	21	29	71
Putrefiers (fish media)	14	19	30	52	11	24	47	9	18	35
Capable of growth at 0°C	7	42	70	91	32	65	84	36	67	86
Sensitivity to 1% Na_2 EDTA	21	24	36	65	28	22	20	24	17	14
No. of cultures identified	70	55	48	62	61	58	64	47	54	59

Table 3. *Pattern of change in the bacterial flora of untreated and EDTA treated prawns (M. dobsoni) during ice storage*

	Percentage of micro-organisms at different intervals									
	Ordinary ice stored				Dip in 0.1% Na_2 EDTA and ordinary ice stored			Dip in 1% Na_2 EDTA and ordinary ice stored		
Days	0	7	12	25	7	12	25	7	12	25
Pseudomonas	10	14	15	19	6	8	12	4	8	8
Moraxella	22	27	36	44	28	30	40	30	42	50
Acinetobacter	16	18	27	30	24	28	28	26	32	36
Vibrio	16	12	8	2	18	12	6	22	10	4
Flavobacter/Cytophaga	7	6	4	0	5	4	2	4	2	0
Micrococcus	8	5	2	2	7	3	2	8	2	2
Others	21	18	8	3	12	15	10	6	4	0
Gelatin liquefiers	68	30	45	90	24	36	70	20	42	60
Putrefiers (fish media)	10	18	28	40	20	20	38	18	22	30
Capable of growth at 1°C	12	50	68	86	40	72	90	50	68	94
Sensitivity to 1% Na_2 EDTA	22	20	26	28	24	20	20	26	18	14
No. of cultures identified	60	52	56	62	48	61	53	65	52	61

perch fillets stored at 0.5°C. While 64% of the flora of the control samples after 8 days of storage was *Pseudomonas* spp., only 4% of the flora of EDTA treated fillets was constituted by *Pseudomonas* spp. and 83% was comprised of by *Achromobacter* spp.

Miyauchi *et al.* (1966) have shown that *Pseudomonas* spp. are the source of trimethyl amine (TMA) production in spoiling fish. Hence, the higher values of TMA in the untreated spoiling fish—both oil sardine and mackerel were due to the higher proportion of *Pseudomonas* spp. in the flora at the time of spoilage. Similarly, the lower percentage of *Pseudomonas* spp. in EDTA treated fish accounted for the lower TMA values in EDTA treated fish.

The findings reported here are in full agreement with the observations made by Pelroy & Seman (1969) in the case of fillets of ocean perch and petrale sole from Canadian waters, that the increased shelf life of the EDTA treated fish is due to the inhibition of growth and concomitant metabolic activity of *Pseudomonas* spp.

3. Flora of prawn (M. dobsoni)

The pattern of qualitative changes in the flora of untreated and EDTA treated prawn *(M. dobsoni)* during ice storage, is presented in Table 3. The flora of spoiling untreated prawn comprised mainly of *Moraxella-Acinetobacter* group, together constituting 74% and the *Pseudomonas* formed 19% of the total flora. Since EDTA is found to eliminate selectively the *Pseudomonas* groups, EDTA treatment did not essentially bring about a significant qualitative change in the succession of the flora of prawns during ice-storage. But, the proportion of *Pseudomonas* spp. was lower compared with the untreated control. At the time of spoilage of 1% Na_2 EDTA treated prawns, 86% of the flora was composed of *Moraxella-Acinetobacter* group. *Pseudomonas* spp. constituted only 8% of the flora.

Much difference in the changes of biochemical groups were not observed between the untreated and EDTA treated prawn during storage. While 90% of the flora of the untreated prawn on 25th day of ice

Table 4. *Na_2 EDTA sensitivity of bacterial cultures isolated from Na_2 EDTA treated oil sardine*

Bacterial genus	Cultures from sardine before treatment				Cultures from sardine dipped in 1% Na_2 EDTA and stored in ice for 8 days				Cultures from sardine dipped in 1% Na_2 EDTA and stored in ice for 25 days			
	Percentage of cultures sensitive to the given Na_2 EDTA levels											
	No. of cultures	Na_2 EDTA 0.1%	1%	2%	No. of cultures	Na_2 EDTA 0.1%	1%	2%	No. of cultures	Na_2 EDTA 0.1%	1%	2%
Pseudomonas	40	30	75	100	45	22	60	80	52	10	40	70
Moraxella	56	12	20	26	48	12	14	22	40	10	15	15
Acinetobacter	48	8	14	22	54	6	14	26	49	2	16	22
Vibrio	64	4	12	16	72	5	10	12	60	8	8	10
Micrococcus	30	9	14	27	25	8	16	20	40	5	10	18
Flavobacter/Cytophaga	18	10	25	35	12	16	16	24	10	0	20	20
Alcaligenes	6	0	16	32	10	0	10	30	6	0	16	16
Arthrobacter	10	10	20	40	4	0	25	25	8	0	12	25

storage, was gelatin liquefiers, the corresponding percentage in the 0.1% Na_2 EDTA treated and 1% Na_2 EDTA treated prawn were 70% and 60% respectively. The percentage of putrefiers in the flora of prawn on the 25th day of ice storage was 40% in the control and 38% and 30% respectively in the 0.1% Na_2EDTA treated and 1% Na_2EDTA treated prawn. The proportion of EDTA sensitive flora decreased with increase in the number of days of ice storage in the case of EDTA treated samples, while in the control samples, the EDTA sensitive fraction remained more or less in the same range.

Thus it is evident that EDTA treatment did not bring about a significant change in the spoilage flora of prawn. As in the control, the major flora at the time of spoilage were the *Moraxella-Acinetobacter* group, the proportion of *Pseudomonas* being less. But, in the control 19% of the spoilage flora was *Pseudomonas* spp. while only 8 to 12% of the spoilage flora of EDTA treated prawns were *Pseudomonas*. Since *Pseudomonas* spp. are known to be the major source of TMA (Miyauchi *et al.*, 1966), in spoiling fish, the low values in TMAN in the EDTA treated samples might be explained as due to low proportions of *Pseudomonas* in them. But, the very low values of TMAN in the treated samples as compared with the control are not so easily explained. For example, by 25th day of ice storage, the TMAN values in the control and 1% Na_2 EDTA treated prawns were 22.86 and 4.92 mg/100g respectively (Surendran, 1980). The corresponding proportions of *Pseudomonas* spp. in the total flora were respectively 19% and 8%. While the ratio of the number of *Pseudomonas* in the treated sample, to that in the control is 8:19 (42%), the corresponding ratio of the TMAN values is 4.92:22.86 (21.5%). TMAN is not exclusively produced by *Pseudomonas* alone, but members of the *Moraxella-Acinetobacter* group and *Vibrio* spp. also produce TMAN; but their contribution is not appreciable in the case of spoilage at low temperature. As major producer of TMA in spoiling fish is *Pseudomonas* spp. itself (Miyauchi *et al.*, 1966) it can be concluded that even among the *Pseudomonas* spp; the TMA producers were more selectively suppressed by EDTA. However, further investigations are required to substantiate this.

4. EDTA sensitivity of cultures from EDTA treated fish and prawn

The sensitivity of cultures towards EDTA was found to undergo considerable changes during the ice storage of EDTA treated fish and prawn. In Table 4, are presented typical results of EDTA sensitivity of bacterial cultures isolated from EDTA treated oil sardine held in ice storage.

In the case of cultures isolated from oil-sardine just before EDTA treatment, 75% of *Pseudomonas* strains, 20% of *Moraxella* strains, 25% of *Flavobacter/Cytophaga* strains, 12% of *Vibrio* strains and 14% of *Acinetobacter* strains were sensitive to 1% Na_2 EDTA. The sensitivity of cultures isolated from EDTA treated fish was found to decrease as the number of days of storage increased. Thus, after 8 days of storage, only 60% of *Pseudomonas* strains, 14% each of *Moraxella* and *Acinetobacter* strains, and 10% of *Vibrio* strains were sensitive to 1% Na_2 EDTA. In the case of strains isolated from treated oil sardine held in ice-storage for 25 days, only 40% of *Pseudomonas* cultures was sensitive to 1% Na_2 EDTA. Thus, it is evident that, although there was a general decrease in the EDTA sensitivity for cultures isolated from treated fish, this decrease was more pronounced in the case of *Pseudomonas* spp. Since, the enhancement of shelf life by EDTA treatment is due to the selective inhibition of growth and concomitant metabolic activity of *Pseudomonas* spp. the development of insensitive *Pseudomonas* strains is of great economic importance, so far as the use of EDTA for fish preservation is concerned.

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