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ORIGINAL ARTICLE

The microflora of fresh and spoiled sardines (*Sardina pilchardus*) caught in Adriatic (Mediterranean) Sea and stored in ice

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A total of 1500 strains were isolated from the skin and gills of fresh, ice-stored (4 days) and spoiled (8 days) Adriatic sardines, and were identified at different taxonomic levels. Fresh sardine microflora found included mostly non-fermenting Gram-negative bacteria, (Pseudomonas, Flavobacterium, Psychrobacter, Acineobacter, Shewanella)*, and a minor proportion of Enterobacteriaceae and Gram-positive bacteria. The highest increase in microflora frequency, after 8 days in ice, was observed for the* Pseudomonas fragi *group (8–30·8%) and* Shewanella putrefaciens *(1·8–11%). A significant presence was also noted for fluorescent* Pseudomonas *(15·6–18·4%) and* Psychrobacter immobilis *(16·4–23·4%). The isolation frequency of the other groups decreased during storage. The most important proteolytic species were* Pseudomonas fluorescens *and* Shewanella putrefaciens *and the most lipolytic bacteria were* Psychrobacter immobilis *and again,* P. fluorescens *and* S. putrefaciens. *A total of 288 isolates, representative of the main groups, were tested for potential spoiling activity (H_2S and off-odour production, TMAO reduction).* Shewanella putrefaciens *was the strongest spoiler, followed by* Pseudomonas fluorescens. *A weaker activity was observed for strains of* Pseudomonas fragi, *enterobacteriaceae and non-saccharolytic pseudomonads. The contribution of weak spoiler bacteria can be undermined by the activity of the strongest spoilers, but in some cases the considerable incidence of the former group suggests their effective role.* Morganella morganii *was the only histamine-producing species among 57 screened strains representative of different taxa.*

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Introduction

Fish spoilage is a complex phenomenon in which different biochemical, microbiological and ecological factors are involved. Bacterial activity is the main cause of spoilage for fish kept at upper freezing temperatures (Anderson 1907, Shewan 1971, Liston 1980, Hobbs and Hodgkiss 1982). Bacterial degradation of soluble, low molecular weight components produces volatile metabolites, which are responsible for the unpleasant and offensive off-odours and off-flavours leading to the sensory rejection of the fish (Herbert et al. 1971, Gillespie and Macrae 1975, Shewan and

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Murray 1979). Among these volatile compounds H_2S, TMA and NH_3 are commonly produced. Other molecules have also been identified (Herbert et al. 1975, Miller et al. 1973a, 1973b, Edwards et al. 1987). Furthermore, the growth and activity of spoilage bacteria can be modulated by competitive and inhibitory mechanisms among cells in the microflora (Gram 1993, 1996).

The shelf-life of raw seafood depends on the storage conditions (for example, temperature and atmosphere), the intrinsic factors of the animals (species, age and size, fat content, feeding and physiological status), and the qualitative and quantitative composition of the initial microflora, related to the environment where the animals live and are caught, the seasonal period, the fishing method and the early handling (Shewan 1971, 1977, Horsley 1973, Gillespie and Macrae 1975, Shewan and Murray 1979, Liston 1980, Ward and Baj 1988, El Marrakchi et al. 1992a, 1992b).

The microbiology of fresh, ice-stored and spoiled fish has been actively studied since the beginning of the nineteenth century. Many reports concerned fish from cold and temperate sea waters, and described non-fermenting Gram-negative bacteria (for example *Pseudomonas*, '*Achromobacter*' *Acinetobacter* and *Psychrobacter*, *Flavobacterium*, *Alteromonas* and *Shewanella*) as the main part of the initial microflora (Shewan et al. 1960, Shewan and Murray 1979, Liston 1980, Hobbs and Hodgkiss 1982, Hobbs 1983). Less extensively, research has been carried out on seafood from tropical and subtropical waters. Newly caught fish from warm waters generally showed a predominance of Gram-positive bacteria (coryneforms, Micrococcaceae) and Enterobacteriacea (Wood 1953, Gillespie and Macrae 1975, Shewan 1977, Fajardo and Marth 1979, Liston 1980, El Marrakchi et al. 1992a, Gram 1990), but in some cases the microflora resembled that of cold and temperate waters (Colwell and Liston 1962, Lima dos Santos, Thesis data unpublished, Gram et al. 1990). Notwithstanding the different initial microflora, *Pseudomonas* and *Shewanella* are the most prolific micro-organisms during the ice storage of fish regardless of its origin (Shewan et al. 1960, Lee and Harrison 1968, Laycock and Regier 1970, Fajardo and Marth 1979, Hobbs 1983, Gram 1990, El Marrakchi et al. 1992a, Drosinos and Nychas 1996).

Our work concerns the microflora of fresh and ice-stored sardines from the Adriatic Sea, which is a part of the Mediterranean Sea located in a median position between the Arctic Polar Circle and the Tropic of Cancer (40–46°N and 12–20°E). It is a non-homogeneous marine environment due to difference in depth, currents, affluents and seasonal water stratification. The pelagic fishes are caught in waters with temperatures ranging annually from 10 to 23°C. Previous work (Gennari et al. 1986, 1988, 1989, 1992, Gennari and Tomaselli 1988, Gennari and Cozzolino 1989) allowed the characterization of single bacterial groups (*Pseudomonas*, *Psychrobacter*, *Flavobacterium* and *Shewanella*). This present paper reports on the overall quantitative and qualitative microbiological results, the degradative activity and the spoiling role of the different taxa.

Materials and Methods

Fish samples, from different coasts and seasonal periods, were collected at the Seafood Market of Milan Italy. They reached the market in ice storage, under commercial conditions. Incidental mishandling of samples before reaching the market could not be ascertained, but the good standard sensory quality of the animals was controlled by the veterinary service during the sampling.

Skin and gills of 10 different samples of sardines were examined after 0, 4 and 8 days of ice storage (the 8 day samples were spoiled). Strains were randomly isolated from TPE agar (Lee and Pfeifer 1974) plate counts and in each analysis 25 colonies from skin and 25 from gills were chosen. Each strain was tested for a first set of phenotypic characters (morphology, motility, Gram and flagella staining, oxidase, catalase, pigment production, metabolism of glucose, penicillin sensitivity, anaerobic growth and H_2S pro-

duction), useful for tentative identification of main generic groups. Additional specific tests (mainly utilization of carbon sources) were carried out for further characterization of pseudomonads, *Moraxella*-like and yellow pigmented strains, on the basis of the relevant taxonomic literature. Sampling, bacteriological analysis and identification of the isolates have been described in detail in previous papers (Gennari and Tomaselli 1988, Gennari and Cozzolino 1989, Gennari et al. 1986, 1988, 1989, 1992).

Gelatin hydrolysis was tested in TPE agar with 0·4% gelatin, spreading the Frazier reagent (Cowan 1974) on the plates after 3 days of incubation; casein hydrolysis was observed in TPE agar containing 15% sterile skimmed milk; Tween-80 hydrolysis was tested by the method of Sierra (1957), using TPE as a nutrient base and tributyrin agar was prepared as described by Cowan (1974), using TPE agar as a base. The medium described by Gram et al. (1987) was employed for TMAO reduction and H_2S production. H_2S production was first observed on Kligler Iron Agar (Difco) on all strains, then on Iron agar (Gram et al. 1987) and by the methods of the Pb papers (Cowan 1974, to show weak production) on part of the strains. Sterile sardine juice (SSJ) was prepared following the method described by Gram et al. (1987) was distributed into tubes (5 ml each). After inoculation, the production of off-odours was estimated by five persons after 1–2 days at 20°C or 7 days at 4°C. Histamine production was tested after growth (24 h at 20°C) on histidine broth containing peptone 0·5%, yeast extract 0·1%, glucose 0·1% and histidine 1%, in artificial sea water (Makemson et al. 1992) at pH 7; a paper chromatography followed as described by Foo (1977). Unless otherwise stated, the culture media were incubated at 20–22°C for 7 days.

Results

Table 1 summarizes the quantitative results for total count, proteolytic bacteria and lipolytic bacteria at different storage times. The reported ranges of values show a certain heterogeneity in the contamination levels of single samples. Incidence of proteolytic and lipolytic strains did not show a constant trend during the storage.

Table 2 reports the isolation frequency of the recognized bacterial groups at different times of ice storage, and their incidence as proteolytic and lipolytic strains. Data concerning skin and gills are pooled due to similar qualitative and quantitative incidence of bacterial groups, which have been previously observed in many cases (Gennari and Tomaselli 1988, Gennari et al. 1988, 1989). However, the increase of pseudomonads appeared more pronounced in skin, and at 8 days the isolation frequences of *P. fragi* group were 39·6% in skin and 19·6% in gills. Consequently, those of *Psycrobacter immobilis* were 16·8% in skin and 30% in gills. The group of non-saccharolytic pseudomonads includes strains of uncertain identification. These are also called *Alteromonas/*

Table 1. Variation of total count, proteolytic and lipolytic bacteria on ice stored sardines

Days in ice	Log total count[a]		% Proteolytic bacteria[b]		% Lipolytic bacteria[b]	
	Skin	Gills	Skin	Gills	Skin	Gills
0	4·08* (4·3–5·7)	5·09 (4·9–7·3)	55·0 (36–72)	53·8 (32–76)	39·6 (20–56)	46·0 (32–56)
4	5·05 (4·6–6·6)	6·05 (5·2–7·6)	44·4 (32–72)	50·0 (32–64)	61·6 (24–76)	60·8 (44–88)
8	6·09 (5·6–8·0)	7·07 (7·5–8·6)	46·8 (24–72)	42·0 (16–72)	44·0 (12–68)	59·2 (16–88)

Units are [a] log cfu cm^{-2} for skin and log cfu g^{-1} for gill, [b] isolation frequency.
* Results, obtained from ten samples of sardines, are means or, in parentheses, ranges among samples.

Table 2. Composition of the microflora of Adriatic sardines after 0 (fresh samples), 4 and 8 (spoiled samples) days of ice storage, and incidence of proteolytic and lipolytic strains*

	% in the microflora (days)				% as proteolytic strains (days)				% as lipolytic strains (days)			
Microflora	0	4	8	All strains	0	4	8	All proteolytic	0	4	8	All lipolytic
Pseudomonas fluorescens	11·8	14·8	11·6	12·7	11·8	14·8	11·6	26·2	6·2	10·2	7·0	15·0
Pseudomonas putida	3·8	6·8	6·8	5·8						0·6	0·4	0·6
Saccharolytic unpigmented Pseudomonads (mainly *P· fragi*)	8·0	12·2	29·6	16·6	4·0	6·2	11·4	14·8	3·0	2·8	3·4	5·9
Non-saccharolytic pseudomonads	6·8	4·8	5·0	5·5	5·0	3·8	4·6	9·2	5·0	4·4	4·2	8·7
Oxidase negative pseudomonads (mainly *Stenotrophomonas maltophilia*)	4·4	2·2	0·2	2·3	4·0	1·6	0·2	4·0	3·2	1·4	0·2	3·1
Shewanella putrefaciens	1·8	7·4	11·8	7·0	1·8	7·4	11·8	14·4	1·8	7·2	11·4	13·1
Psychrobacter immobilis	16·4	26·2	23·4	22·0					14·6	26·0	22·2	40·4
Acinetobacter	2·2	4·4	1·0	2·5					2·0	3·8	0·8	4·2
Flavobacterium/Citophagaceae[a]	19·0	9·6	3·4	10·7	16·8	8·0	2·2	18·5	2·4	1·0	0·4	2·5
Gram-, nonfermenters, peritrichous (tentatively *Alcaligenes*)	0·6	0·8	0·2	0·5		0·2	0·2	0·3		0·2	0·2	0·3
Enterobacteriaceae[b]	8·6	2·6	1·6	4·3	3·2	1·0	1·0	3·5	2·4	1·0	0·8	2·7
Vibrionaceae (Gram-, fermenters, oxidase +)		0·4	0·2	0·2		0·4	0·2	0·4		0·4	0·2	0·4
Micrococcus	2·6	0·8	0·3	1·3	2·2	0·4		1·8	0·4	0·2		0·4
Staphylococcus	1·8	0·6	0·6	1·0	1·2	0·2	0·2	1·1	0·6	0·2		0·5
Streptococcaceae	0·4	0·6	0·2	0·4	0·4	0·2	0·2	0·5				
Coryneforms	5·2	1·8	1·4	2·8	3·4	1·8	0·8	4·1	0·4	0·2	0·2	0·5
Other gram + (motile, asporigenous)	2·2	0·4		0·9	0·2	0·4		0·4				
Yeasts	0·8	1·8	0·6	1·1		0·2		0·1	0·4	0·2	0·2	0·5
Unidentified	3·6	1·8	2·0	2·4	0·4	0·6		0·7	0·4	1·4		1·2
Total	100	100	100	100	54·4	47·2	44·4	100	42·8	61·2	51·6	100

Units are isolation frequencies. Strains from skin and gills are pooled, 500 isolates for each storage time.

*This table is a revision and combination of previous identification work (Gennari and Cozzolino 1989, Gennari and Tomaselli 1988, Gennari et al. 1986, 1988, 1989, 1992).

[a] The ratio *Flavobacterium*: Citophagaceae was 83:14 on a sample of 100 strains.

[b] A part of the Enterobacteriaceae (50 strains) were identified by commercial kits as follows: 52% *Enterobacter agglomerans*, 40% *Enterobacter cloacae*, 2% *Enterobacter aerogenes*, 4% *Citrobacter freundii*, 2% *Morganella morganii*.

Shewanella-like (Gennari et al. 1988) because of some resemblance (that is, salt requiring, presence of a polar flagellum) with those marine bacteria. Strains previously classified in this group but showing slight pink pigmentation and ornithine decarboxylase are considered *Shewanella putrefaciens* in this paper, even if negative for H_2S production.

In the literature on seafood microbiology, the psychrotrophic, unpigmented, non-motile and non-fermenting oxidase-positive, Gram-negative coccobacilli, were first identified in the obsolete genus '*Achromobacter*'. Subsequently they were considered '*Moraxella*' or '*Moraxella*'-like in tentative identification or as a result of commercial bacterial identification systems (Shewan et al. 1960, Lee and Harrison 1968, Laycock and Regier 1970, Gillespie and Macrae 1975, Liston 1980). These names do not appear in Table 2 because we have demonstrated previously that nearly all these strains belong to the species *Psychrobacter immobilis* (Gennari et al. 1989), which was the most prominent in all isolates.

The plate count medium used gave good results, as shown by Lee and Pfeifer (1974), but it is lacking a sea water mineral base, causing the genus *Vibrio* and other bacteria requiring sea water to be underestimated.

Of 1500 bacterial isolates, 730 (48·7%) showed proteolytic activity; 88·2% of these strains were active both on gelatine and casein, 9·3% on gelatine alone and 2·5% only on casein. In Table 2, all strains are positive in at least one of the tests.

The incidence of proteolytic taxa during storage reflected the qualitative changes in the microflora. In fresh fish we noted various groups (most numerously flavobacteria, but also pseudomonads, Enterobacteriaceae and Gram-positive strains). After 8 days in ice, most proteolytic strains belonged to the predominating pseudomonads (*Pseudomonas fragi*, *Pseudomonas fluorescens* and *Shewanella putrefaciens*). The most common proteolytic species was *P. fluorescens* due to its high presence in both fresh and spoiled fish.

Seven hundred and seventy-eight isolates, 51·9%, hydrolysed Tween-80. Eighty of these, representative of the different taxonomic groups, were positive on Tributyrin agar. *Psychrobacter immobilis* was the most common lipolytic species. Differences were observed in the intensity and time of appearance of Tween-80 hydrolysis on the agar plates. In general, *P. fluorescens* and *S. putrefaciens* performed better than *Psychrobacter* strains. Differences were also observed in proteolytic activity. *In vitro*, *S. putrefaciens* and *P. fluorescens* showed an activity stronger than *P. fragi* and flavobacteria. Some *P. fluorescens* and *S. putrefaciens* strains were tested for gelatin lysis at 0·5°C; growth became visible on the plate after 4–5 days, and proteolysis after 5–6 days.

In Table 3, we report the observations on the potential spoiling activity of strains chosen from different groups. *S. putrefaciens* was the only strong H_2S-producing organism. Weak sulphide production (showed by Pb paper) occurred in some other groups (*Stenotrophomonas maltophilia*, non-saccharolytic pseudomonads, Enterobacteriaceae, flavobacteria, *Psychrobacter*, coryneforms and *Micrococcus*), but it was not related to the off-odour production. TMAO reduction is a usual activity of *S. putrefaciens*. Most strains of Enterobacteriaceae were also positive.

Strains inoculated in SSJ produced odours to different extent. In some cases, the type of odour was different for the same group. Most strains of *S. putrefaciens* produced strong and offensive off-odours (fecal, sulphide, putrid, cabbage, rotten egg) whereas many strains of *P. fluorescens* produced offensive odours (rotten, ammoniacal, putrid), generally to a lesser extent than *S. putrefaciens*. Some non-saccharolytic pseudomonads also produced weak odour (rotten, cheese, humid ground). One third of Enterobacteriaceae gave putrid odours, some *P. fragi* strains gave light fruity, musty odour (more rarely rotten fruit, or ammonia) and *Psychrobacter immobilis* did not produce odour (except for some strains giving a weak mushroom-like aroma). The off-odour production described in Table 3 was recognized after 24 h at 20°C. A reduced number of strains (results not

Table 3. Potential spoiling activity of bacterial strains isolated from Adriatic sardines

Bacterial strains	No. tested strains	Sulphide production	TMAO reduction	Off-odour production in sterile sardine juice
Pseudomonas fluorescens	27	−[a]/−[b]	−	Weak rotten (12)[c], putrid (6), strong ammoniacal (5)
Pseudomonas putida	9	−/−	−	Weak rotten (1)
Saccharolytic unpigmented pseudomonads (mainly *P. fragi*)	60	−/−	−	Strong (4) or weak (19) fruity, weak rotten fruit or musty (9), weak ammoniacal (7)
Non-saccharolytic pseudomonads (*Alteromonas/Shewanella*-like)	25	−/+	−	Weak rotten (12), weak ammoniacal (1), cheese (2), fishy (1), humid ground (1)
Shewanella putrefaciens	47	+/+	+	Sulphide, rotten egg, cabbage; generally strong (38), sometimes weak (9)
Stenotrophomonas maltophilia	2	−/+	−	Weak musty (1)
Psychrobacter immobilis	35	−/−	−	Mushroom aroma (6)
Acinetobacter	20	−/−	−	No offensive odour
Flavobacterium	17	−/+(2)	−	Weak fruity (2)
Citophagaceae	8	−/−	−	No offensive odour
Gram-, nonfermenters, peitrichous (tentatively *Alcaligenes*)	1	−/+	−	No offensive odour
Enterobacteriaceae:				
Enterobacter cloacae	7	−/+(6)	+(6)	Strong (1) or weak (3) rotten, fruity (1)
Enterobacter agglomerans	5	−/+(4)	+(1)	Rotten (1), mushroom (1)
Citrobacter freundii	1	−/+	+	Rotten
Morganella morganii	1	−/+	+	Strong rotten
Vibrionaceae (Gram-, fermenters, oxidase +)	1	−/+	−	No offensive odour
Micrococcus	3	−/+(1)	−	No offensive odour
Staphylococcus	2	−/+(1)	−	No offensive odour
Streptococcaceae	1	−/−	−	No offensive odour
Coryneforms	10	−/+(4)	−	No offensive odour
Motile, asporigenous, Gram +	5	−/−	−	No offensive odour
Yeasts	1	−/−	−	No offensive odour

[a] Tested on Kligler Iron Agar or Iron Agar.
[b] Weak production shown on Pb paper.
[c] When different results are observed among strains of a group, the number of positives are reported in parentheses.

shown) were also tested at 4°C (7 days incubation). The same odour production was confirmed for psychrotrophic strains (*Pseudomonas* and *Shewanella*) within 5–7 days, but Enterobacteriaceae showed weak or no growth and activity at low temperatures.

The production of histamine from histidine was screened on 57 representative strains (six *P. fluorescens* strains, three *P. putida*, five *P. fragi*, four non-saccharolytic pseudomonads, six *S. putrefaciens*, three *Stenotrophomonas maltophilia*, two *Alcaligenes*, four *Flavobacterium*, one *Cithophaga*, six *Psychrobacter immobilis*, three *Acinetobacter*, two *Enterobacter cloacae*, two *Enterobacter agglomerans*, one *Morganella morganii*, one *Vibrionaceae*, three coryneforms, two *Micrococcus*, two *Staphyloccus*, one yeast). *M. morganii* was found to be the only histamine-producing strain.

Discussion

Observation on strain identification and intra-species biodiversity

Most isolates were satisfactorily identified in this work at different taxonomic levels. However, although bacteriological techniques and bacterial taxonomy have improved the identification of bacteria, several studies, and our work, have demonstrated the difficulty in identifying bacteria isolated from fish by conventional phenotypical methods. This is due to many reasons including large variability in phenotypical features of strains of the same species, evolutive or adaptive characters, undescribed species or subspecies. Moreover, phenotypical characterization of some species are based on a limited number of strains in taxonomic papers, which does not permit the identification of less frequent characters.

Genetic and cellular component analyses are useful for microbial identification but they have not been applicable until recently for the study of a large number and variety of isolates. It is only recently that molecular techniques for DNA analysis have been made available for routine use. Commercial kits for bacterial identification, considered in the beginning of our study, are more reliable in clinical microbiology. Some of them do not take into consideration species and biovars commonly present in the environment and food, such as *P. fragi*, which may lead to species being unidentified or misidentified. For example, *Psychrobacter* is usually misidentified as *Moraxella*. Thus, additional tests are often needed for a more satisfactory identification. For these reasons we used different identification sets of conventional methods which focused on the specific bacterial groups as recommended by the relevant literature, except for Enterobacteriaceae.

Subspecies' phenotypical differences were often observed by strain characterization. Different *P. fluorescens* biovars were identified, in particular biovars V1, V6 and III-1 (Gennari et al. 1988, Gennari and Dragotto 1992). Different *S. putrefaciens* biotypes were isolated, as previously observed in fish, other food and clinical isolates (Owen et al. 1978, Holmes et al. 1975, Levin 1972, Strenström and Molin 1990 and Gennari and Campanini 1991). In particular, non- or weak H_2S producing strains of *S. putrefaciens* were reported in this work and by other researchers (Levin 1975, Gillespie 1981, Molin and Ternström 1982, Sternström and Molin 1990, Gennari and Campanini 1991, Dalgaard 1995). These strains can generally be identified by other key characters (pink salmon pigment and ornithine decarboxylase). Furthermore, different physiological and spoiling activity was observed among strains of *S. putrefaciens* isolated from fish (Jørgensen and Huss 1989, Gram et al. 1990).

Concerning the subspecies classification of *P. fragi*, we recognized strains fitting the A, B1 and B2 subclusters as described by Molin and Ternström (1986), but some strains showed atypical characters (Gennari et al. 1988). Strains recognizable as *P. fragi* but having atypical characters were also described by Gram et al. (1990).

Off-odour producing and non-odour producing strains of *P. fragi* group were not distinguishable by conventional biochemical tests in this work or in other similar research (Gillespie 1981, Gram et al. 1990).

Considerations on proteolytic and lipolytic strains

This work showed a considerable presence of proteolytic and lipolytic bacteria in fresh, ice-stored and spoiled sardines, although there were qualitative changes in the microflora during the ice storage (Table 1).

Strains that hydrolysed Tween-80 were also capable of tributyrin hydrolysis, but not all gelatinolytic strains were caseinolytic. Variation in bacterial activities on different lipid and protein substrates have been described (Kazanaz 1968, Tom and Crisan 1975, Sikes and Maxcy 1979, El Marrakchi et al. 1992b), in particular, gelatinolytic or caseinolytic strains can be inactive on heat-sterilized fish proteins (Chandrasekaran et al. 1985, El Marrakchi et al. 1992b). However, Kazanaz (1968) described most proteolytic strains active both on casein and fish proteins sterilized by irradiation. In a recent study (data not shown) we observed gelatinolytic strains of *S. putrefaciens* and *P. fluorescens* active, to varying extents, on cytoplasmic fish proteins sterilized by filtration, but inactive on autoclaved fish proteins (Chandrasekaran et al. 1985). Moreover only in the case of *S. putrefaciens* were all tested strains active. However, proteolytic bacteria contribute, in varying degrees, to the degradation of fish tissues, in extent of activity and substrate specificities on cytoplasmic and fibrillar proteins (Venugopal 1990, Odagami et al. 1994) but the variety of proteolytic activities shown by different bacteria have not been subjected to extensive and comparative studies. It is probable that, as for the odour-producing bacteria, only a reduced part of proteolytic bacteria play a main degradative role.

Rancid smell was recognized as a component of the off-odours of two spoiled samples. Ababouch et al. (1996) observed that rancidity can sometimes be the main reason for spoilage of Moroccan sardines. The inconstant rancidity development in sardine spoilage may be due to the large range of fat content of this fish (1·2–9·8%), and the variable incidence of lipolytic flora. Most research on fish spoilage addresses the sulphide or putrid degradation but little information is available on rancid spoilage. Although spoilage is mainly due to off-odour production from small soluble components, the attack of macromolecules by proteolytic and lipolytic enzymes of psychrotrophic bacteria causes quality deterioration of the fish muscles (Venugopal 1990, Makarios-Lahman and Lee 1993). Furthermore, it provides utilizable metabolites in addition to the initial pool (which come from living tissues and post-mortem autolytic processes), to support the growth of the microflora after the first days of storage (Lerke et al. 1967, Chandrasekaran et al. 1985, Venugopal 1990).

Spoiling role of main bacterial groups

The microflora of fresh and spoiled sardines includes a large variety of potentially degradative bacteria. The importance of a single taxonomic group depends on its activity and quantitative incidence during the storage. Considering the potential activity reported in Table 3 and the isolation frequency (Table 2), bacterial groups can be classified as follows:

Strong and specific spoilers. *S. putrefaciens*. This species is capable of specific spoiling activity, that is, H_2S and unpleasant off-odour production and TMAO reduction. It is normally present in the marine environment and its growth rate at low temperature permits a notable increase during ice storage. It is capable of anaerobic respiration of TMAO and other compounds. Most strains show strong activity but isolates with lower activity can be encountered. *S. putrefaciens* is considered the strongest spoiler of seafood from cold and temperate water (Levin 1968, Miller et al. 1973b, Van Spreekens 1977, Gram et al. 1987). The present work indicates a lower incidence when compared to other research carried out on different fishes stored in ice (Chai et al. 1968, Laycock and Regier 1970, Herbert et al. 1971, Gram et al. 1987, Stenström and Molin 1990, El Marrakchi et al. 1992a, Liston 1992).

Other active spoilers. *P. fluorescens* is the second most important off-odour producer and the most isolated proteolytic species. It does not produce H_2S but is capable of producing other volatile metabolites (Edwards et al. 1987). Its incidence, higher than that of *S. putrefaciens*, was important both in fresh and stored sardines. Not all strains are active spoilers and the extent of spoilage differs among isolates. *P. fluorescens* has been considered as a spoiler organism in many other studies concerning fishes of cold and temperate waters (Chai et al. 1968, Levin 1968, Herbert et al. 1971, Shewan and Murray 1979). In some cases it did not appear as a prominent spoiler or it was not isolated (Lerke et al. 1965, Gram et al. 1987).

Bacteria with low, limited or occasional spoiling activity. (a) Unpigmented saccharolytic pseudomonads. Mainly *P. fragi*, these are the group best adapted to ice storage as they showed the greatest increase after 8 days. The weak offensive, fruity off-odour production of some strains is well known (Castell et al. 1959, Miller et al. 1973a, Shewan and Murray 1979, Gram et al. 1990), but strong active strains attributed to this species have also been described (Herbert et al. 1971). In our case, its high incidence could support some effective spoiling activity.

(b) Non-saccharolytic pseudomonads. Some strains developed weak off-odours. They showed a low global incidence (about 5%), but an abundant presence was noted in some samples.

(c) Enterobacteriaceae. Some strains of this group (in particular *Enterobacter cloacae*) were capable of TMAO reduction and production of putrid odour if tested at 20°C, but their activity appeared scarce at low temperatures. Although many strains can grow at low temperatures, their abundance decreases during ice storage, possibly because their growth rate is lower than that of Gram-negative non-fermentative psychrotrophic spoilers. Enterobacteriaceae were absent in some samples, but they occasionally represented a consistent part of the isolates of fresh samples (20% on skin and 44% on gills). This suggests that they may contribute to spoilage, especially in the case of polluted water or delay in chilling after the catch.

Bacteria with no sensory spoilage activity. (a) *Psychrobacter* and *Acinetobacter* (formerly *Achromobacter*, *Moraxella* and *Moraxella*-like). This is the most consistent group (mainly *Psychrobacter*) in all isolates, lacking effective activity on the sulphur or putrid spoilage. These organisms are generally considered non-spoilers (Gillespie and Macrae 1975, Shewan and Murray 1979) and active strains have been rarely described (Lerke et al. 1965, El Marrakchi et al. 1992b). Their eventual role in rancid spoilage should be further investigated, considering their predominance as a lipolytic group.

(b) Flavobacteria are the most consistent proteolytic component of the fresh samples, but they seem to have no effective spoiling action.

(c) *P. putida* was frequently isolated from fresh and stored samples, but only a weakly active strain was observed in this work. Likewise spoiler strains have rarely been described (Herbert et al. 1971).

(d) Other Gram-negative bacteria such as *Stenotrophomonas maltophilia* and *Alcaligenes*, showed low incidence in fresh fish, which decreased further during storage and did not show spoiling activity.

(e) Gram-positive bacteria (for example, coryneforms, *Micrococcus*, *Staphylococcus*) and yeasts were observed mainly in fresh fish. They showed some lytic activity but no off-odour production.

Although the present work shows *S. putrefaciens* and *P. fluorescens* as the main spoilers, the two species represent, respectively only, 11·8% and 11·6% of all isolates at spoilage (Table 2). Other bacterial groups show weak spoilage potential. Their single contributions seem negligible and their action could be undermined by the stronger activity of *S. putrefaciens* and *P. fluorescens*, nevertheless, in some samples we observed low incidence of strong spoilers and

a remarkable presence of weak spoilers (*P. fragi* group and non-saccharolytic pseudomonads). Thus, their contribution can be implied.

P. morganii, the only histamine producer found in this work, is well known for its decarboxylative activity and it has already been isolated from the sardine by Ababouch et al. (1991).

Comparison between the microflora of Adriatic sardines and those of similar fish from different environments

The microflora of fresh Adriatic sardines appeared similar to those described for many fishes from cold and temperate water, where Gram-negative, non-fermenting psychrotrophic micro-organisms (pseudomonads, *Psychrobacter* and flavobacteria) predominate and the presence of Enterobacteriaceae and Gram-positive bacteria is less prominent. The spoilage microbiology of the Adriatic iced sardines also shows analogy with those of various fish from different cold and temperate areas, where *S. putrefaciens* and *P. fluorescens* appear as the main spoilers (Shewan and Murray 1979, Liston 1980, Hobbs and Hodgkiss 1982).

The keeping time of fish (7–8 days) was similar or slightly shorter than that observed for Atlantic Moroccan sardines (8–11 days), caught in local, but warmer water (El Marrakchi et al. 1990, 1992a, 1993, Ababouch et al. 1996). A more prolonged shelf-life (20 days) was observed on Senegalese sardinelles (Diouf et al. 1982, Gram 1990). In Atlantic Moroccan sardines (El Marrakchi et al. 1992a, 1992b, El Marrakchi 1993) *S. putrefaciens* was recognized as an important spoiler (22·7% of the total microflora at spoilage), *P. fluorescens* was poorly isolated, and other taxa (Enterobacteriaceae, Vibrionaceae and some Gram-positives) were observed to be active on proteins and TMAO, suggesting a relevant role in the spoilage.

In other research on Atlantic Moroccan sardines (Ababouch et al. 1996) a moderate incidence of *S. putrefaciens* (2–10%) was observed, and the effective contribution to spoilage of non-H_2S producing pseudomonads was suggested. This research appears closer to our work due to similar observations on the initial contamination of skin and gills, and the qualitative incidence of spoilers.

Investigations on the spoilage of sardines and other sea and fresh water tropical fish in ice (Gram et al. 1989, 1990) recognized the presence of *S. putrefaciens*, but mesophilic strains from those fishes appeared less active in spoilage than psychrotrophic strains from cold water seafood. Fluorescent *Pseudomonas* were also isolated, but the main spoiling role was attributed to unpigmented, H_2S and TMA non-producing pseudomonads (possibly *P. fragi*). These bacteria were shown to produced weak, fruity and musty off-odours, and they caused slow spoilage in the absence of other, stronger spoilers.

Observation on the spoilage pattern of fish from the Adriatic Sea and nearby marine areas

The literature concerning fish from North European waters (in particular North Sea, Kattegat and Baltic Sea) shows *S. putrefaciens* as the main spoiler of aerobic, cold storage. For this reason it is considered a specific spoilage organism (SSO), and so can be a useful microbial index for quality control, and an effective target of rapid and alternative methods for shelf-life assessment and prediction (Gram et al. 1987, Jørgensen et al. 1988, Gram 1992). Conversely, the spoiling role of *S. putrefaciens* was found to be less marked in tropical iced fish. The shelf-life is more extended and other bacteria are involved in the spoilage (Shewan 1977, Poulter et al. 1981, Diouf et al. 1982, Gram et al. 1989).

The role of *S. putrefaciens* in the spoilage of Adriatic iced sardines appears intermediate between that described for fish from cold and tropical sea water, as the geographical and climatic area is also intermediate. Different incidence ranges of active bacteria were observed on different samples at spoilage (2–28% *S. putrefaciens*, 2–40% *P. fluorescens*, 8–60% *P. fragi*). A similar observa-

tion was reported by Ababouch et al. (1996) on Atlantic Moroccan sardines (33°N–34°N) where different kinetics of TMA and TVB-N production were suggested as a result of alternative main proliferation of *Shewanella* or *Pseudomonas*. The heterogeneous microbiological pattern (within a limited and well known group of spoiling taxa) appears to be characteristic of the spoilage of iced fish from Mediterranean temperate water and similar climatic areas. Thus, we can considered *S. putrefaciens* as the main SSO, *P. fluorescens* an important SO, and *P. fragi* a secondary (or weak) SO.

Although *S. putrefaciens* counts can be useful in quality determination of fish from the Adriatic sea, it is probably not a sufficient parameter for shelf-life prediction. The different spoilage pattern observed for different samples reflects the effect of natural factors (geographical and seasonal origins), and commercial practices such as fishing technique, handling practices and transportation. Furthermore mishandling, which can not be excluded, even if improbable, may incidentally introduce extraneous bacteria or irregular growth rates.

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