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Carnobacterium spp. in seafood packaged in modified atmosphere

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Abstract - The composition of lactic acid population in five kinds of seafood (salmon, tuna, shrimps, swordfish and cuttlefish) packaged in two different modified atmospheres, MAP1 (80 O_2/20 N_2) and MAP2 (40 CO_2/60 N_2), at 4 °C for 6 days was investigated. The isolates were heterofermentative rods belonging to *Carnobacterium*, *Lactobacillus*, and cocci of the *Leuconostoc* genus. The determination of phenotypical characters and a new polymerase chain reaction primer were used to distinguish *Carnobacterium* from *Lactobacillus*. The microorganisms found varied with the kind of seafood and the gas composition of the modified atmospheres: in MAP1, richer of oxygen than MAP2, *Carnobacterium* spp. represents the prevalent microbial group, especially in tuna, shrimps and swordfish, whereas MAP2 seems to favour *Lactobacillus* spp. Cocci, belonging to *Leuconostoc* spp., were dominant in salmon and cuttlefish independently of gas composition.

Key words: lactic acid bacteria, seafood products, modified atmosphere packaging.

INTRODUCTION

The storage of seafood products is problematic, because of their high water activity, post mortem pH value and high content of non-protein nitrogen. The rate of deterioration processes depends on initial microbial count, temperature of storage and modality of packaging (Huss, 1995). Packaging under modified atmosphere with high concentration of CO_2 associated with refrigeration, represent a good solution to preserve from spoilage processes and to extend the shelf life of these products (Galli *et al*., 1993; Bosku and Delbevere, 1997; Gray *et al*., 1983).

Psychrotrophic Gram-negative rods are the principal responsible for spoilage of seafood products and the presence of CO_2 exerts a delaying effect proportional to its concentration and temperature of storage (Galli *et al*., 1993; Lyhs *et al*., 2001). Usually seafood products are considered unacceptable when the microbial count is higher than 10^6 CFU/g (Gray *et al*., 1983). During the storage Gram-posi-

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tive microorganisms develop and almost always become the dominant population at the end of shelf-life. They consist of lactic acid bacteria and non-aciduric lactic acid bacteria ascribable to *Carnobacterium* genus (Baya *et al*., 1991; Paludan-Müller *et al.*, 1998).

Proliferation of these microorganisms often found on skin, gills and in the gastro-intestinal tract of fish products (Evelyn and Dermott, 1961; Ringø and Gatesaupe, 1998) is not negative as they prevent the development of undesired bacteria thanks to their ability to produce acid metabolites, reduce the redox potential and bacteriocines synthesis. However they can negatively affect food safety by means of production of bygones amine (Masson *et al*., 1997; Stoffels *et. al*., 1992).

Differentiation of *Carnobacterium* from *Lactobacillus* is not always easy and rapid, in fact it is based on complex traditional biochemical and physiological tests, often tedious, time consuming and not always providing appropriate results; for this reason the use of tests based on molecular characteristics is to be preferred (Collins *et al*., 1987; Lyhs *et al*., 2002). The aim of our work was to individuate the lactic acid population in seafood packaged under modified atmosphere and to distinguish *Carnobacterium* spp. from *Lactobacillus* spp. using traditional techniques, based on phenotypical tests (Montel *et al.*, 1991), and PCR-based systems.

MATERIALS AND METHODS

Samples. Five kinds of seafood, different in origin and size: slice of tuna, salmon and swordfish, whole cuttlefish and shrimps were used. The seafood products were bought clean at the Milan fish market, transported under refrigerated conditions (4-6 °C ± 1 °C) to the laboratory where they were packaged within 4 hours from the purchase. All products were packaged under two different atmospheres, MAP1 (80 O_2/20 N_2) and MAP2 (40 CO_2/60 N_2) into high barrier bags (PO_2<0.5 cm^3/24 h bar m^2 at 23°C 0%U.R.) by means of a laboratory vacuum packaging machine (S-100 digit Tecnovac)**,** and the bags were stored at 4.0 ± 0.5 °C and analysed shortly after packaging and after 3 and 6 days.

Microbiological determinations. 10 g of seafood were aseptically removed from each package, put into a sterile stomacher bag, homogenised with 90 ml of sterile 0.85% tryptone salt solution and blended for two minutes at 230 rpm in a Stomacher (Seward 400 circulator, PBI International).

Decimal progressive dilutions were prepared and the following microbial determinations were performed:

Mesophylic aerobic bacterial Count (TBC) by pour plates, in Tryptic Soy Agar (TSA, Merck, Germany), incubation at 30 °C for 48-72 h (APHA, 1992).

H_2S producing non-spore forming bacteria by pour plates with an agar overlay, in Iron Agar, incubation at 20 °C for 72 h (Jensen and Shulze, 1980).

Lactic acid bacteria on Man Rogosa Sharpe (MRS, Difco, USA) by pour plates, incubation under anaerobic conditions in gas-pack jar at 30 °C for 48-72 h (De Man Rogosa *et al.*, 1963).

All microbiological determinations were carried out in triplicate and the numerical values reported in each table are the results of arithmetical average.

Isolation. All colonies grown on the last countable dilution of MRS were isolated. The isolates were, at the beginning, differentiated by observation of their cellular morphology using phase contrast microscopy (480X), Gram staining and catalase test.

The isolates were kept under refrigerated conditions (4 °C) in MRS broth and at –80 °C in MRS broth with 20% glycerol (w/v) for long-term maintenance.

Phenotypical identification. Phenotypical identification of the isolates, at genus level was carried out according to the scheme of Fig. 1. All isolates were tested for the production of gas from glucose and NH_3 from arginine, growth at 45 and 10 °C, enantiomorph of lactic acid were detected spectrophotometrically in the surnatant fluids of 24 h cultures by enzymatic methods (Boehringer Mannheim, Germany). Only for rods, grown on MRS agar at pH 9.5 (Hammes *et al.*, 1991), but not on acetate agar, the presence of m-diaminopimelic acid in the cell wall was determined (Bousfield *et al*. 1985). Fermentative patterns for putative *Carnobacterium* was performed by means of the fermentation test API CH 50 gallery and CHL (bioMériux, France).

FIG. 1 – Key used for preliminary identification of *Carnobacterium* genus isolated from seafood packaged under modified atmosphere and stored at 5 °C.

DNA extraction and PCR protocols. Total DNA extraction was performed on 100 µl of overnight broth culture as previously described (Mora, *et al*. 2000). DNA solutions were stored at -20 °C. All PCR reactions were performed in a volume of 50 µl containing approximately 50-100 ng of bacterial genomic DNA solution, 5 µl of 10X PCR reaction buffer, 200 µM of each dNTP, 2 mM of $MgCl_2$, 0,5 µM of each primer and 0.5 U of Taq Polymerase (Amersham-Pharmacia). Amplification of a 16S rDNA fragment of about 280 bp from the species belonging to the genus *Carnobacterium*, was carried out using a selection of seven primers, constituted on the primer set described by Scarpellini *et al*. (2002), (Carno1R 5'-AGC-CACCTTTCCTTCAAG-3'; Carno2R 5'-AACCGTCTTTTATCCATCC-3'; Carno3R 5'-AGCCACCTTTCATCCGTTC-3'; Carno4R 5'-AGCGGTAGCC-GAAGCCAC-3';16SF 5'-AGAGTTTGATCCTGGCTCAG-3'), added with two new primers as forward Carno1F and Carno2F (Carno1F 5'-ATACATGCAAGTC-GAACGCT-3'; Carno2F 5'-ATACATGCAAGTCGAACGCA-3').

DNA 16S-23S intergenic spacer (ITS) region amplification was performed using the primer set ITSF-ITSR (ITSF 5'-GTCGTAACAAGGTAGCCGTA-3'; ITSR 5'-CAAGGCATCCACCGT-3') and the following thermal profile: 5 cycles consisting of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and 30 cycles consisting of 92 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min. For all amplifications the final extension was continued for 7 min at 72 °C. All amplification reactions were performed in a T-Gradient 96 thermocycle (Biometra, Germany). Following the amplification 7 µl product was electrophoresed at 5 Vcm^{-1} (1,5 % agarose gel, 0,2 µg of ethidium bromide ml^{-1}) in TAE buffer. Reference strains and isolates used are reported in Table 1.

RESULTS AND DISCUSSION

The results of the microbiological determinations, performed on the different kinds of seafood, are shown in Fig. 2 and 3.

All products at the beginning of storage were of good quality: the starting level of microbial population were within the normal range for fresh products (Fig. 2 and 3). The high concentration of CO_2 and the absence of oxygen in MAP2 delayed the microbial growth of aerobic forms and Gram-negative H_2S producing bacteria, considered to be responsible for seafood spoilage under modified atmosphere. Under these conditions, at the end of storage, the counts were almost two orders of magnitude lower in comparison with MAP1.

The positive action of CO_2 was more evident during the first 3 days, afterwards counts higher than 10^6 CFU/g and evident alteration phenomena were observed.

The presence of CO_2 did not seem to influence lactic acid bacteria, that showed a gradual increasing during the storage Thanks to their metabolic activity they can potentiate the CO_2 antagonist action against less acid tolerant microorganisms, like Gram negative rods, considered to be responsible of alterative processes.

Microorganisms identification

All strains isolated on the base of phenotypic tests were heterofermentative, with a predominance of the rods form. *Lactobacillus* spp. and cocci were classified at the genus level, while *Carnobacterium* spp. were tested with PCR method according to

TABLE 1 – PCR results for *Carnobacterium* and reference strains

Origin	Strain	PCR primer set		ITS classification
		16SF-Carn1-4R[1]	CarnF1-2-Carn1-4R[2]	
CRAYFISH	G1; G4; G5; G6; G7; G10; G11; G12; G15	+	+	*C. divergens*
	G2; G3; G8; G9; G13; G14; G16; G17	+	+	*C. piscicola*[5]
SWORDFISH	SP1; SP2; SP4; SP5; SP6; SP7; SP8; SP9; SP10	+	+	*C. divergens*
TUNA	T1;T2; T3; T4; T5; T6; T11; T12	+	+	*C. divergens*
SHRIMPS	P1; P2; P3	+	+	*C. piscicola*[5]
Lactobacillus acidophilus ATCC[a] 43121		–	–	
Lactobacillus brevis CNRZ[b] 214		+	+[3]	
Lactobacillus bulgaricus NCFB[c]1373		–	–	
Lactobacillus casei NCFB 207		–	–	
Lactobacillus lactis NCFB 970		–	–	
Lactobacillus lindneri DSM[d] 20690[T]		–	–	
Lactobacillus maltaromicus DSM 20344[5]		+	+	
Lactobacillus plantarum ATCC 10241		+	–	
Carnobacterium piscicola DSM 20722[T5]		+	+	
Carnobacterium gallinarum DSM 4847[T]		+	+	
Carnobacterium mobile DSM 4848[T]		+	+	
Carnobacterium divergens DSM 20623[T]		+	+	
Carnobacterium alterfunditum DSM 5973		+	+	
Carnobacterium funditum DSM 5971		+	+	
Carnobacterium inhibens CCUG[e] 31728		+	+	
Desemzia incerta DSM 20581[T]		–	–	
Vagococcus salmonarium DSM 6633[T]		–	–	
Enterococcus hirae DSM 20160		–	–	
Streptococcus thermophilus DSM 20617[T]		–	–	
Escherichia coli NCTC[f] 12079[T]		+[4]	+[4]	

d: DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.
e: CCUG, Colture Collection University of Gotemborg, Dept. Clinical Bacteriology Gotemborg, Sweden.
f: NCTC, National Type Colture Collection, London, England.
a: ATCC, American Type Colture Collection, Rockville, MD.
b: CNRZ, INRA, Collection Française de Bactéries Lactiques. Station de Recherches Laitiéres Jouy-En Josas, France.
c: NCFB (NCDO), National Collection of Food Bacteria c/o NCIMB Ltd., Aberdeen, Scotland, UK.
1. The PCR was carried out using the 16SF as forward primer and Carno1R, Carno2R, Carno3R and Carno4R as reverse primers (280 pb).
2. The PCR was carried out using Carno1F and Carno2F as forward primers and Carno1R, Carno2R, Carno3R and Carno4R as reverse primers (200 pb).
3. Positive signal but of different length (250 bp).
4. Positive signal but of different length (900 bp).
5. Now reclassified as *Carnobacterium maltaromaticum* (Mora *et al*. 2003).

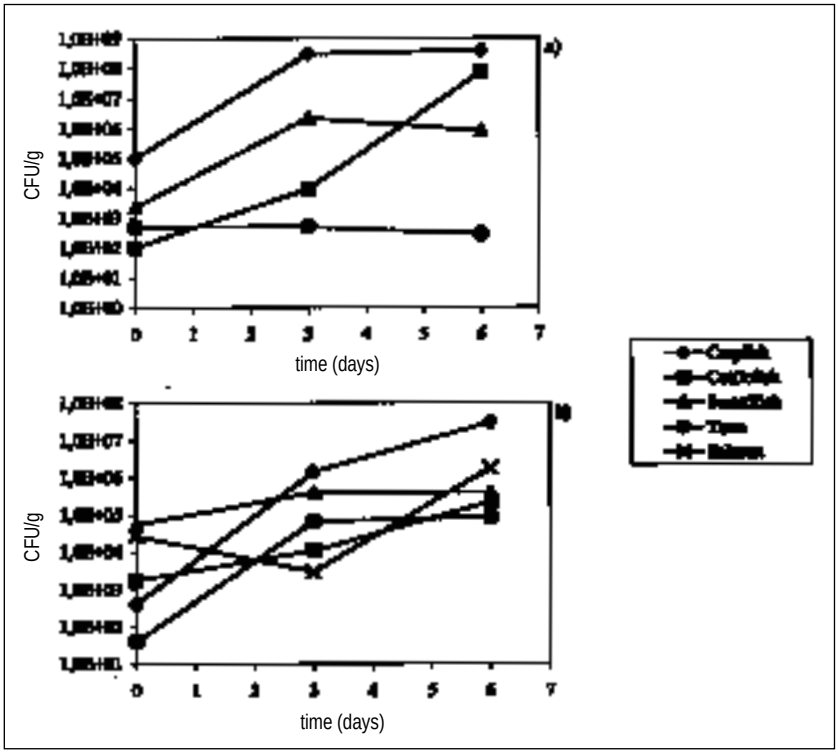

FIG. 2 – Evolution of H_2S producing bacteria (a) and lactic acid bacteria (b) during storage of seafood under MAP1. H_2S producing bacteria were not found in salmon.

Scarpellini *et al.* (2002). With this primer set a fragment of the same length (about 280 bp) for *Lactobacillus brevis* and *Lactobacillus plantarum* was obtained. A fragment was also observed for *Escherichia coli*, but with different length (900 bp). The addition of two new primers, as described in Materials and Methods, permitted to eliminate this undesirable fragment. For this reason the final amplification was performed with a mix of six primers Carno1R, Carno2R, Carno3R and Carno4R as reverse primers and Carno1F and Carno2F as forward primers. The length of the signal obtained was 200 bp for all *Carnobacterium* tested. With this primer set *Lactobacillus plantarum* resulted negative, while *Lactobacillus brevis* showed a fragment with a different length, about 250 bp. The comparison between the two primer sets for the selective identification of the genus *Carnobacterium* is shown in Fig. 4, where *Streptococcus thermophilus* was considered as negative control.

The identification of the species was performed by the amplification of 16S-23S intergenic spacer (ITS) (Scarpellini *et al.*, 2002). The results are shown in Table 1 and Fig. 5.

The lactic acid forms found at the end of storage seemed to be influenced by the type of seafood and by gas composition of atmosphere.

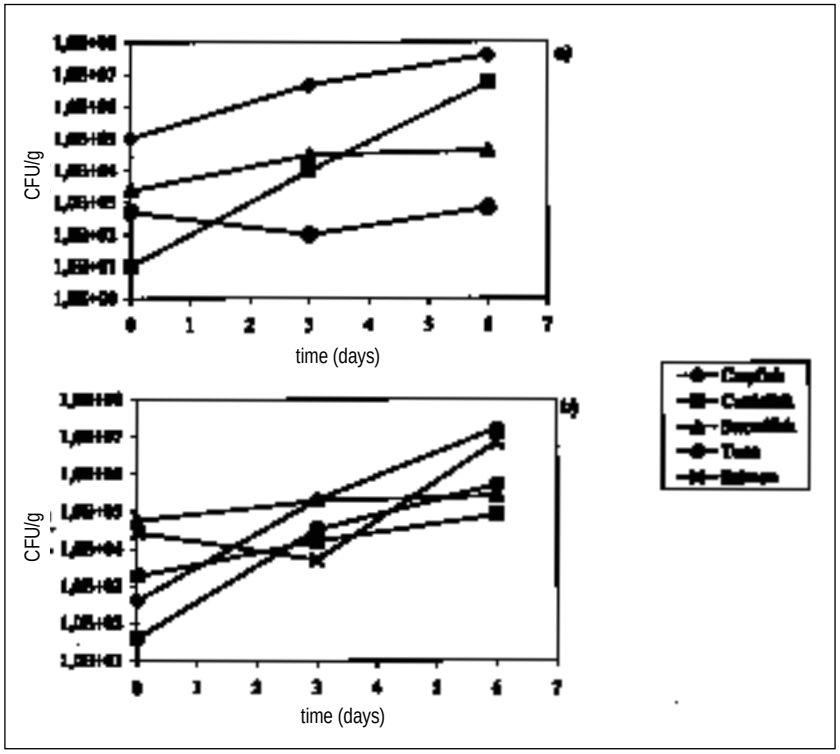

FIG. 3 – Evolution of H_2S producing bacteria (a) and lactic acid bacteria (b) during storage of seafood under MAP2. H_2S producing bacteria were not found in salmon.

The genus *Carnobacterium*, was the most frequently found, however its presence was more abundant in MAP1 products. In tuna in MAP1, *Carnobacterium divergens* represented 100% of lactic acid population, while in MAP2 *Lactobacillus* genus was prevalent (Fig. 6a). In shrimps in MAP1 the genus *Carnobacterium* was almost equally subdivided between *C. divergens* e *Carnobacterium piscicola* (Fig. 6b), while in MAP2 the presence of *Carnobacterium* decreased and *Lactobacillus* spp. was present. In swordfish *C. divergens* was dominant in both atmospheres; in MAP1 *Lactobacillus* spp. was found, while in MAP2 this genus was substituted with *Leuconostoc* spp. (Fig. 6c)**.** In cuttlefish and salmon under both atmospheres, cocci were prevalent: they produced gas from glucose, they were negative to the arginine test and produced only D(-)lactic acid; they grown at 10 °C but not at 40 °C and were included in the genus *Leuconostoc*.

In conclusion genus *Carnobacterium* was dominant in MAP1 where the more acid environment of MAP2, due to the solubilization of CO_2 into seafood texture, realizes more favourable conditions to *Lactobacillus*, while *Carnobacterium* becomes trascurable.

The new set of primers used to identify *Carnobacterium* at the genus level is more selective and specific than the set proposed by Scarpellini *et al.* (2002). We

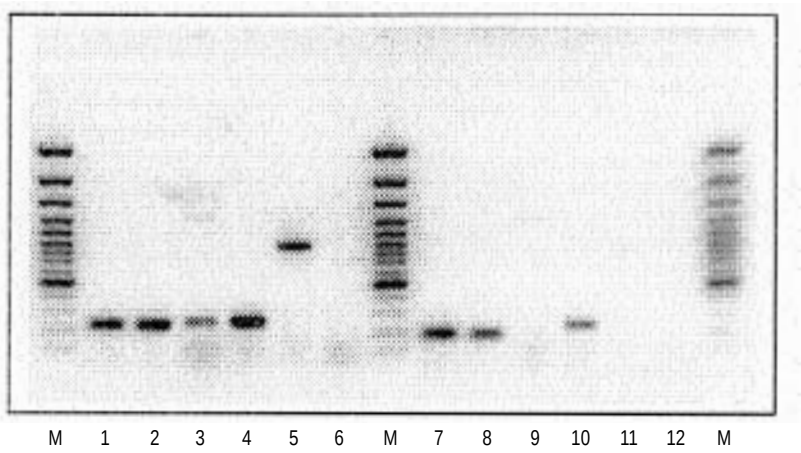

FIG. 4 – PCR products from *C. divergens, C. piscicola, L. plantarun, L. brevis, E. coli, S. thermophilus,* obtained using set primer 16SF-Carno1-4R (lane 1-6) and -Carno1-2F-Carno1-4R (lane 7-12) Lane 1, 7, *C. divergens* DSM 20623; lane 2, 8, *C. piscicola* DSM 20722T; lane 3, 9, *Lactobacillus plantarum* ATCC 10241, lane 4, 10, *Lactobacillus brevis* CNRZ 214; lane 5, 11, *Escherichia coli* NCTC 12079; lane 6, 12, *Streptococcos thermophilus* DSM 20617. M = DNA molecular weight marker 100bp ladder (MBI Fermentas).

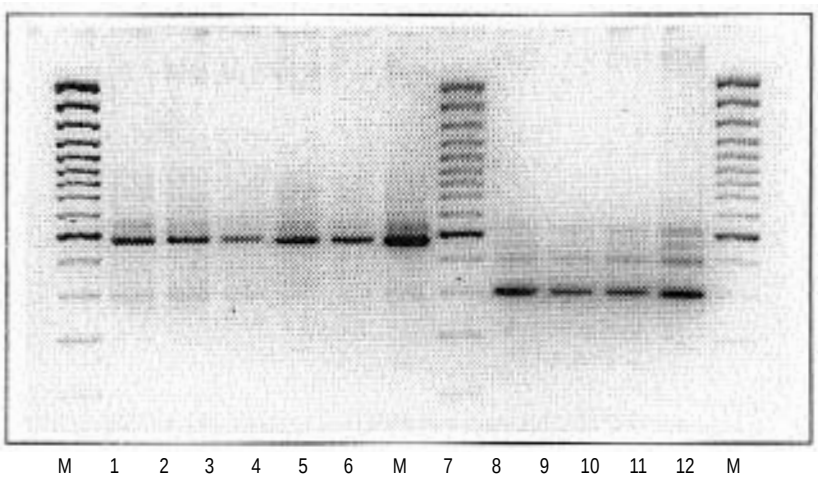

FIG. 5 – 16S-23S rDNA spacer amplification pattern of *C. piscicola, C. divergens*. Lane 1, *C. piscicola* DSM 20722^{T}; lane 2, G16; lane 3, SC6; lane 4, P1; lane 5, N2, lane 6, SO2; lane 7; *C. divergens* DSM4847 ^{T}; lane 8, G15; lane 9, SP10; lane 10, T11. M = DNA molecular weight marker 100bp ladder (MBI Fermentas).

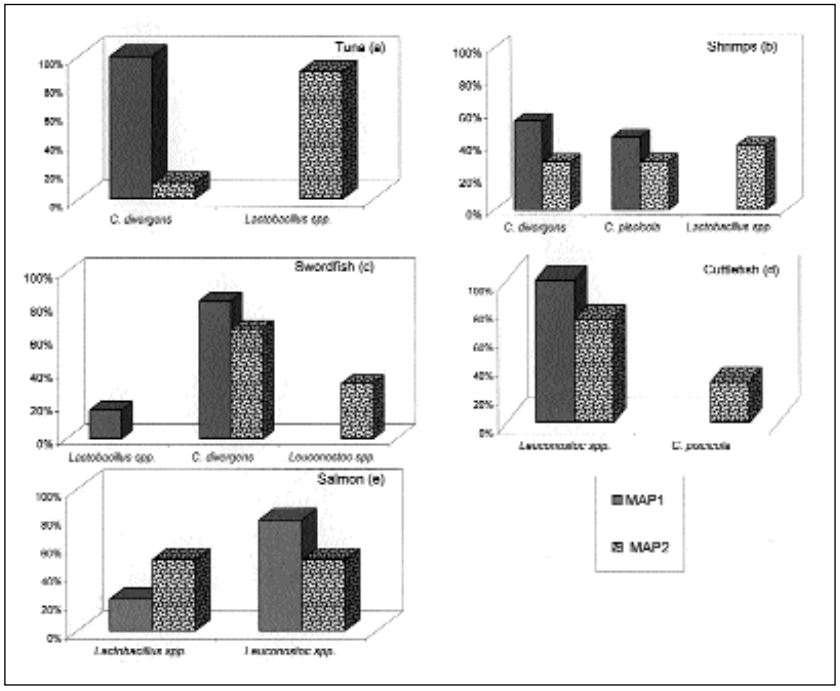

FIG. 6 – Lactic acid population found on seafood packaged in modified atmosphere (MAP1 and MAP2) and stored at 4 °C for 6 days.

therefore suggest the use of CarnoF1 and CarnoF2 as forward primers instead of 16SF universal forward primer, in order to eliminate possible false positive, during the screening for the detection of the genus *Carnobacterium* with PCR method.

For the identification of carnobacteria at the species level the amplification of intergenic spacer between 16S and 23S rDNA was found to be exhaustive and can therefore be used for this scope as already suggested by Scarpellini *et al*. (2002).

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