Microbiological quality of ready-to-eat pickled fish products

Short title: **Bacteriological quality of RTE products**

Abstract

The bacteriological quality of 18 commercially available ready-to-eat fish products containing *Engraulidae* was evaluated through application of the corresponding ISO procedures for total mesophilic aerobic microbial counts, detection and enumeration of *Enterobacteriaceae* and detection of *Staphylococcus* spp. All isolates were identified to the species level using two different biochemical methods as the API® test and the Biolog® identification system. The most commonly occurring contaminants found were *Enterobacteriaceae* like *Citrobacter freundii* and other *Citrobacter* species, *Enterobacter cloacae, Cronobacter sakazakii, Hafnia alvei, Pantoea*, *Proteus ssp.* and *Escherichia coli.* In terms of total enterobacterial counts 3 products were considered not apt for consumption.

Keywords

Ready-to-eat fish products, *Engraulidae*, bacteriological quality, *Enterobacteriaceae*, opportunistic pathogens

1. Introduction

In modern intensive everyday life the availability, quality and safety of ready-to-eat (RTE) foods has been increasingly gaining importance. Novel and attractive preparations are constantly provided to the market and restaurant sector, products which require detailed study of the microflora present as well as strict safety control. According to the European legislation

(Commission Regulation No 2073/2005 and its modification No 1441/2007) "ready-to-eat" food means food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level the microorganisms of concern. RTE food safety control implies the application of internationally validated analytical protocols (corresponding ISO procedures) for detection and quantification of indicator and pathogenic microorganisms as according to the microbiological criteria fixed in the current national and European legislations (Commission Regulations No 2073/2005 and No 1441/2007). Most commonly adopted indicators include coliforms, *Enterobacteriaceae*, total viable counts, though this criterion is rather general and gives no detailed information on the identity of the microflora present, coagulase positive Staphylococci and pathogenic microorganisms like *Salmonella* and *Listeria monocytogenes*. In the case of RTE products elaborated with fish and/or seafood the only existing legislative criteria from a microbiological point of view are fixed for cooked crustaceans and molluscan shellfish. For products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine, limits are also fixed for the amounts of histamine present in the final product. However, the safety of all RTE foods may be affected by the microbiological quality of the starting materials as well as by the handling, processing, transportation and storage conditions of the finished product before its actual use by the consumer (Angelidis *et al.* 2006).

In the international scientific literature several studies exist on the problematic microbiological quality and characteristics of traditional RTE foods from different countries (Yeboah-Manu *et al.* 2010; Haryani *et al.* 2008; Angelidis *et al.* 2006; Thapa *et al.* 2006; Fang *et al.* 2003). Nine bacterial pathogens were isolated from various RTEs distributed at the University of Ghana campus (Yeboah-Manu *et al.* 2010) namely *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae*, *Streptococcus* sp., *Enterobacter cloacae (E. cloacae)*, *Bacillus* sp.,

Pseudomonas aeruginosa, *Staphilococcus aureus (S. aureus)*, *Enterococcus faecalis* and *Proteus* sp. Moreover, high occurrence of *E. cloacae* (7 strains with proven antibiotic resistance) in several typical Malaysian RTE products sold in the street has been reported (Haryani *et al.* 2008). In a broad study of commercially available traditional Greek RTE foods 3.4 % of the products were unacceptable for consumption due to exceeding pathogen levels of *S. aureus* and *E. coli* while a considerable percentage of samples was found to contain high concentrations of contaminating microflora (Angelidis *et al.* 2006). In a similar research carried out with a wide number of commercial RTEs from central Taiwan incidences as high as 7.9 % for *E. coli*, 49.8 % for *Bacillus cereus* and 17.9 % for *S. aureus* were detected (Fang *et al.* 2003). *Cronobacter* (*Enterobacter sakazakii)*, an opportunistic human pathogen associated mainly with infant infections but also in some cases causative agent of adult infection outbreaks (Lai 2001; Ray *et al.* 2007; See *et al.* 2007; Gosney 2008) has been found in different categories of Swiss ready-to-eat foods other than infant formula (Baumgartner *et al.* 2009).

One of the most typical Spanish RTE fish products, the anchovies in brine or in oil, belongs to the group of the semi-preserved RTE foods, obtained without any heating process to stabilize the end product. These are prepared from fresh *Engraulis* sp. by salting and ripening during prolonged periods of time, usually for more than 3 months. Further on, the ripened fish is desalted, filleted and immersed in brine or oil, with final presentation at the market in glass jars or cans. This end product must be kept chilled and even if manufacturer's storage instructions are respected, proteolytic and other quality changes may occur before consumption as previously demonstrated (Veciana-Nogués *et al.* 1997; Rodriguez-Jerez *et al.* 1994). The desalting and filleting procedures prior to packaging increase the risk of secondary bacterial contamination of the end product. To our knowledge there are not many works

published on the diversity of enterobacteria and their evolution upon refrigerated storage in this type of marketed Spanish traditional RTE.

The aim of our study was to investigate the microbiological quality and the evolution of the enterobacterial content upon storage of different *Engraulis* based ready-to-eat products from the Spanish market. The final objective was to evaluate the existing risks for the consumer's health in the selected products thus providing important information to the manufacturers of this type of fish foods since currently there are no fixed legal limits for microbiological indicators. European legislation in vigour (Commission Regulations No 2073/2005 and No 1441/2007) allows manufacturers to establish their proper control limits depending on the specific production processes and in function of their Hazard Analysis and Critical Control Points (HACCP) programmes.

2. Materials and Methods

2.1. Food samples

Eighteen samples of ready-to-eat foods prepared with *Engraulidae* were purchased in Spanish retailers and supermarkets between December 2009 and April 2010 (3 products per batch from each commercial brand). These comprised 11 samples of anchovies in olive oil, 2 samples of anchovies in sunflower oil and 3 sample of anchovies in another type of vegetable oil, one sample of snacks of anchovies with onion, red peppers and olives (presented immersed in oil) and one sample of snacks of *Engraulis* with onion, red peppers and olives (also immersed in vegetable oil). All samples were produced by 14 different manufacturers, some wearing brands well known to the Spanish consumer.

2.2. Bacteriological analysis of ready-to-eat foods

The analysis of the commercial samples was done according to the following ISO procedures: ISO 4833:2003, ISO 21528-1:2004 and ISO 6888-3:2003, taking into account the ISO 7218:2007. The enrichment step for the initial detection and isolation of *Enterobacteriaceae* in all samples analyzed was done in tubes with 9 ml Buffered Peptone Water (Biokar Diagnostics, Beauvais, France) inoculated with 1 g from the respective sample. After 24h incubation at 37ºC 1ml from each of these tubes was passed to a tube with 10 ml E.E. Broth (Oxoid Ltd., Basingstoke, UK). These tubes were further incubated for 24h at 37ºC and then used for the inoculation of three plates per sample of Violet Red Bile Glucose Agar (VRBG) (Cultimed, Panreac, Castellar del Valles, Spain). Colonies with different morphologies observed on VRBG were then isolated separately by passing to plates with Nutrient Agar at 2% (Biokar Diagnostics, Beauvais, France) containing 5 g/l NaCl. A biochemical confirmation was done of the ability of each pure isolate to ferment glucose in tubes with 10 ml of Dextrose Tryptone Yeast Bromocresol Agar (Glucose agar) (Laboratorios Microkit, Madrid, Spain). The change of the colour to yellow at the end of the incubation time (24h ± 2 at 37ºC) was considered as positive result. The oxidase reaction of each isolate was also verified by applying a drop from the Oxydase reagent (BioMerieux SA, Marcy l'Etoile, France) on a single colony material as according to the instructions of the reagent's producer.

2.2.1. Definition of Total Viable Counts

For the enumeration of the TVCs 5 g per product were weighed from the corresponding sample in stomacher bags Fbag-03 (AES Chemunex SA, Barcelona, Spain) in aseptic conditions. The samples were then diluted with 45 ml of sterile Buffered Peptone Water (BPW) (Biokar Diagnostics, Beauvais, France) and homogenized during 30 sec. in a Laboratory Blender Stomacher 400 (Seward Ltd., West Sussex, UK). Serial ten-fold dilutions up to 10^{-7} were prepared per sample in tubes with 9 ml BPW. Two Petri dishes with agar were inoculated from each dilution. In the case of aerobic mesophiles the agar medium applied was

Plate Count Agar (PCA) (Biokar Diagnostics, Beauvais, France), with a second layer of Bacteriological agar type E at 18 g/l (Biokar Diagnostics, Beauvais, France). For the enumeration of the anaerobic mesophilic microflora agar Schaedler (Biokar Diagnostics, Beauvais, France) with defibrinated sheep blood at 5 % (E&O Laboratories Ltd., UK) was used. Aerobic TVC were cultivated for 72 h at 30 ± 1 ºC and anaerobic TVC – at 37 ± 1 ºC for 72 h at anaerobic conditions, assured by the GENbag anaer system (BioMerieux SA, Marcy l'Etoile, France). The different morphologies obtained on the TVC agar plates were further isolated on Tryptone Soy Agar (TSA) (Biokar Diagnostics, Beauvais, France) and Nutrient Agar at 2 % (Biokar Diagnostics, Beauvais, France) for Gram staining and identification.

2.2.2. Definition of the Total Enterobacterial Counts

For the enumeration of the total enterobacterial counts the samples from the same batch and brand were opened, mixed and 25 g per product were weighted in TEMPO® bags (BioMerieux SA, Marcy l'Etoile, France) in aseptic conditions. These were diluted with 225 ml of sterile Tryptone-Salt broth (Biokar Diagnostics, Beauvais, France) and homogenized during 30 sec. in our Laboratory Blender Stomacher 400. Further on this material was used for the inoculation of TEMPO®EB cards (BioMerieux SA, Marcy l'Etoile, France) in the TEMPO® preparation station as according to the manufacturer's instructions. The cards were incubated during 24 h at 35 ± 1 ºC in an incubator and then results were read and interpreted with the TEMPO® Reader station and associated software. This method represents a miniaturized MPN assay.

2.3. Identification of the isolates

Gram staining was done for all pure enterobacterial and mesophillic isolates. Catalase reaction was tested for the TVC isolates on single colony material by treatment with a

hydrogen peroxide (H_2O_2) solution at 3 %. All cultures were then identified to the species level by the application of API® test and Biolog® identification systems.

2.3.1. Identification by API® tests

Fresh (24 h) colonies of all enterobacterial isolates were homogenized in sterile distilled water and inoculated into API 20E galleries (BioMerieux SA, Marcy l'Etoile, France) following the instructions of the manufacturer. For some TVC isolates identification was done with the API 20A (Anaerobes) and API 50CHB (*Bacillus*) galleries, where applicable, as in accordance with the list of genera/species included in the identification range of the corresponding type of gallery. Staphylococcal isolates were identified by the API Staph test. Results were expressed in percentages of probability for the identification as according to the ApiWeb software.

2.3.2. Biolog® Identification

In parallel, the Biolog® MicroStation system and related software (Biolog, USA) were used for the identification of all enterobacterial and TVC isolates obtained. For the purpose the pure isolates were inoculated on TSA plates prior to Biolog® analysis and incubated at the corresponding conditions for each microbial group. Anaerobiosis was applied in the case of the anaerobic TVC isolates using the GENbag anaer system. The cultures were consequently resuspended in tubes with 10 ml of GN/GP-IF inoculation fluid (Biolog, USA) using the Biolog® turbidity standards and with addition of Biolog® sodium thioglycolate reagent as according to the instructions of the manufacturer. These tubes were used for the inoculation of Biolog® GN2 plates in the case of Gram negative isolates and Biolog® GP2 plates in the case of Gram positive isolates. All plates were inoculated during 24 h at the cultivation temperatures required (37 ± 1 °C for Enterobacteria and 30 °C for mesophiles) and then results were read in the Biolog® MicroStation plate reader. Interpretation of results was done with the MicroLog3 4.20.04 software and these were expressed in percentages of probability

for the identification or in their corresponding similarity index values (SIM) whenever less than 0.5 SIM was obtained for the identification. According to the instructions of the manufacturer a value close to 0.5 means high probability of identification and to each SIM higher than 0.5 a probability percentage corresponds.

2.4. Shelf life study

Thirteen commercial products of anchovies in oil were selected at random for the shelf life study in refrigeration, at 4 - 8 °C, storage conditions adequate for this type of semi-preserves (products stabilized for a limited period by appropriate treatment and sealed in containers, light tight under normal pressure; or not sealed in containers, the shelf life being extended by chill storage). TVC, total enterobacterial counts by TEMPO® and the qualitative enterobacterial content of the selected products were followed at three-month intervals up to 9 months of refrigerated storage. Thus, four experimental points were done – right after the acquisition of the products (time 0) and at 3, 6 and 9 months of refrigerated storage.

2.5. Definition of the histamine content

Histamine content was measured by HPLC reverse phase chromatography as according to Vieites and colleagues (Ben-Gigirey *et al.* 1999). 4 g of homogenized sample were mixed with 20 ml perchloric acid 0.4 M, centrifuged for 20 minutes at 4000 rpm, and filtered. 1 ml of the extracted sample was derivatized by adding 200 µl NaOH 2 N, 300 µl sodium bicarbonate (saturated solution) and 2 ml dansyl chloride. Tubes were incubated for 45 minutes at 46 ºC. Then dansyl chloride residues were removed with 100 µl ammonia, centrifuged 5 minutes at 4000 rpm, and filtered. Derivatized samples were injected in an HPLC/PDA system (Waters Corporation, MA, USA), using a Nucleosil C18 5 µm reverse phase column and monitored at λ = 254 mn. Histamine dihydrochloride, mínimum 99 % purity, was purchased by SIGMA-ALDRICH and was used as standard. The detection

limit of this method is 10 mg histamine/ kg. Three of the products from the shelf life study that showed highest enterobacterial counts and provided highest number of enterobacterial isolates were selected for analysis by this method after 9 months of storage in refrigeration.

3. Results

3.1. Bacteriological analysis of ready-to-eat foods

Table 1 presents all food products examined as according to their covering sauce along with the total enterobacterial counts and the corresponding identification of the isolates obtained on selective media (E.E. broth, VRBG) after enrichment. Seven out of the 18 products (approx. 39 %) did not contain any Enterobacteria, among these four products of anchovies in olive oil, one product of anchovies in vegetable oil and two products of anchovy/ *Engraulis* spp. snacks. From the rest of the products a total of 30 different Gram-negative, oxidase negative isolates were obtained. Although in most positive samples enterobacteria were present in amounts below the limit of detection of the TEMPO® method (10 cfu/g), 3 samples showed significant enterobacterial counts (640, 33 and 21 cfu/g). The sample with highest enterobacterial counts showed highest number of different isolates. The prevailing genera among all isolates were *Enterobacter* (represented by 8 isolates), *Citrobacter* (7 isolates), *Proteus* (3 isolates), *Cronobacter* (*Enterobacter sakazakii*) (3 isolates), *Hafnia* (2 isolates) and *Escherichia* (2 isolates, among which one identified as *E. coli* O157:H7). *Klebsiella*, *Serratia*, *Raoultella* and *Pantoea* were represented by single species each. *Citrobacter freundii* was the most common Enterobacterial contaminant of the studied products. *Cronobacter sakazakii* was detected in 3 products.

TVC related isolates, including some of the staphylococci, obtained on selective media from the studied products are shown in Table 2. These all were Gram-positive, both catalase

positive or negative. In contrast to enterobacterial counts TVC were present in all the samples; however, in 5 products (all anchovies in olive oil, approx. 28 % of the total) only lactic acid bacteria (LAB) were found, namely *Pediococcus pentosaceus*, a species renowned for its probiotic properties (Osmanagaoglu *et al.* 2010; Semjonovs & Zikmanis 2008). In the rest of the products the contaminating (non-LAB) microflora was more important in quantities. The mesophillic aerobic isolates included several representatives of the coagulase negative Staphylococci (among which *Staphylococcus xylosus*), *Cellulomonas*, *Rhodococcus*, *Bacillus* and *Corynebacterium*. Among the anaerobic isolates *Desulfomonile tiedjei* and *Fusobacterium mortiferum* were detected. Certain *Actinomyces* isolates were also found in three of the products as identified by both API and Biolog.

3.2. Comparison of the methods for identification of the isolates

Both methods used by us for the identification of the isolates were based on biochemical characterization of each microorganism's carbohydrate metabolism. The Biolog system implies testing for 95 different carbohydrates while the API system is in general more limited in the number of substrates analysed, depending on outreach of each gallery. Therefore, the API system has a more limited database for comparison and certain species are excluded from possible identification as stated in the instruction manuals of the corresponding galleries. We have tried to apply both systems for each isolate, whenever available, in order to reach better identification. In most of the cases the results obtained by the two methods were in good concordance, however, certain discrepancies existed for some of the enterobacterial isolates. In such cases the method providing higher identification probability was selected and included as final identification result in Tables 1 and 2. The identification probabilities given by the Biolog system were very often expressed in similarity index values since no sufficient growth was reached during the maximum incubation period advised by the manufacturer (24

h for most microbial groups), thus, no percentage of identification was obtained by using this method.

3.3. Shelf life study

The shelf life study concerning microbiological parameters was realized on thirteen products designated by numbers 4 - 16 in Tables 1 and 2. The initial enterobacterial counts and total number of enterobacterial isolates at time 0 were highest, followed by a prominent decrease after 3 months of storage. At the experimental point of 6 months no enterobacterial counts were found in any of the tested products and only two isolates were obtained after enrichment from the product with highest initial counts (640 cfu/g) (Fig. 1). No enterobacterial counts /isolates were obtained after 9 months of storage. The most persistent species at refrigeration conditions belonged to the genera *Enterobacter* and *Pantoea* (data not shown). *Cronobacter* (*Enterobacter sakazakii*) had generally poor viability under the selected conditions. The same reduction in counts was not observed for the TVC. The number of TVC isolates increased for most samples with the increase of the storage period (Fig. 2). The catalase negative staphylococcal isolates (*Staph. lentus, Staph. warneri, Staph. saprophyticus, Staph. arlettae, etc.*) were the most resistant group at the selected storage conditions. The histamine quantities detected (two values below the detection limit of the method of 10 mg/kg and one value of 84 ± 22 mg/kg) in the samples analyzed were below the officially allowed limit for histamine (200 mg/kg) in products manufactured from fish species associated with high histidine amounts (Commission Regulations No 2073/2005 and No1441/2007).

4. Discussion

In this report 18 RTE food products were included, selected and acquired after a preliminary market study. Semi-preserves elaborated with *Engraulidae* and presented in various types of

covering sauces are highly appreciated by local and international consumers; therefore, their systematic safety control is of great relevance to avoid public health risks. After application of the established ISO procedures for the detection and quantification of indicator and pathogenic microorganisms or alternative validated methods (TEMPO), presence of certain opportunistic or obligate bacterial pathogens, mainly belonging to the *Enterobacteriaceae* family, was confirmed in 61 % of the foods sampled. Three of the products (17 %) could be considered unsafe for consumption due to their high enterobacterial counts. Thirty different enterobacterial species were isolated from all products analyzed.

Enterobacteriaceae are generally accepted as the primary cause of food spoilage and histamine development in different food products. Species like *Citrobacter freundii*, found to be one of the predominant enterobacterial isolates in the current research work, together with *Morganella morganii*, *Proteus* spp., *Enterobacter* spp., *Klebsiella pneumoniae*, *Escherichia* spp., *Hafnia alvei*, *Raoultella* spp. and *Serratia* spp. have previously been reported as biogenic amine producers in various foods – tuna sandwiches (Kung *et al.* 2009), packaged and processed meat (Durlu - Özkaya *et al.* 2001), seafood (Kim *et al*. 2003), salted mackerel (Tsai *et al.* 2005), etc., causing indigestion, intoxications and food allergies. *Morganella*, *Citrobacter*, *Proteus* spp., *Klebsiella pneumoniae* and *Hafnia alvei* have been known for their important histamine production while *Enterobacter amnigenus* and *Enterobacter cloacae* have been shown to produce particularly high amounts of putrescine (Lavizzari *et al*. 2010). Although we have not isolated any *Morganella morganii* or *Klebsiella pneumoniae* species from *Engraulis* spp. containing RTE foods we have identified several isolates with known histidine decarboxilase activities as *Proteus penneri*, *Proteus mirabilis*, *Proteus vulgaris, Pantoea* spp., *Raoultella planticola/ ornithinolytica, Raoultella terrigena* and *Serratia ficaria.* The presence of these microorganisms could suppose certain degree of filet softening, proteolysis, histamine accumulation and spoilage during the storage of the sampled foods.

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Keywords

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1. Introduction

In modern intensive everyday life the availability, quality and safety of ready-to-eat (RTE) foods has been increasingly gaining importance. Novel and attractive preparations are constantly provided to the market and restaurant sector, products which require detailed study of the microflora present as well as strict safety control. According to the European legislation

(Commission Regulation No 2073/2005 and its modification No 1441/2007) "ready-to-eat" food means food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level the microorganisms of concern. RTE food safety control implies the application of internationally validated analytical protocols (corresponding ISO procedures) for detection and quantification of indicator and pathogenic microorganisms as according to the microbiological criteria fixed in the current national and European legislations (Commission Regulations No 2073/2005 and No 1441/2007). Most commonly adopted indicators include coliforms, *Enterobacteriaceae*, total viable counts, though this criterion is rather general and gives no detailed information on the identity of the microflora present, coagulase positive Staphylococci and pathogenic microorganisms like *Salmonella* and *Listeria monocytogenes*. In the case of RTE products elaborated with fish and/or seafood the only existing legislative criteria from a microbiological point of view are fixed for cooked crustaceans and molluscan shellfish. For products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine, limits are also fixed for the amounts of histamine present in the final product. However, the safety of all RTE foods may be affected by the microbiological quality of the starting materials as well as by the handling, processing, transportation and storage conditions of the finished product before its actual use by the consumer (Angelidis *et al.* 2006).

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Pseudomonas aeruginosa, *Staphilococcus aureus (S. aureus)*, *Enterococcus faecalis* and *Proteus* sp. Moreover, high occurrence of *E. cloacae* (7 strains with proven antibiotic resistance) in several typical Malaysian RTE products sold in the street has been reported (Haryani *et al.* 2008). In a broad study of commercially available traditional Greek RTE foods 3.4 % of the products were unacceptable for consumption due to exceeding pathogen levels of *S. aureus* and *E. coli* while a considerable percentage of samples was found to contain high concentrations of contaminating microflora (Angelidis *et al.* 2006). In a similar research carried out with a wide number of commercial RTEs from central Taiwan incidences as high as 7.9 % for *E. coli*, 49.8 % for *Bacillus cereus* and 17.9 % for *S. aureus* were detected (Fang *et al.* 2003). *Cronobacter* (*Enterobacter sakazakii)*, an opportunistic human pathogen associated mainly with infant infections but also in some cases causative agent of adult infection outbreaks (Lai 2001; Ray *et al.* 2007; See *et al.* 2007; Gosney 2008) has been found in different categories of Swiss ready-to-eat foods other than infant formula (Baumgartner *et al.* 2009).

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hydrogen peroxide (H_2O_2) solution at 3 %. All cultures were then identified to the species level by the application of API® test and Biolog® identification systems.

2.3.1. Identification by API® tests

Fresh (24 h) colonies of all enterobacterial isolates were homogenized in sterile distilled water and inoculated into API 20E galleries (BioMerieux SA, Marcy l'Etoile, France) following the instructions of the manufacturer. For some TVC isolates identification was done with the API 20A (Anaerobes) and API 50CHB (*Bacillus*) galleries, where applicable, as in accordance with the list of genera/species included in the identification range of the corresponding type of gallery. Staphylococcal isolates were identified by the API Staph test. Results were expressed in percentages of probability for the identification as according to the ApiWeb software.

2.3.2. Biolog® Identification

In parallel, the Biolog® MicroStation system and related software (Biolog, USA) were used for the identification of all enterobacterial and TVC isolates obtained. For the purpose the pure isolates were inoculated on TSA plates prior to Biolog® analysis and incubated at the corresponding conditions for each microbial group. Anaerobiosis was applied in the case of the anaerobic TVC isolates using the GENbag anaer system. The cultures were consequently resuspended in tubes with 10 ml of GN/GP-IF inoculation fluid (Biolog, USA) using the Biolog® turbidity standards and with addition of Biolog® sodium thioglycolate reagent as according to the instructions of the manufacturer. These tubes were used for the inoculation of Biolog® GN2 plates in the case of Gram negative isolates and Biolog® GP2 plates in the case of Gram positive isolates. All plates were inoculated during 24 h at the cultivation temperatures required (37 ± 1 °C for Enterobacteria and 30 °C for mesophiles) and then results were read in the Biolog® MicroStation plate reader. Interpretation of results was done with the MicroLog3 4.20.04 software and these were expressed in percentages of probability

for the identification or in their corresponding similarity index values (SIM) whenever less than 0.5 SIM was obtained for the identification. According to the instructions of the manufacturer a value close to 0.5 means high probability of identification and to each SIM higher than 0.5 a probability percentage corresponds.

2.4. Shelf life study

Thirteen commercial products of anchovies in oil were selected at random for the shelf life study in refrigeration, at 4 - 8 °C, storage conditions adequate for this type of semi-preserves (products stabilized for a limited period by appropriate treatment and sealed in containers, light tight under normal pressure; or not sealed in containers, the shelf life being extended by chill storage). TVC, total enterobacterial counts by TEMPO® and the qualitative enterobacterial content of the selected products were followed at three-month intervals up to 9 months of refrigerated storage. Thus, four experimental points were done – right after the acquisition of the products (time 0) and at 3, 6 and 9 months of refrigerated storage.

2.5. Definition of the histamine content

Histamine content was measured by HPLC reverse phase chromatography as according to Vieites and colleagues (Ben-Gigirey *et al.* 1999). 4 g of homogenized sample were mixed with 20 ml perchloric acid 0.4 M, centrifuged for 20 minutes at 4000 rpm, and filtered. 1 ml of the extracted sample was derivatized by adding 200 µl NaOH 2 N, 300 µl sodium bicarbonate (saturated solution) and 2 ml dansyl chloride. Tubes were incubated for 45 minutes at 46 ºC. Then dansyl chloride residues were removed with 100 µl ammonia, centrifuged 5 minutes at 4000 rpm, and filtered. Derivatized samples were injected in an HPLC/PDA system (Waters Corporation, MA, USA), using a Nucleosil C18 5 µm reverse phase column and monitored at λ = 254 mn. Histamine dihydrochloride, mínimum 99 % purity, was purchased by SIGMA-ALDRICH and was used as standard. The detection

limit of this method is 10 mg histamine/ kg. Three of the products from the shelf life study that showed highest enterobacterial counts and provided highest number of enterobacterial isolates were selected for analysis by this method after 9 months of storage in refrigeration.

3. Results

3.1. Bacteriological analysis of ready-to-eat foods

Table 1 presents all food products examined as according to their covering sauce along with the total enterobacterial counts and the corresponding identification of the isolates obtained on selective media (E.E. broth, VRBG) after enrichment. Seven out of the 18 products (approx. 39 %) did not contain any Enterobacteria, among these four products of anchovies in olive oil, one product of anchovies in vegetable oil and two products of anchovy/ *Engraulis* spp. snacks. From the rest of the products a total of 30 different Gram-negative, oxidase negative isolates were obtained. Although in most positive samples enterobacteria were present in amounts below the limit of detection of the TEMPO® method (10 cfu/g), 3 samples showed significant enterobacterial counts (640, 33 and 21 cfu/g). The sample with highest enterobacterial counts showed highest number of different isolates. The prevailing genera among all isolates were *Enterobacter* (represented by 8 isolates), *Citrobacter* (7 isolates), *Proteus* (3 isolates), *Cronobacter* (*Enterobacter sakazakii*) (3 isolates), *Hafnia* (2 isolates) and *Escherichia* (2 isolates, among which one identified as *E. coli* O157:H7). *Klebsiella*, *Serratia*, *Raoultella* and *Pantoea* were represented by single species each. *Citrobacter freundii* was the most common Enterobacterial contaminant of the studied products. *Cronobacter sakazakii* was detected in 3 products.

TVC related isolates, including some of the staphylococci, obtained on selective media from the studied products are shown in Table 2. These all were Gram-positive, both catalase

positive or negative. In contrast to enterobacterial counts TVC were present in all the samples; however, in 5 products (all anchovies in olive oil, approx. 28 % of the total) only lactic acid bacteria (LAB) were found, namely *Pediococcus pentosaceus*, a species renowned for its probiotic properties (Osmanagaoglu *et al.* 2010; Semjonovs & Zikmanis 2008). In the rest of the products the contaminating (non-LAB) microflora was more important in quantities. The mesophillic aerobic isolates included several representatives of the coagulase negative Staphylococci (among which *Staphylococcus xylosus*), *Cellulomonas*, *Rhodococcus*, *Bacillus* and *Corynebacterium*. Among the anaerobic isolates *Desulfomonile tiedjei* and *Fusobacterium mortiferum* were detected. Certain *Actinomyces* isolates were also found in three of the products as identified by both API and Biolog.

3.2. Comparison of the methods for identification of the isolates

Both methods used by us for the identification of the isolates were based on biochemical characterization of each microorganism's carbohydrate metabolism. The Biolog system implies testing for 95 different carbohydrates while the API system is in general more limited in the number of substrates analysed, depending on outreach of each gallery. Therefore, the API system has a more limited database for comparison and certain species are excluded from possible identification as stated in the instruction manuals of the corresponding galleries. We have tried to apply both systems for each isolate, whenever available, in order to reach better identification. In most of the cases the results obtained by the two methods were in good concordance, however, certain discrepancies existed for some of the enterobacterial isolates. In such cases the method providing higher identification probability was selected and included as final identification result in Tables 1 and 2. The identification probabilities given by the Biolog system were very often expressed in similarity index values since no sufficient growth was reached during the maximum incubation period advised by the manufacturer (24

h for most microbial groups), thus, no percentage of identification was obtained by using this method.

3.3. Shelf life study

The shelf life study concerning microbiological parameters was realized on thirteen products designated by numbers 4 - 16 in Tables 1 and 2. The initial enterobacterial counts and total number of enterobacterial isolates at time 0 were highest, followed by a prominent decrease after 3 months of storage. At the experimental point of 6 months no enterobacterial counts were found in any of the tested products and only two isolates were obtained after enrichment from the product with highest initial counts (640 cfu/g) (Fig. 1). No enterobacterial counts /isolates were obtained after 9 months of storage. The most persistent species at refrigeration conditions belonged to the genera *Enterobacter* and *Pantoea* (data not shown). *Cronobacter* (*Enterobacter sakazakii*) had generally poor viability under the selected conditions. The same reduction in counts was not observed for the TVC. The number of TVC isolates increased for most samples with the increase of the storage period (Fig. 2). The catalase negative staphylococcal isolates (*Staph. lentus, Staph. warneri, Staph. saprophyticus, Staph. arlettae, etc.*) were the most resistant group at the selected storage conditions. The histamine quantities detected (two values below the detection limit of the method of 10 mg/kg and one value of 84 ± 22 mg/kg) in the samples analyzed were below the officially allowed limit for histamine (200 mg/kg) in products manufactured from fish species associated with high histidine amounts (Commission Regulations No 2073/2005 and No1441/2007).

4. Discussion

In this report 18 RTE food products were included, selected and acquired after a preliminary market study. Semi-preserves elaborated with *Engraulidae* and presented in various types of

covering sauces are highly appreciated by local and international consumers; therefore, their systematic safety control is of great relevance to avoid public health risks. After application of the established ISO procedures for the detection and quantification of indicator and pathogenic microorganisms or alternative validated methods (TEMPO), presence of certain opportunistic or obligate bacterial pathogens, mainly belonging to the *Enterobacteriaceae* family, was confirmed in 61 % of the foods sampled. Three of the products (17 %) could be considered unsafe for consumption due to their high enterobacterial counts. Thirty different enterobacterial species were isolated from all products analyzed.

Enterobacteriaceae are generally accepted as the primary cause of food spoilage and histamine development in different food products. Species like *Citrobacter freundii*, found to be one of the predominant enterobacterial isolates in the current research work, together with *Morganella morganii*, *Proteus* spp., *Enterobacter* spp., *Klebsiella pneumoniae*, *Escherichia* spp., *Hafnia alvei*, *Raoultella* spp. and *Serratia* spp. have previously been reported as biogenic amine producers in various foods – tuna sandwiches (Kung *et al.* 2009), packaged and processed meat (Durlu - Özkaya *et al.* 2001), seafood (Kim *et al*. 2003), salted mackerel (Tsai *et al.* 2005), etc., causing indigestion, intoxications and food allergies. *Morganella*, *Citrobacter*, *Proteus* spp., *Klebsiella pneumoniae* and *Hafnia alvei* have been known for their important histamine production while *Enterobacter amnigenus* and *Enterobacter cloacae* have been shown to produce particularly high amounts of putrescine (Lavizzari *et al*. 2010). Although we have not isolated any *Morganella morganii* or *Klebsiella pneumoniae* species from *Engraulis* spp. containing RTE foods we have identified several isolates with known histidine decarboxilase activities as *Proteus penneri*, *Proteus mirabilis*, *Proteus vulgaris, Pantoea* spp., *Raoultella planticola/ ornithinolytica, Raoultella terrigena* and *Serratia ficaria.* The presence of these microorganisms could suppose certain degree of filet softening, proteolysis, histamine accumulation and spoilage during the storage of the sampled foods.

Table 1. Identification of all enterobacterial isolates obtained from the products containing *Engraulis* spp.

Covering sauce	Total counts [cfu/g]	Identification	Method	
			API [%]	Biolog ID [%] or SIM†
Olive oil	≤ 10	*Proteus penneri* *Proteus mirabilis*	99.2	 0.367
	≤ 10	*Citrobacter freundii*	99	99
	≤ 10	*E. coli* 1	99	99
	≤ 10	*Enterobacter gergoviae* *Serratia ficaria*	99.8	99.8 0.265
	640	*Enterobacter aerogenes* *Klebsiella oxytoca* *Pantoea* spp. *Enterobacter sakazakii* *Raoultella terrigena*	96	 99 90.5 0.212 99
	≤ 10	*E.coli* O157:H7 *Proteus vulgaris*	 99.9	0.328
	≤ 10	*Enterobacter hormaechei*		88
Vegetable oil	33	*Hafnia alvei* *Citrobacter freundii* *Enterobacter sakazakii*	99.6 84 77.7	
	≤ 10	*Citrobacter spp.* *Citrobacter braakii* *Enterobacter amnigenus*	 77.8 85.4	0.376
Sunflower oil	≤ 10	*Enterobacter amnigenus* *Enterobacter cancerogenus* *Hafnia alvei* *Raoultella planticola/ ornithinolytica*	65.4 85 99.7	 85 99
	21	*Citrobacter freundii* *Enterobacter cloacae*	99	 0.136

†SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system. Good identification by this system is normally considered for SIM index values above 0.3.

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†SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system. Good identification by this system is normally considered for SIM index values above 0.3.

Table 2. Identification of the total viable count (TVC) isolates from the ready-to-eat products analyzed.

Covering sauce	Identification		Method	
	Aerobes	Anaerobes	API [%]	Biolog ID [%] or SIM†
Olive oil	*Pediococcus pentosaceus*	-----		99
	Staphilococcus epidermidis	-----		75
	Cellulomonas flavigena	-----		0.228
	Brevibacterium otitidis *Staphilococcus lentus* *Staphilococcus warneri*	-----	94	0.350 0.410
	Staphilococcus saprophyticus *Staphilococcus arlettae*	-----		0.361 0.789
Vegetable oil	*Staphilococcus lutrae*	-----		0.220
	Staphilococcus equorum *Bacillus psychrosaccharolyticus*	-----		0.310 0.325
	Corynebacterium nitrophilus *Clavibacter agropyri*	-----		0.440 90
Sunflower oil	*Cellulomonas flavigena* *Rhodococcus rhodochrous*	-----		0.370 0.337
	Actinomyces hyovaginalis *Clavibacter agropyri*	-----		0.280 98
Snacks of anchovies	*Tsukumurella inchonensis* *Actinomyces naeslundii* *Staphylococcus xylosus*	*Desulfomonile tiedjei*	60	99 98,5 0.182
Snacks of *Engraulis* spp.	*Tsukumurella inchonensis* *Actinomyces naeslundii* *Cellulomonas cellasea*	*Fusobacterium mortiferum*	60 90.6	0.273 98

†SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system. Good identification by this system is normally considered for SIM index values above 0.3.

Fig. 1.

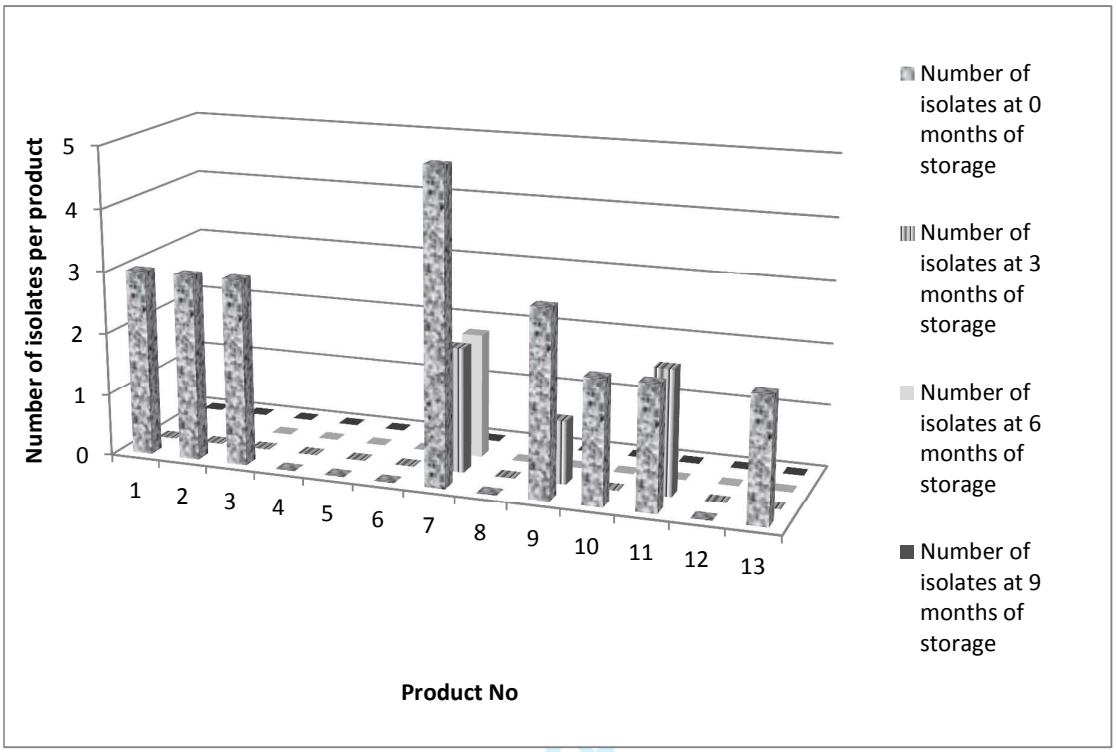
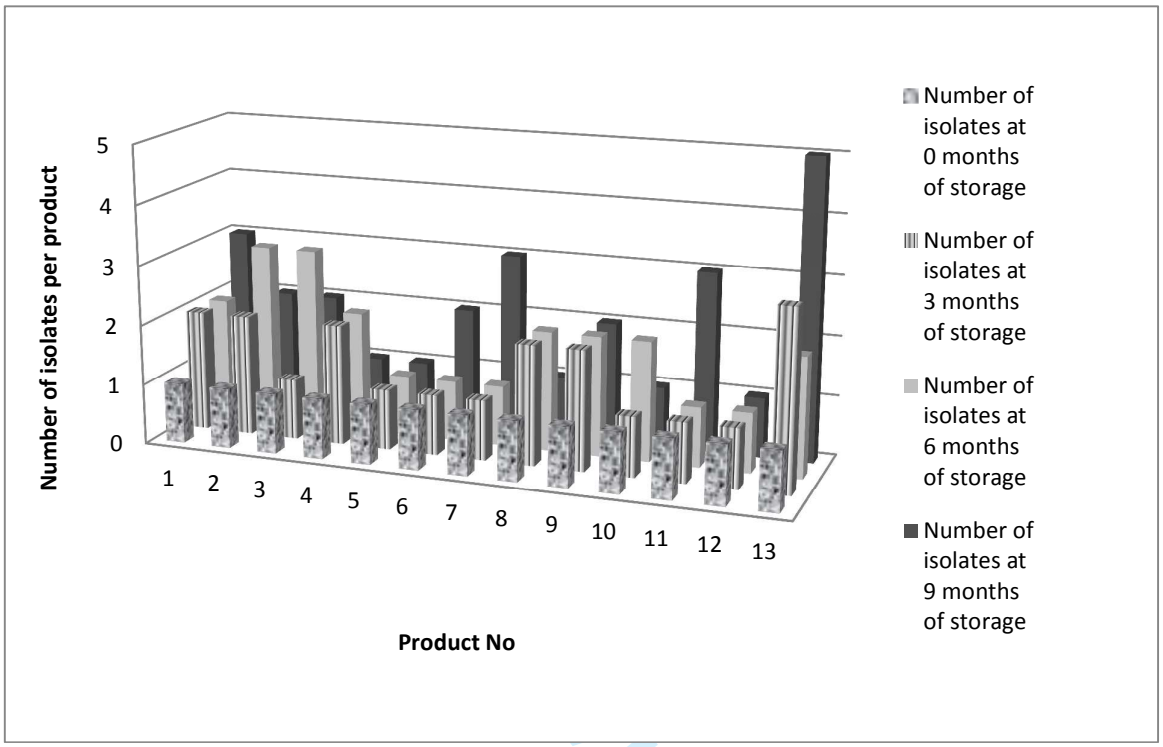

Number of isolates per product
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11
12
13
Product No
Number of isolates at 0 months of storage
Number of isolates at 3 months of storage
Number of isolates at 6 months of storage
Number of isolates at 9 months of storage

Fig. 2.



Therefore, the histamine content of the samples with highest enterobacterial counts and detected presence of known histamine formers as *Hafnia alvei*, *Citrobacter freundii*, *Enterobacter aerogenes, E. amnigenus* and *Raoultella* spp. was checked by analytical chromatography. No dependence between the enterobacterial counts and histamine amount in our samples was established, a result previously seen in other research works (Rodriguez-Jerez *et al.* 1994). Neither were found in the samples analyzed any amounts surpassing the legally fixed histamine levels for this type of foods.

Our results are in good correlation with the enterobacterial species isolated from other RTE and seafood products (Yeboah-Manu *et al.* 2010; Haryani *et al*. 2008; Angelidis *et al*. 2006; Thapa *et al*. 2006; Fang *et al*. 2003; Hsu *et al*. 2009; Rodriguez-Jerez *et al*. 1994). However, to our knowledge there are no many studies published on the isolation and exhaustive identification of the enterobacterial microflora of traditional Spanish anchovies in oil RTE products.

In the current study we have isolated 8 different *Enterobacter* spp. isolates, identified as *Enterobacter cloacae*, *E. gergoviae*, *E. amnigenus, E. aerogenes, E. cancerogenus* and *E. hormaechei. Enterobacter* spp. are generally considered as the sixth most frequent cause of nosocomial infections with antibiotic-resistant strains being reported in literature with increasing frequency (Haryani *et al*. 2008). Due to the high incidences of antibiotic resistance in *E. cloacae* it is one of the few bacteria which by official rules have to be monitored in all healthcare facilities in Europe. Several reports exist on bacteremia and bloodstream infections in hospitalized patients caused by *E. cloacae*, *E. aerogenes* and *Citrobacter freundii* (Deal *et al*. 2007; Chen *et al*. 2009; Badger *et al*. 1999). *Hafnia alvei*, another representative of the *Enterobacteriaceae*, that has been detected in the RTE foods analyzed in this work (2 isolates), has been shown to be predominantly associated with several intestinal disorders,

gastroenteritis, respiratory tract infections, sepsis, peritonitis, urinary infection and other illnesses (Stock *et al*. 2005).

The medical importance and toxin production by enterohemorrhagic *Escherichia coli* strains is well known and has already been widely reviewed (Kawasaki *et al*. 2009; Jothikumar *et al*. 2002). In our study we have reached preliminary identification for two isolates as belonging to *E. coli*, one of which identified by the database of the Biolog MicroStation system as pathogenic *E.coli* O157:H7. Although further confirmation of the *E. coli* identifications is neccessary by the application of the corresponding specific ISO procedures these results are already an indication for posible food-borne illness transmission.

In relation to the other bacterial groups studied the staphylococcal isolates were prevailing among all TVC isolates (26 %, 8 different cultures). No coagulase positive *Staphylococci* were found in any of the food products analyzed. Nevertheless, some of the coagulase negative staphylococcal isolates obtained on selective and non-selective media (BP-RPF, PCA) represented certain potential hazard to consumer health. *Staphylococcus xylosus*, isolated from one of the studied products, has been considered by some medical scientists an emerging human pathogen which can cause nosocomial infections and bacteraemia in hospitalized patients (Esper *et al*. 2000). Among the other mesophillic bacteria identified on the TVC plates *Tsukumurella inchonensis* is a newly defined species that is related to corynebacteria, *Mycobacterium*, *Nocardia* and *Rhodococcus*. It was originally isolated from blood cultures of different clinical cases (Yassin *et al*. 1995) and to our knowledge has not previously been reported in food samples. One isolate was identified as *Corynebacterium nitrophilus* and one as *Rhodococcus rhodochrous,* belonging to the same group of clinically significant microorganisms.

We have not applied any standard protocols for *Listeria monocytogenes* detection since the growth of this pathogen was not supported at the low humidity conditions (35 %, water activity Aw of 0.657) and high content of chlorinated compounds (12.5 %) experimentally defined in the analyzed products (data not shown).

In summary, during the 9-month shelf life study, intended to determine the possible qualitative and quantitative changes in the discovered bacterial microflora at storage conditions recommended by the manufacturers of the selected RTE foods on their respective packaging (approx. 1 year at temperatures between 5 and 12 ºC), we have seen that most of the enterobacterial species present were still viable over a three month storage period with only two viable isolates obtained after 6 months of storage. This tendency was further confirmed at the last point of 9 months of storage when absolutely no counts and no isolates were obtained after the two-step enrichment procedure on selective media. We considered that the observed decline in enterobacterial numbers and loss of viability might be due to the preserving effect of the salt (high content of chlorinated compounds in the samples) and the plant oil dressing. The antimicrobial effect of plant oils has been known for long and the bactericidal action of some essential plant oils on enterobacterial pathogens (*E.coli* and various *Salmonella* species) has previously been demonstrated (Peñalver *et al*. 2005).

Thus, it has been demonstrated that these traditional RTE foods, elaborated with *Engraulis* sp. and preserved under cold storage in salt and oil dressing, are at high risk of being contaminated by enterobacteria and staphylococci. Due to recent legislative changes introduced at EU level the manufacturers need to obtain detailed information on possible microbiological risks in their production lines and to design additional control actions in order to assure the supply of safe and high quality products to the market.

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Figure 1. Evolution of the total number of enterobacterial isolates in the products analyzed during a 9-month shelf life study.

Figure 2. Evolution of the TVC isolates in the products analyzed during a 9-month shelf life study.

Table 1. Identification of all enterobacterial isolates obtained from the products containing *Engraulis* spp.

Covering sauce	Total counts [cfu/g]	Identification	Method	
			API [%]	Biolog ID [%] or SIM†
Olive oil	≤ 10	*Proteus penneri* *Proteus mirabilis*	99.2	 0.367
	≤ 10	*Citrobacter freundii*	99	99
	≤ 10	*E. coli* 1	99	99
	≤ 10	*Enterobacter gergoviae* *Serratia ficaria*	99.8	99.8 0.265
	640	*Enterobacter aerogenes* *Klebsiella oxytoca* *Pantoea* spp. *Enterobacter sakazakii* *Raoultella terrigena*	96	 99 90.5 0.212 99
	≤ 10	*E.coli* O157:H7 *Proteus vulgaris*	 99.9	0.328
	≤ 10	*Enterobacter hormaechei*		88
Vegetable oil	33	*Hafnia alvei* *Citrobacter freundii* *Enterobacter sakazakii*	99.6 84 77.7	
	≤ 10	*Citrobacter spp.* *Citrobacter braakii* *Enterobacter amnigenus*	 77.8 85.4	0.376
Sunflower oil	≤ 10	*Enterobacter amnigenus* *Enterobacter cancerogenus* *Hafnia alvei* *Raoultella planticola/ ornithinolytica*	65.4 85 99.7	 85 99
	21	*Citrobacter freundii* *Enterobacter cloacae*	99	 0.136

†SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system. Good identification by this system is normally considered for SIM index values above 0.3.

Table 2. Identification of the total viable count (TVC) isolates from the ready-to-eat products analyzed.

Covering sauce	Identification		Method	
	Aerobes	Anaerobes	API [%]	Biolog ID [%] or SIM†
Olive oil	*Pediococcus pentosaceus*	-----		99
	Staphilococcus epidermidis	-----		75
	Cellulomonas flavigena	-----		0.228
	Brevibacterium otitidis *Staphilococcus lentus* *Staphilococcus warneri*	-----	94	0.350 0.410
	Staphilococcus saprophyticus *Staphilococcus arlettae*	-----		0.361 0.789
Vegetable oil	*Staphilococcus lutrae*	-----		0.220
	Staphilococcus equorum *Bacillus psychrosaccharolyticus*	-----		0.310 0.325
	Corynebacterium nitrophilus *Clavibacter agropyri*	-----		0.440 90
Sunflower oil	*Cellulomonas flavigena* *Rhodococcus rhodochrous*	-----		0.370 0.337
	Actinomyces hyovaginalis *Clavibacter agropyri*	-----		0.280 98
Snacks of anchovies	*Tsukumurella inchonensis* *Actinomyces naeslundii* *Staphylococcus xylosus*	*Desulfomonile tiedjei*	60	99 98,5 0.182
Snacks of *Engraulis* spp.	*Tsukumurella inchonensis* *Actinomyces naeslundii* *Cellulomonas cellasea*	*Fusobacterium mortiferum*	60 90.6	0.273 98

†SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system. Good identification by this system is normally considered for SIM index values above 0.3.

Fig. 1.

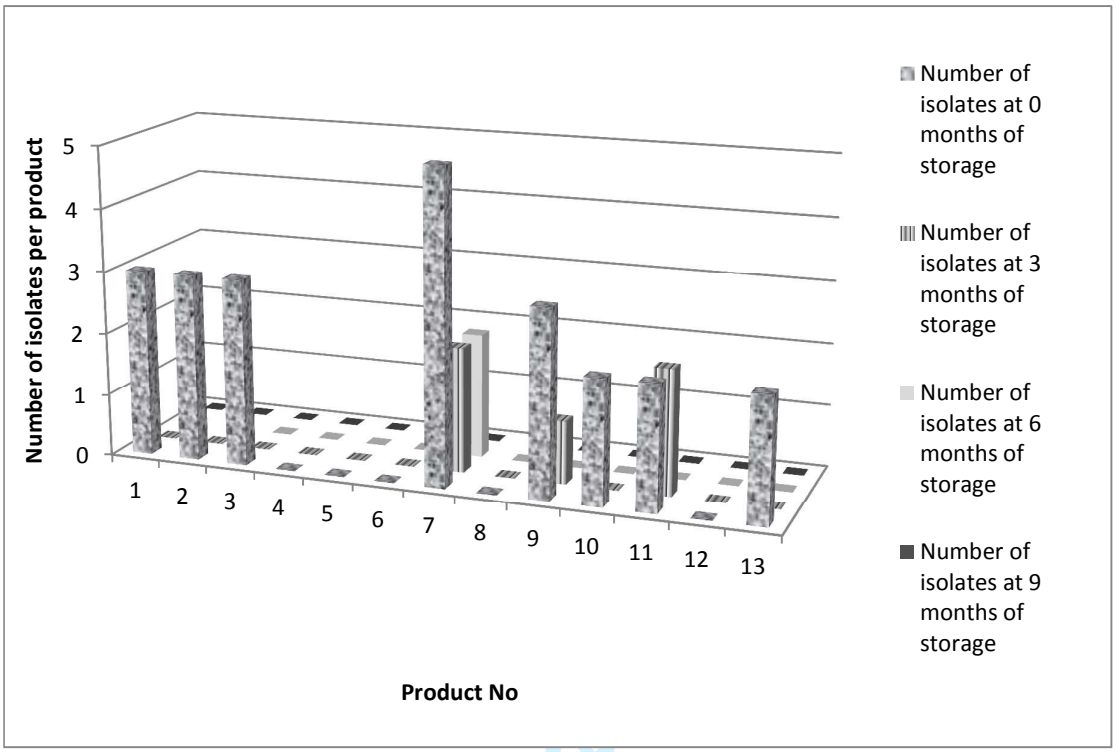
Number of isolates per product
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11
12
13
Product No
Number of isolates at 0 months of storage
Number of isolates at 3 months of storage
Number of isolates at 6 months of storage
Number of isolates at 9 months of storage

Fig. 2.