

# 1 Microbiological quality of ready-to-eat pickled fish products

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2 Short title: Bacteriological quality of RTE products

## 3 Abstract

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

## 14 Keywords

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

## 18 1. Introduction

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

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3 23 (Commission Regulation No 2073/2005 and its modification No 1441/2007) “ready-to-eat”  
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5 24 food means food intended by the producer or the manufacturer for direct human consumption  
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7 25 without the need for cooking or other processing effective to eliminate or reduce to an  
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9 26 acceptable level the microorganisms of concern. RTE food safety control implies the  
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11 27 application of internationally validated analytical protocols (corresponding ISO procedures)  
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13 28 for detection and quantification of indicator and pathogenic microorganisms as according to  
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15 29 the microbiological criteria fixed in the current national and European legislations  
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17 30 (Commission Regulations No 2073/2005 and No 1441/2007). Most commonly adopted  
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19 31 indicators include coliforms, *Enterobacteriaceae*, total viable counts, though this criterion is  
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21 32 rather general and gives no detailed information on the identity of the microflora present,  
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23 33 coagulase positive Staphylococci and pathogenic microorganisms like *Salmonella* and  
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25 34 *Listeria monocytogenes*. In the case of RTE products elaborated with fish and/or seafood the  
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27 35 only existing legislative criteria from a microbiological point of view are fixed for cooked  
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29 36 crustaceans and molluscan shellfish. For products which have undergone enzyme maturation  
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31 37 treatment in brine, manufactured from fish species associated with a high amount of histidine,  
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33 38 limits are also fixed for the amounts of histamine present in the final product. However, the  
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35 39 safety of all RTE foods may be affected by the microbiological quality of the starting  
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37 40 materials as well as by the handling, processing, transportation and storage conditions of the  
38  
39 41 finished product before its actual use by the consumer (Angelidis *et al.* 2006).  
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42 In the international scientific literature several studies exist on the problematic  
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44 43 microbiological quality and characteristics of traditional RTE foods from different countries  
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46 44 (Yeboah-Manu *et al.* 2010; Haryani *et al.* 2008; Angelidis *et al.* 2006; Thapa *et al.* 2006;  
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48 45 Fang *et al.* 2003). Nine bacterial pathogens were isolated from various RTEs distributed at the  
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50 46 University of Ghana campus (Yeboah-Manu *et al.* 2010) namely *Escherichia coli* (*E. coli*),  
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52 47 *Klebsiella pneumoniae*, *Streptococcus* sp., *Enterobacter cloacae* (*E. cloacae*), *Bacillus* sp.,  
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3 48 *Pseudomonas aeruginosa*, *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecalis* and  
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5 49 *Proteus* sp. Moreover, high occurrence of *E. cloacae* (7 strains with proven antibiotic  
6  
7 50 resistance) in several typical Malaysian RTE products sold in the street has been reported  
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9 51 (Haryani *et al.* 2008). In a broad study of commercially available traditional Greek RTE foods  
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11 52 3.4 % of the products were unacceptable for consumption due to exceeding pathogen levels of  
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13 53 *S. aureus* and *E. coli* while a considerable percentage of samples was found to contain high  
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15 54 concentrations of contaminating microflora (Angelidis *et al.* 2006). In a similar research  
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17 55 carried out with a wide number of commercial RTEs from central Taiwan incidences as high  
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19 56 as 7.9 % for *E. coli*, 49.8 % for *Bacillus cereus* and 17.9 % for *S. aureus* were detected (Fang  
20  
21 57 *et al.* 2003). *Cronobacter* (*Enterobacter sakazakii*), an opportunistic human pathogen  
22  
23 58 associated mainly with infant infections but also in some cases causative agent of adult  
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25 59 infection outbreaks (Lai 2001; Ray *et al.* 2007; See *et al.* 2007; Gosney 2008) has been found  
26  
27 60 in different categories of Swiss ready-to-eat foods other than infant formula (Baumgartner *et*  
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29 61 *al.* 2009).

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34 62 One of the most typical Spanish RTE fish products, the anchovies in brine or in oil, belongs to  
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36 63 the group of the semi-preserved RTE foods, obtained without any heating process to stabilize  
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38 64 the end product. These are prepared from fresh *Engraulis* sp. by salting and ripening during  
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40 65 prolonged periods of time, usually for more than 3 months. Further on, the ripened fish is  
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42 66 desalted, filleted and immersed in brine or oil, with final presentation at the market in glass  
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44 67 jars or cans. This end product must be kept chilled and even if manufacturer's storage  
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46 68 instructions are respected, proteolytic and other quality changes may occur before  
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48 69 consumption as previously demonstrated (Veciana-Nogués *et al.* 1997; Rodriguez-Jerez *et al.*  
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50 70 1994). The desalting and filleting procedures prior to packaging increase the risk of secondary  
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52 71 bacterial contamination of the end product. To our knowledge there are not many works  
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3 72 published on the diversity of enterobacteria and their evolution upon refrigerated storage in  
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5 73 this type of marketed Spanish traditional RTE.  
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8 74 The aim of our study was to investigate the microbiological quality and the evolution of the  
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10 75 enterobacterial content upon storage of different *Engraulis* based ready-to-eat products from  
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12 76 the Spanish market. The final objective was to evaluate the existing risks for the consumer's  
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14 77 health in the selected products thus providing important information to the manufacturers of  
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16 78 this type of fish foods since currently there are no fixed legal limits for microbiological  
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18 79 indicators. European legislation in vigour (Commission Regulations No 2073/2005 and No  
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20 80 1441/2007) allows manufacturers to establish their proper control limits depending on the  
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22 81 specific production processes and in function of their Hazard Analysis and Critical Control  
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24 82 Points (HACCP) programmes.  
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## 32 **2. Materials and Methods**

### 33 34 35 85 *2.1. Food samples*

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38 86 Eighteen samples of ready-to-eat foods prepared with *Engraulidae* were purchased in Spanish  
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40 87 retailers and supermarkets between December 2009 and April 2010 (3 products per batch  
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42 88 from each commercial brand). These comprised 11 samples of anchovies in olive oil, 2  
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44 89 samples of anchovies in sunflower oil and 3 sample of anchovies in another type of vegetable  
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46 90 oil, one sample of snacks of anchovies with onion, red peppers and olives (presented  
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48 91 immersed in oil) and one sample of snacks of *Engraulis* with onion, red peppers and olives  
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50 92 (also immersed in vegetable oil). All samples were produced by 14 different manufacturers,  
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52 93 some wearing brands well known to the Spanish consumer.  
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### 56 94 *2.2. Bacteriological analysis of ready-to-eat foods*

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3 95 The analysis of the commercial samples was done according to the following ISO procedures:  
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5 96 ISO 4833:2003, ISO 21528-1:2004 and ISO 6888-3:2003, taking into account the ISO  
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7 97 7218:2007. The enrichment step for the initial detection and isolation of *Enterobacteriaceae*  
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9 98 in all samples analyzed was done in tubes with 9 ml Buffered Peptone Water (Biokar  
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11 99 Diagnostics, Beauvais, France) inoculated with 1 g from the respective sample. After 24h  
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13 100 incubation at 37°C 1ml from each of these tubes was passed to a tube with 10 ml E.E. Broth  
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15 101 (Oxoid Ltd., Basingstoke, UK). These tubes were further incubated for 24h at 37°C and then  
16  
17 102 used for the inoculation of three plates per sample of Violet Red Bile Glucose Agar (VRBG)  
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19 103 (Cultimed, Panreac, Castellar del Valles, Spain). Colonies with different morphologies  
20  
21 104 observed on VRBG were then isolated separately by passing to plates with Nutrient Agar at  
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23 105 2% (Biokar Diagnostics, Beauvais, France) containing 5 g/l NaCl. A biochemical  
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25 106 confirmation was done of the ability of each pure isolate to ferment glucose in tubes with 10  
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27 107 ml of Dextrose Tryptone Yeast Bromocresol Agar (Glucose agar) (Laboratorios Microkit,  
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29 108 Madrid, Spain). The change of the colour to yellow at the end of the incubation time (24h ± 2  
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31 109 at 37°C) was considered as positive result. The oxidase reaction of each isolate was also  
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33 110 verified by applying a drop from the Oxydase reagent (BioMerieux SA, Marcy l'Etoile,  
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35 111 France) on a single colony material as according to the instructions of the reagent's producer.  
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#### 41 2.2.1. Definition of Total Viable Counts

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44 113 For the enumeration of the TVCs 5 g per product were weighed from the corresponding  
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46 114 sample in stomacher bags Fbag-03 (AES Chemunex SA, Barcelona, Spain) in aseptic  
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48 115 conditions. The samples were then diluted with 45 ml of sterile Buffered Peptone Water  
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50 116 (BPW) (Biokar Diagnostics, Beauvais, France) and homogenized during 30 sec. in a  
51  
52 117 Laboratory Blender Stomacher 400 (Seward Ltd., West Sussex, UK). Serial ten-fold dilutions  
53  
54 118 up to 10<sup>-7</sup> were prepared per sample in tubes with 9 ml BPW. Two Petri dishes with agar were  
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56 119 inoculated from each dilution. In the case of aerobic mesophiles the agar medium applied was  
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3 120 Plate Count Agar (PCA) (Biokar Diagnostics, Beauvais, France), with a second layer of  
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5 121 Bacteriological agar type E at 18 g/l (Biokar Diagnostics, Beauvais, France). For the  
6  
7 122 enumeration of the anaerobic mesophilic microflora agar Schaedler (Biokar Diagnostics,  
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9 123 Beauvais, France) with defibrinated sheep blood at 5 % (E&O Laboratories Ltd., UK) was  
10  
11 124 used. Aerobic TVC were cultivated for 72 h at  $30 \pm 1$  °C and anaerobic TVC – at  $37 \pm 1$  °C  
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13 125 for 72 h at anaerobic conditions, assured by the GENbag anaer system (BioMerieux SA,  
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15 126 Marcy l’Etoile, France). The different morphologies obtained on the TVC agar plates were  
16  
17 127 further isolated on Tryptone Soy Agar (TSA) (Biokar Diagnostics, Beauvais, France) and  
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19 128 Nutrient Agar at 2 % (Biokar Diagnostics, Beauvais, France) for Gram staining and  
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21 129 identification.

### 22 23 24 25 26 130 *2.2.2. Definition of the Total Enterobacterial Counts*

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29 131 For the enumeration of the total enterobacterial counts the samples from the same batch and  
30  
31 132 brand were opened, mixed and 25 g per product were weighted in TEMPO<sup>®</sup> bags  
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33 133 (BioMerieux SA, Marcy l’Etoile, France) in aseptic conditions. These were diluted with 225  
34  
35 134 ml of sterile Tryptone-Salt broth (Biokar Diagnostics, Beauvais, France) and homogenized  
36  
37 135 during 30 sec. in our Laboratory Blender Stomacher 400. Further on this material was used  
38  
39 136 for the inoculation of TEMPO<sup>®</sup>EB cards (BioMerieux SA, Marcy l’Etoile, France) in the  
40  
41 137 TEMPO<sup>®</sup> preparation station as according to the manufacturer’s instructions. The cards were  
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43 138 incubated during 24 h at  $35 \pm 1$  °C in an incubator and then results were read and interpreted  
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45 139 with the TEMPO<sup>®</sup> Reader station and associated software. This method represents a  
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47 140 miniaturized MPN assay.

### 48 49 50 51 52 141 *2.3. Identification of the isolates*

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55 142 Gram staining was done for all pure enterobacterial and mesophilic isolates. Catalase  
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57 143 reaction was tested for the TVC isolates on single colony material by treatment with a

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3 144 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution at 3 %. All cultures were then identified to the species  
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5 145 level by the application of API<sup>®</sup> test and Biolog<sup>®</sup> identification systems.  
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8 146 *2.3.1. Identification by API<sup>®</sup> tests*  
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11 147 Fresh (24 h) colonies of all enterobacterial isolates were homogenized in sterile distilled water  
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13 148 and inoculated into API 20E galleries (BioMerieux SA, Marcy l'Etoile, France) following the  
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15 149 instructions of the manufacturer. For some TVC isolates identification was done with the API  
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17 150 20A (Anaerobes) and API 50CHB (*Bacillus*) galleries, where applicable, as in accordance  
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19 151 with the list of genera/species included in the identification range of the corresponding type of  
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21 152 gallery. Staphylococcal isolates were identified by the API Staph test. Results were expressed  
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23 153 in percentages of probability for the identification as according to the ApiWeb software.  
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27 154 *2.3.2. Biolog<sup>®</sup> Identification*  
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30 155 In parallel, the Biolog<sup>®</sup> MicroStation system and related software (Biolog, USA) were used  
31  
32 156 for the identification of all enterobacterial and TVC isolates obtained. For the purpose the  
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34 157 pure isolates were inoculated on TSA plates prior to Biolog<sup>®</sup> analysis and incubated at the  
35  
36 158 corresponding conditions for each microbial group. Anaerobiosis was applied in the case of  
37  
38 159 the anaerobic TVC isolates using the GENbag anaer system. The cultures were consequently  
39  
40 160 resuspended in tubes with 10 ml of GN/GP-IF inoculation fluid (Biolog, USA) using the  
41  
42 161 Biolog<sup>®</sup> turbidity standards and with addition of Biolog<sup>®</sup> sodium thioglycolate reagent as  
43  
44 162 according to the instructions of the manufacturer. These tubes were used for the inoculation of  
45  
46 163 Biolog<sup>®</sup> GN2 plates in the case of Gram negative isolates and Biolog<sup>®</sup> GP2 plates in the case  
47  
48 164 of Gram positive isolates. All plates were inoculated during 24 h at the cultivation  
49  
50 165 temperatures required (37 ± 1 °C for Enterobacteria and 30 °C for mesophiles) and then  
51  
52 166 results were read in the Biolog<sup>®</sup> MicroStation plate reader. Interpretation of results was done  
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54 167 with the MicroLog3 4.20.04 software and these were expressed in percentages of probability  
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3 168 for the identification or in their corresponding similarity index values (SIM) whenever less  
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5 169 than 0.5 SIM was obtained for the identification. According to the instructions of the  
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7 170 manufacturer a value close to 0.5 means high probability of identification and to each SIM  
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9 171 higher than 0.5 a probability percentage corresponds.  
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#### 12 172 *2.4. Shelf life study*

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15 173 Thirteen commercial products of anchovies in oil were selected at random for the shelf life  
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17 174 study in refrigeration, at 4 - 8 °C, storage conditions adequate for this type of semi-preserves  
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19 175 (products stabilized for a limited period by appropriate treatment and sealed in containers,  
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21 176 light tight under normal pressure; or not sealed in containers, the shelf life being extended by  
22  
23 177 chill storage). TVC, total enterobacterial counts by TEMPO<sup>®</sup> and the qualitative  
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25 178 enterobacterial content of the selected products were followed at three-month intervals up to 9  
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27 179 months of refrigerated storage. Thus, four experimental points were done – right after the  
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29 180 acquisition of the products (time 0) and at 3, 6 and 9 months of refrigerated storage.  
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#### 33 181 *2.5. Definition of the histamine content*

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36 182 Histamine content was measured by HPLC reverse phase chromatography as according to  
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38 183 Vieites and colleagues (Ben-Gigirey *et al.* 1999). 4 g of homogenized sample were mixed  
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40 184 with 20 ml perchloric acid 0.4 M, centrifuged for 20 minutes at 4000 rpm, and filtered. 1  
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42 185 ml of the extracted sample was derivatized by adding 200 µl NaOH 2 N, 300 µl sodium  
43  
44 186 bicarbonate (saturated solution) and 2 ml dansyl chloride. Tubes were incubated for 45  
45  
46 187 minutes at 46 °C. Then dansyl chloride residues were removed with 100 µl ammonia,  
47  
48 188 centrifuged 5 minutes at 4000 rpm, and filtered. Derivatized samples were injected in an  
49  
50 189 HPLC/PDA system (Waters Corporation, MA, USA), using a Nucleosil C18 5 µm reverse  
51  
52 190 phase column and monitored at  $\lambda = 254$  nm. Histamine dihydrochloride, minimum 99 %  
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54 191 purity, was purchased by SIGMA-ALDRICH and was used as standard. The detection  
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3 192 limit of this method is 10 mg histamine/ kg. Three of the products from the shelf life study  
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5 193 that showed highest enterobacterial counts and provided highest number of  
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7 194 enterobacterial isolates were selected for analysis by this method after 9 months of  
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9 195 storage in refrigeration.  
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### 12 196 **3. Results**

#### 13 14 15 197 *3.1. Bacteriological analysis of ready-to-eat foods*

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17  
18 198 Table 1 presents all food products examined as according to their covering sauce along with  
19  
20 199 the total enterobacterial counts and the corresponding identification of the isolates obtained on  
21  
22 200 selective media (E.E. broth, VRBG) after enrichment. Seven out of the 18 products (approx.  
23  
24 201 39 %) did not contain any Enterobacteria, among these four products of anchovies in olive oil,  
25  
26 202 one product of anchovies in vegetable oil and two products of anchovy/ *Engraulis* spp.  
27  
28 203 snacks. From the rest of the products a total of 30 different Gram-negative, oxidase negative  
29  
30 204 isolates were obtained. Although in most positive samples enterobacteria were present in  
31  
32 205 amounts below the limit of detection of the TEMPO<sup>®</sup> method (10 cfu/g), 3 samples showed  
33  
34 206 significant enterobacterial counts (640, 33 and 21 cfu/g). The sample with highest  
35  
36 207 enterobacterial counts showed highest number of different isolates. The prevailing genera  
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38 208 among all isolates were *Enterobacter* (represented by 8 isolates), *Citrobacter* (7 isolates),  
39  
40 209 *Proteus* (3 isolates), *Cronobacter* (*Enterobacter sakazakii*) (3 isolates), *Hafnia* (2 isolates)  
41  
42 210 and *Escherichia* (2 isolates, among which one identified as *E. coli* O157:H7). *Klebsiella*,  
43  
44 211 *Serratia*, *Raoultella* and *Pantoea* were represented by single species each. *Citrobacter*  
45  
46 212 *freundii* was the most common Enterobacterial contaminant of the studied products.  
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48 213 *Cronobacter sakazakii* was detected in 3 products.  
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54 214 TVC related isolates, including some of the staphylococci, obtained on selective media from  
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56 215 the studied products are shown in Table 2. These all were Gram-positive, both catalase  
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3 216 positive or negative. In contrast to enterobacterial counts TVC were present in all the  
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5 217 samples; however, in 5 products (all anchovies in olive oil, approx. 28 % of the total) only  
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7 218 lactic acid bacteria (LAB) were found, namely *Pediococcus pentosaceus*, a species renowned  
8  
9 219 for its probiotic properties (Osmanagaoglu *et al.* 2010; Semjonovs & Zikmanis 2008). In the  
10  
11 220 rest of the products the contaminating (non-LAB) microflora was more important in  
12  
13 221 quantities. The mesophilic aerobic isolates included several representatives of the coagulase  
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15 222 negative Staphylococci (among which *Staphylococcus xylosus*), *Cellulomonas*, *Rhodococcus*,  
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17 223 *Bacillus* and *Corynebacterium*. Among the anaerobic isolates *Desulfomonile tiedjei* and  
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19 224 *Fusobacterium mortiferum* were detected. Certain *Actinomyces* isolates were also found in  
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21 225 three of the products as identified by both API and Biolog.  
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### 26 3.2. Comparison of the methods for identification of the isolates

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29 227 Both methods used by us for the identification of the isolates were based on biochemical  
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31 228 characterization of each microorganism's carbohydrate metabolism. The Biolog system  
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33 229 implies testing for 95 different carbohydrates while the API system is in general more limited  
34  
35 230 in the number of substrates analysed, depending on outreach of each gallery. Therefore, the  
36  
37 231 API system has a more limited database for comparison and certain species are excluded from  
38  
39 232 possible identification as stated in the instruction manuals of the corresponding galleries. We  
40  
41 233 have tried to apply both systems for each isolate, whenever available, in order to reach better  
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43 234 identification. In most of the cases the results obtained by the two methods were in good  
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45 235 concordance, however, certain discrepancies existed for some of the enterobacterial isolates.  
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47 236 In such cases the method providing higher identification probability was selected and  
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49 237 included as final identification result in Tables 1 and 2. The identification probabilities given  
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51 238 by the Biolog system were very often expressed in similarity index values since no sufficient  
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53 239 growth was reached during the maximum incubation period advised by the manufacturer (24  
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240 h for most microbial groups), thus, no percentage of identification was obtained by using this  
241 method.

### 242 3.3. Shelf life study

243 The shelf life study concerning microbiological parameters was realized on thirteen products  
244 designated by numbers 4 - 16 in Tables 1 and 2. The initial enterobacterial counts and total  
245 number of enterobacterial isolates at time 0 were highest, followed by a prominent decrease  
246 after 3 months of storage. At the experimental point of 6 months no enterobacterial counts  
247 were found in any of the tested products and only two isolates were obtained after enrichment  
248 from the product with highest initial counts (640 cfu/g) (Fig. 1). No enterobacterial counts  
249 /isolates were obtained after 9 months of storage. The most persistent species at refrigeration  
250 conditions belonged to the genera *Enterobacter* and *Pantoea* (data not shown). *Cronobacter*  
251 (*Enterobacter sakazakii*) had generally poor viability under the selected conditions. The same  
252 reduction in counts was not observed for the TVC. The number of TVC isolates increased for  
253 most samples with the increase of the storage period (Fig. 2). The catalase negative  
254 staphylococcal isolates (*Staph. lentus*, *Staph. warneri*, *Staph. saprophyticus*, *Staph. arlettae*,  
255 *etc.*) were the most resistant group at the selected storage conditions. The histamine quantities  
256 detected (two values below the detection limit of the method of 10 mg/kg and one value of 84  
257  $\pm$  22 mg/kg) in the samples analyzed were below the officially allowed limit for histamine  
258 (200 mg/kg) in products manufactured from fish species associated with high histidine  
259 amounts (Commission Regulations No 2073/2005 and No1441/2007).

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## 261 4. Discussion

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

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3 264 covering sauces are highly appreciated by local and international consumers; therefore, their  
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5 265 systematic safety control is of great relevance to avoid public health risks. After application of  
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7 266 the established ISO procedures for the detection and quantification of indicator and  
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9 267 pathogenic microorganisms or alternative validated methods (TEMPO), presence of certain  
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11 268 opportunistic or obligate bacterial pathogens, mainly belonging to the *Enterobacteriaceae*  
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13 269 family, was confirmed in 61 % of the foods sampled. Three of the products (17 %) could be  
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15 270 considered unsafe for consumption due to their high enterobacterial counts. Thirty different  
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17 271 enterobacterial species were isolated from all products analyzed.

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21 272 *Enterobacteriaceae* are generally accepted as the primary cause of food spoilage and  
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23 273 histamine development in different food products. Species like *Citrobacter freundii*, found to  
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25 274 be one of the predominant enterobacterial isolates in the current research work, together with  
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27 275 *Morganella morganii*, *Proteus* spp., *Enterobacter* spp., *Klebsiella pneumoniae*, *Escherichia*  
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29 276 spp., *Hafnia alvei*, *Raoultella* spp. and *Serratia* spp. have previously been reported as  
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31 277 biogenic amine producers in various foods – tuna sandwiches (Kung *et al.* 2009), packaged  
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33 278 and processed meat (Durlu - Özkaya *et al.* 2001), seafood (Kim *et al.* 2003), salted mackerel  
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35 279 (Tsai *et al.* 2005), etc., causing indigestion, intoxications and food allergies. *Morganella*,  
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37 280 *Citrobacter*, *Proteus* spp., *Klebsiella pneumoniae* and *Hafnia alvei* have been known for their  
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39 281 important histamine production while *Enterobacter amnigenus* and *Enterobacter cloacae*  
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41 282 have been shown to produce particularly high amounts of putrescine (Lavizzari *et al.* 2010).  
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43 283 Although we have not isolated any *Morganella morganii* or *Klebsiella pneumoniae* species  
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45 284 from *Engraulis* spp. containing RTE foods we have identified several isolates with known  
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47 285 histidine decarboxylase activities as *Proteus penneri*, *Proteus mirabilis*, *Proteus vulgaris*,  
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49 286 *Pantoea* spp., *Raoultella planticola/ ornithinolytica*, *Raoultella terrigena* and *Serratia ficaria*.  
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51 287 The presence of these microorganisms could suppose certain degree of filet softening,  
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53 288 proteolysis, histamine accumulation and spoilage during the storage of the sampled foods.  
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# 1 Microbiological quality of ready-to-eat pickled fish products

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

## 7 8 9 **Abstract**

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

51 Ready-to-eat fish products, *Engraulidae*, bacteriological quality, *Enterobacteriaceae*,  
52 opportunistic pathogens  
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## 61 **1. Introduction**

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

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3 23 (Commission Regulation No 2073/2005 and its modification No 1441/2007) “ready-to-eat”  
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5 24 food means food intended by the producer or the manufacturer for direct human consumption  
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7 25 without the need for cooking or other processing effective to eliminate or reduce to an  
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9 26 acceptable level the microorganisms of concern. RTE food safety control implies the  
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11 27 application of internationally validated analytical protocols (corresponding ISO procedures)  
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13 28 for detection and quantification of indicator and pathogenic microorganisms as according to  
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15 29 the microbiological criteria fixed in the current national and European legislations  
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17 30 (Commission Regulations No 2073/2005 and No 1441/2007). Most commonly adopted  
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19 31 indicators include coliforms, *Enterobacteriaceae*, total viable counts, though this criterion is  
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21 32 rather general and gives no detailed information on the identity of the microflora present,  
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23 33 coagulase positive Staphylococci and pathogenic microorganisms like *Salmonella* and  
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25 34 *Listeria monocytogenes*. In the case of RTE products elaborated with fish and/or seafood the  
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27 35 only existing legislative criteria from a microbiological point of view are fixed for cooked  
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29 36 crustaceans and molluscan shellfish. For products which have undergone enzyme maturation  
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31 37 treatment in brine, manufactured from fish species associated with a high amount of histidine,  
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33 38 limits are also fixed for the amounts of histamine present in the final product. However, the  
34  
35 39 safety of all RTE foods may be affected by the microbiological quality of the starting  
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37 40 materials as well as by the handling, processing, transportation and storage conditions of the  
38  
39 41 finished product before its actual use by the consumer (Angelidis *et al.* 2006).  
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42 In the international scientific literature several studies exist on the problematic  
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44 43 microbiological quality and characteristics of traditional RTE foods from different countries  
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46 44 (Yeboah-Manu *et al.* 2010; Haryani *et al.* 2008; Angelidis *et al.* 2006; Thapa *et al.* 2006;  
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48 45 Fang *et al.* 2003). Nine bacterial pathogens were isolated from various RTEs distributed at the  
49  
50 46 University of Ghana campus (Yeboah-Manu *et al.* 2010) namely *Escherichia coli* (*E. coli*),  
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52 47 *Klebsiella pneumoniae*, *Streptococcus* sp., *Enterobacter cloacae* (*E. cloacae*), *Bacillus* sp.,  
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3 48 *Pseudomonas aeruginosa*, *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecalis* and  
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5 49 *Proteus* sp. Moreover, high occurrence of *E. cloacae* (7 strains with proven antibiotic  
6  
7 50 resistance) in several typical Malaysian RTE products sold in the street has been reported  
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9 51 (Haryani *et al.* 2008). In a broad study of commercially available traditional Greek RTE foods  
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11 52 3.4 % of the products were unacceptable for consumption due to exceeding pathogen levels of  
12  
13 53 *S. aureus* and *E. coli* while a considerable percentage of samples was found to contain high  
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15 54 concentrations of contaminating microflora (Angelidis *et al.* 2006). In a similar research  
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17 55 carried out with a wide number of commercial RTEs from central Taiwan incidences as high  
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19 56 as 7.9 % for *E. coli*, 49.8 % for *Bacillus cereus* and 17.9 % for *S. aureus* were detected (Fang  
20  
21 57 *et al.* 2003). *Cronobacter* (*Enterobacter sakazakii*), an opportunistic human pathogen  
22  
23 58 associated mainly with infant infections but also in some cases causative agent of adult  
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25 59 infection outbreaks (Lai 2001; Ray *et al.* 2007; See *et al.* 2007; Gosney 2008) has been found  
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27 60 in different categories of Swiss ready-to-eat foods other than infant formula (Baumgartner *et*  
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29 61 *al.* 2009).

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35 62 One of the most typical Spanish RTE fish products, the anchovies in brine or in oil, belongs to  
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37 63 the group of the semi-preserved RTE foods, obtained without any heating process to stabilize  
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39 64 the end product. These are prepared from fresh *Engraulis* sp. by salting and ripening during  
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41 65 prolonged periods of time, usually for more than 3 months. Further on, the ripened fish is  
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43 66 desalted, filleted and immersed in brine or oil, with final presentation at the market in glass  
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45 67 jars or cans. This end product must be kept chilled and even if manufacturer's storage  
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47 68 instructions are respected, proteolytic and other quality changes may occur before  
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49 69 consumption as previously demonstrated (Veciana-Nogués *et al.* 1997; Rodriguez-Jerez *et al.*  
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51 70 1994). The desalting and filleting procedures prior to packaging increase the risk of secondary  
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53 71 bacterial contamination of the end product. To our knowledge there are not many works  
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3 72 published on the diversity of enterobacteria and their evolution upon refrigerated storage in  
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5 73 this type of marketed Spanish traditional RTE.  
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8 74 The aim of our study was to investigate the microbiological quality and the evolution of the  
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10 75 enterobacterial content upon storage of different *Engraulis* based ready-to-eat products from  
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12 76 the Spanish market. The final objective was to evaluate the existing risks for the consumer's  
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14 77 health in the selected products thus providing important information to the manufacturers of  
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16 78 this type of fish foods since currently there are no fixed legal limits for microbiological  
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18 79 indicators. European legislation in vigour (Commission Regulations No 2073/2005 and No  
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20 80 1441/2007) allows manufacturers to establish their proper control limits depending on the  
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22 81 specific production processes and in function of their Hazard Analysis and Critical Control  
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24 82 Points (HACCP) programmes.  
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## 32 **2. Materials and Methods**

### 33 34 35 85 *2.1. Food samples*

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38 86 Eighteen samples of ready-to-eat foods prepared with *Engraulidae* were purchased in Spanish  
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40 87 retailers and supermarkets between December 2009 and April 2010 (3 products per batch  
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42 88 from each commercial brand). These comprised 11 samples of anchovies in olive oil, 2  
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44 89 samples of anchovies in sunflower oil and 3 sample of anchovies in another type of vegetable  
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46 90 oil, one sample of snacks of anchovies with onion, red peppers and olives (presented  
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48 91 immersed in oil) and one sample of snacks of *Engraulis* with onion, red peppers and olives  
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50 92 (also immersed in vegetable oil). All samples were produced by 14 different manufacturers,  
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52 93 some wearing brands well known to the Spanish consumer.  
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### 56 94 *2.2. Bacteriological analysis of ready-to-eat foods*

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3 95 The analysis of the commercial samples was done according to the following ISO procedures:  
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5 96 ISO 4833:2003, ISO 21528-1:2004 and ISO 6888-3:2003, taking into account the ISO  
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7 97 7218:2007. The enrichment step for the initial detection and isolation of *Enterobacteriaceae*  
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9 98 in all samples analyzed was done in tubes with 9 ml Buffered Peptone Water (Biokar  
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11 99 Diagnostics, Beauvais, France) inoculated with 1 g from the respective sample. After 24h  
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13 100 incubation at 37°C 1ml from each of these tubes was passed to a tube with 10 ml E.E. Broth  
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15 101 (Oxoid Ltd., Basingstoke, UK). These tubes were further incubated for 24h at 37°C and then  
16  
17 102 used for the inoculation of three plates per sample of Violet Red Bile Glucose Agar (VRBG)  
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19 103 (Cultimed, Panreac, Castellar del Valles, Spain). Colonies with different morphologies  
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21 104 observed on VRBG were then isolated separately by passing to plates with Nutrient Agar at  
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23 105 2% (Biokar Diagnostics, Beauvais, France) containing 5 g/l NaCl. A biochemical  
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25 106 confirmation was done of the ability of each pure isolate to ferment glucose in tubes with 10  
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27 107 ml of Dextrose Tryptone Yeast Bromocresol Agar (Glucose agar) (Laboratorios Microkit,  
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29 108 Madrid, Spain). The change of the colour to yellow at the end of the incubation time (24h ± 2  
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31 109 at 37°C) was considered as positive result. The oxidase reaction of each isolate was also  
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33 110 verified by applying a drop from the Oxydase reagent (BioMerieux SA, Marcy l'Etoile,  
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35 111 France) on a single colony material as according to the instructions of the reagent's producer.  
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#### 41 2.2.1. Definition of Total Viable Counts

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44 113 For the enumeration of the TVCs 5 g per product were weighed from the corresponding  
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46 114 sample in stomacher bags Fbag-03 (AES Chemunex SA, Barcelona, Spain) in aseptic  
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48 115 conditions. The samples were then diluted with 45 ml of sterile Buffered Peptone Water  
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50 116 (BPW) (Biokar Diagnostics, Beauvais, France) and homogenized during 30 sec. in a  
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52 117 Laboratory Blender Stomacher 400 (Seward Ltd., West Sussex, UK). Serial ten-fold dilutions  
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54 118 up to 10<sup>-7</sup> were prepared per sample in tubes with 9 ml BPW. Two Petri dishes with agar were  
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56 119 inoculated from each dilution. In the case of aerobic mesophiles the agar medium applied was  
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3 120 Plate Count Agar (PCA) (Biokar Diagnostics, Beauvais, France), with a second layer of  
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5 121 Bacteriological agar type E at 18 g/l (Biokar Diagnostics, Beauvais, France). For the  
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7 122 enumeration of the anaerobic mesophilic microflora agar Schaedler (Biokar Diagnostics,  
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9 123 Beauvais, France) with defibrinated sheep blood at 5 % (E&O Laboratories Ltd., UK) was  
10  
11 124 used. Aerobic TVC were cultivated for 72 h at  $30 \pm 1$  °C and anaerobic TVC – at  $37 \pm 1$  °C  
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13 125 for 72 h at anaerobic conditions, assured by the GENbag anaer system (BioMerieux SA,  
14  
15 126 Marcy l’Etoile, France). The different morphologies obtained on the TVC agar plates were  
16  
17 127 further isolated on Tryptone Soy Agar (TSA) (Biokar Diagnostics, Beauvais, France) and  
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19 128 Nutrient Agar at 2 % (Biokar Diagnostics, Beauvais, France) for Gram staining and  
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21 129 identification.  
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### 26 130 *2.2.2. Definition of the Total Enterobacterial Counts*

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29 131 For the enumeration of the total enterobacterial counts the samples from the same batch and  
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31 132 brand were opened, mixed and 25 g per product were weighted in TEMPO<sup>®</sup> bags  
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33 133 (BioMerieux SA, Marcy l’Etoile, France) in aseptic conditions. These were diluted with 225  
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35 134 ml of sterile Tryptone-Salt broth (Biokar Diagnostics, Beauvais, France) and homogenized  
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37 135 during 30 sec. in our Laboratory Blender Stomacher 400. Further on this material was used  
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39 136 for the inoculation of TEMPO<sup>®</sup>EB cards (BioMerieux SA, Marcy l’Etoile, France) in the  
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41 137 TEMPO<sup>®</sup> preparation station as according to the manufacturer’s instructions. The cards were  
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43 138 incubated during 24 h at  $35 \pm 1$  °C in an incubator and then results were read and interpreted  
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45 139 with the TEMPO<sup>®</sup> Reader station and associated software. This method represents a  
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47 140 miniaturized MPN assay.  
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### 52 141 *2.3. Identification of the isolates*

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55 142 Gram staining was done for all pure enterobacterial and mesophilic isolates. Catalase  
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57 143 reaction was tested for the TVC isolates on single colony material by treatment with a  
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3 144 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution at 3 %. All cultures were then identified to the species  
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5 145 level by the application of API<sup>®</sup> test and Biolog<sup>®</sup> identification systems.  
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8 146 *2.3.1. Identification by API<sup>®</sup> tests*  
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11 147 Fresh (24 h) colonies of all enterobacterial isolates were homogenized in sterile distilled water  
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13 148 and inoculated into API 20E galleries (BioMerieux SA, Marcy l'Etoile, France) following the  
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15 149 instructions of the manufacturer. For some TVC isolates identification was done with the API  
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17 150 20A (Anaerobes) and API 50CHB (*Bacillus*) galleries, where applicable, as in accordance  
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19 151 with the list of genera/species included in the identification range of the corresponding type of  
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21 152 gallery. Staphylococcal isolates were identified by the API Staph test. Results were expressed  
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23 153 in percentages of probability for the identification as according to the ApiWeb software.  
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27 154 *2.3.2. Biolog<sup>®</sup> Identification*  
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30 155 In parallel, the Biolog<sup>®</sup> MicroStation system and related software (Biolog, USA) were used  
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32 156 for the identification of all enterobacterial and TVC isolates obtained. For the purpose the  
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34 157 pure isolates were inoculated on TSA plates prior to Biolog<sup>®</sup> analysis and incubated at the  
35  
36 158 corresponding conditions for each microbial group. Anaerobiosis was applied in the case of  
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38 159 the anaerobic TVC isolates using the GENbag anaer system. The cultures were consequently  
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40 160 resuspended in tubes with 10 ml of GN/GP-IF inoculation fluid (Biolog, USA) using the  
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42 161 Biolog<sup>®</sup> turbidity standards and with addition of Biolog<sup>®</sup> sodium thioglycolate reagent as  
43  
44 162 according to the instructions of the manufacturer. These tubes were used for the inoculation of  
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46 163 Biolog<sup>®</sup> GN2 plates in the case of Gram negative isolates and Biolog<sup>®</sup> GP2 plates in the case  
47  
48 164 of Gram positive isolates. All plates were inoculated during 24 h at the cultivation  
49  
50 165 temperatures required (37 ± 1 °C for Enterobacteria and 30 °C for mesophiles) and then  
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52 166 results were read in the Biolog<sup>®</sup> MicroStation plate reader. Interpretation of results was done  
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54 167 with the MicroLog3 4.20.04 software and these were expressed in percentages of probability  
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3 168 for the identification or in their corresponding similarity index values (SIM) whenever less  
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5 169 than 0.5 SIM was obtained for the identification. According to the instructions of the  
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7 170 manufacturer a value close to 0.5 means high probability of identification and to each SIM  
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9 171 higher than 0.5 a probability percentage corresponds.  
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#### 12 172 *2.4. Shelf life study*

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15 173 Thirteen commercial products of anchovies in oil were selected at random for the shelf life  
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17 174 study in refrigeration, at 4 - 8 °C, storage conditions adequate for this type of semi-preserves  
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19 175 (products stabilized for a limited period by appropriate treatment and sealed in containers,  
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21 176 light tight under normal pressure; or not sealed in containers, the shelf life being extended by  
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23 177 chill storage). TVC, total enterobacterial counts by TEMPO<sup>®</sup> and the qualitative  
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25 178 enterobacterial content of the selected products were followed at three-month intervals up to 9  
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27 179 months of refrigerated storage. Thus, four experimental points were done – right after the  
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29 180 acquisition of the products (time 0) and at 3, 6 and 9 months of refrigerated storage.  
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#### 33 181 *2.5. Definition of the histamine content*

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36 182 Histamine content was measured by HPLC reverse phase chromatography as according to  
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38 183 Vieites and colleagues (Ben-Gigirey *et al.* 1999). 4 g of homogenized sample were mixed  
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40 184 with 20 ml perchloric acid 0.4 M, centrifuged for 20 minutes at 4000 rpm, and filtered. 1  
41  
42 185 ml of the extracted sample was derivatized by adding 200 µl NaOH 2 N, 300 µl sodium  
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44 186 bicarbonate (saturated solution) and 2 ml dansyl chloride. Tubes were incubated for 45  
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46 187 minutes at 46 °C. Then dansyl chloride residues were removed with 100 µl ammonia,  
47  
48 188 centrifuged 5 minutes at 4000 rpm, and filtered. Derivatized samples were injected in an  
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50 189 HPLC/PDA system (Waters Corporation, MA, USA), using a Nucleosil C18 5 µm reverse  
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52 190 phase column and monitored at  $\lambda = 254$  nm. Histamine dihydrochloride, minimum 99 %  
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54 191 purity, was purchased by SIGMA-ALDRICH and was used as standard. The detection  
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3 192 limit of this method is 10 mg histamine/ kg. Three of the products from the shelf life study  
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5 193 that showed highest enterobacterial counts and provided highest number of  
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7 194 enterobacterial isolates were selected for analysis by this method after 9 months of  
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9 195 storage in refrigeration.  
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### 12 196 **3. Results**

#### 13 14 15 197 *3.1. Bacteriological analysis of ready-to-eat foods*

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18 198 Table 1 presents all food products examined as according to their covering sauce along with  
19  
20 199 the total enterobacterial counts and the corresponding identification of the isolates obtained on  
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22 200 selective media (E.E. broth, VRBG) after enrichment. Seven out of the 18 products (approx.  
23  
24 201 39 %) did not contain any Enterobacteria, among these four products of anchovies in olive oil,  
25  
26 202 one product of anchovies in vegetable oil and two products of anchovy/ *Engraulis* spp.  
27  
28 203 snacks. From the rest of the products a total of 30 different Gram-negative, oxidase negative  
29  
30 204 isolates were obtained. Although in most positive samples enterobacteria were present in  
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32 205 amounts below the limit of detection of the TEMPO<sup>®</sup> method (10 cfu/g), 3 samples showed  
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34 206 significant enterobacterial counts (640, 33 and 21 cfu/g). The sample with highest  
35  
36 207 enterobacterial counts showed highest number of different isolates. The prevailing genera  
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38 208 among all isolates were *Enterobacter* (represented by 8 isolates), *Citrobacter* (7 isolates),  
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40 209 *Proteus* (3 isolates), *Cronobacter* (*Enterobacter sakazakii*) (3 isolates), *Hafnia* (2 isolates)  
41  
42 210 and *Escherichia* (2 isolates, among which one identified as *E. coli* O157:H7). *Klebsiella*,  
43  
44 211 *Serratia*, *Raoultella* and *Pantoea* were represented by single species each. *Citrobacter*  
45  
46 212 *freundii* was the most common Enterobacterial contaminant of the studied products.  
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48 213 *Cronobacter sakazakii* was detected in 3 products.  
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54 214 TVC related isolates, including some of the staphylococci, obtained on selective media from  
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56 215 the studied products are shown in Table 2. These all were Gram-positive, both catalase  
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3 216 positive or negative. In contrast to enterobacterial counts TVC were present in all the  
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5 217 samples; however, in 5 products (all anchovies in olive oil, approx. 28 % of the total) only  
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7 218 lactic acid bacteria (LAB) were found, namely *Pediococcus pentosaceus*, a species renowned  
8  
9 219 for its probiotic properties (Osmanagaoglu *et al.* 2010; Semjonovs & Zikmanis 2008). In the  
10  
11 220 rest of the products the contaminating (non-LAB) microflora was more important in  
12  
13 221 quantities. The mesophilic aerobic isolates included several representatives of the coagulase  
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15 222 negative Staphylococci (among which *Staphylococcus xylosus*), *Cellulomonas*, *Rhodococcus*,  
16  
17 223 *Bacillus* and *Corynebacterium*. Among the anaerobic isolates *Desulfomonile tiedjei* and  
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19 224 *Fusobacterium mortiferum* were detected. Certain *Actinomyces* isolates were also found in  
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21 225 three of the products as identified by both API and Biolog.  
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### 26 3.2. Comparison of the methods for identification of the isolates

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29 227 Both methods used by us for the identification of the isolates were based on biochemical  
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31 228 characterization of each microorganism's carbohydrate metabolism. The Biolog system  
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33 229 implies testing for 95 different carbohydrates while the API system is in general more limited  
34  
35 230 in the number of substrates analysed, depending on outreach of each gallery. Therefore, the  
36  
37 231 API system has a more limited database for comparison and certain species are excluded from  
38  
39 232 possible identification as stated in the instruction manuals of the corresponding galleries. We  
40  
41 233 have tried to apply both systems for each isolate, whenever available, in order to reach better  
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43 234 identification. In most of the cases the results obtained by the two methods were in good  
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45 235 concordance, however, certain discrepancies existed for some of the enterobacterial isolates.  
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47 236 In such cases the method providing higher identification probability was selected and  
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49 237 included as final identification result in Tables 1 and 2. The identification probabilities given  
50  
51 238 by the Biolog system were very often expressed in similarity index values since no sufficient  
52  
53 239 growth was reached during the maximum incubation period advised by the manufacturer (24  
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240 h for most microbial groups), thus, no percentage of identification was obtained by using this  
241 method.

### 242 3.3. Shelf life study

243 The shelf life study concerning microbiological parameters was realized on thirteen products  
244 designated by numbers 4 - 16 in Tables 1 and 2. The initial enterobacterial counts and total  
245 number of enterobacterial isolates at time 0 were highest, followed by a prominent decrease  
246 after 3 months of storage. At the experimental point of 6 months no enterobacterial counts  
247 were found in any of the tested products and only two isolates were obtained after enrichment  
248 from the product with highest initial counts (640 cfu/g) (Fig. 1). No enterobacterial counts  
249 /isolates were obtained after 9 months of storage. The most persistent species at refrigeration  
250 conditions belonged to the genera *Enterobacter* and *Pantoea* (data not shown). *Cronobacter*  
251 (*Enterobacter sakazakii*) had generally poor viability under the selected conditions. The same  
252 reduction in counts was not observed for the TVC. The number of TVC isolates increased for  
253 most samples with the increase of the storage period (Fig. 2). The catalase negative  
254 staphylococcal isolates (*Staph. lentus*, *Staph. warneri*, *Staph. saprophyticus*, *Staph. arlettae*,  
255 *etc.*) were the most resistant group at the selected storage conditions. The histamine quantities  
256 detected (two values below the detection limit of the method of 10 mg/kg and one value of 84  
257  $\pm$  22 mg/kg) in the samples analyzed were below the officially allowed limit for histamine  
258 (200 mg/kg) in products manufactured from fish species associated with high histidine  
259 amounts (Commission Regulations No 2073/2005 and No1441/2007).

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## 261 4. Discussion

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

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3 264 covering sauces are highly appreciated by local and international consumers; therefore, their  
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5 265 systematic safety control is of great relevance to avoid public health risks. After application of  
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7 266 the established ISO procedures for the detection and quantification of indicator and  
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9 267 pathogenic microorganisms or alternative validated methods (TEMPO), presence of certain  
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11 268 opportunistic or obligate bacterial pathogens, mainly belonging to the *Enterobacteriaceae*  
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13 269 family, was confirmed in 61 % of the foods sampled. Three of the products (17 %) could be  
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15 270 considered unsafe for consumption due to their high enterobacterial counts. Thirty different  
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17 271 enterobacterial species were isolated from all products analyzed.

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21 272 *Enterobacteriaceae* are generally accepted as the primary cause of food spoilage and  
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23 273 histamine development in different food products. Species like *Citrobacter freundii*, found to  
24  
25 274 be one of the predominant enterobacterial isolates in the current research work, together with  
26  
27 275 *Morganella morganii*, *Proteus* spp., *Enterobacter* spp., *Klebsiella pneumoniae*, *Escherichia*  
28  
29 276 spp., *Hafnia alvei*, *Raoultella* spp. and *Serratia* spp. have previously been reported as  
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31 277 biogenic amine producers in various foods – tuna sandwiches (Kung *et al.* 2009), packaged  
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33 278 and processed meat (Durlu - Özkaya *et al.* 2001), seafood (Kim *et al.* 2003), salted mackerel  
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35 279 (Tsai *et al.* 2005), etc., causing indigestion, intoxications and food allergies. *Morganella*,  
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37 280 *Citrobacter*, *Proteus* spp., *Klebsiella pneumoniae* and *Hafnia alvei* have been known for their  
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39 281 important histamine production while *Enterobacter amnigenus* and *Enterobacter cloacae*  
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41 282 have been shown to produce particularly high amounts of putrescine (Lavizzari *et al.* 2010).  
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43 283 Although we have not isolated any *Morganella morganii* or *Klebsiella pneumoniae* species  
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45 284 from *Engraulis* spp. containing RTE foods we have identified several isolates with known  
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47 285 histidine decarboxylase activities as *Proteus penneri*, *Proteus mirabilis*, *Proteus vulgaris*,  
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49 286 *Pantoea* spp., *Raoultella planticola/ ornithinolytica*, *Raoultella terrigena* and *Serratia ficaria*.  
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51 287 The presence of these microorganisms could suppose certain degree of filet softening,  
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53 288 proteolysis, histamine accumulation and spoilage during the storage of the sampled foods.  
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483 **Table 1. Identification of all enterobacterial isolates obtained from the products**  
 484 **containing *Engraulis* spp.**

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

485 †SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system.

486 Good identification by this system is normally considered for SIM index values above 0.3.

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485 †SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system.

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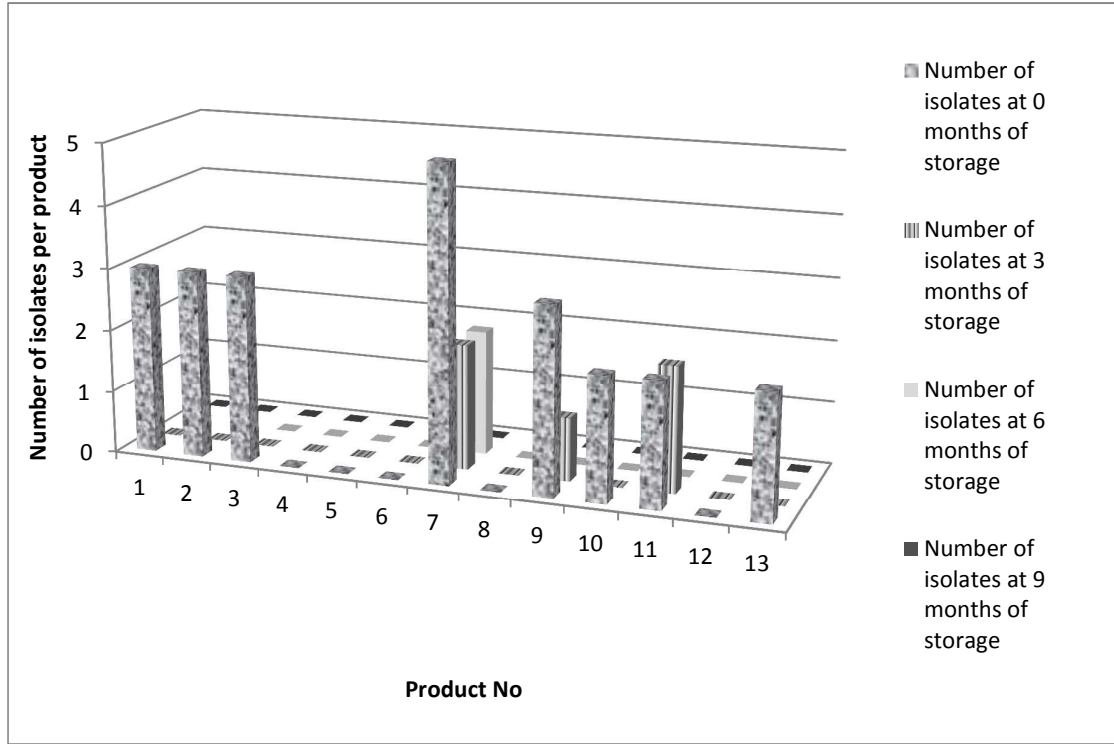
**Table 2. Identification of the total viable count (TVC) isolates from the ready-to-eat products analyzed.**

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

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3 490 †SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system.  
4 491 Good identification by this system is normally considered for SIM index values above 0.3.  
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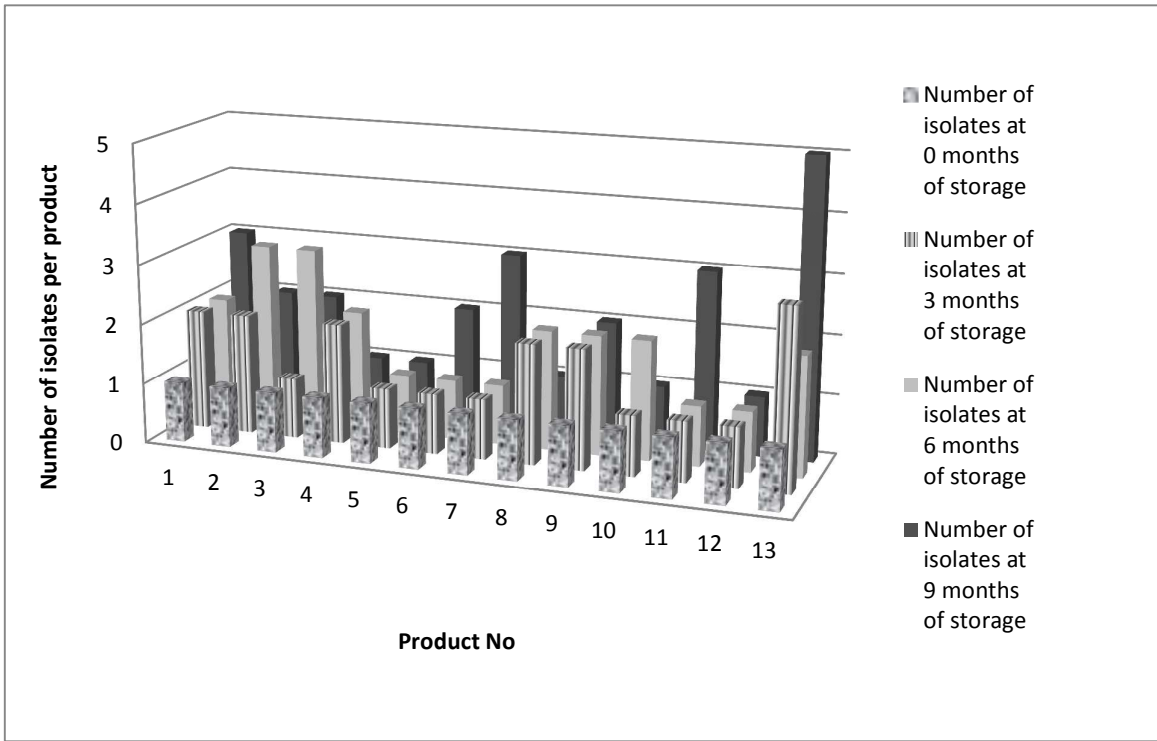
Fig. 1.



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**Fig. 2.**



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3 289 Therefore, the histamine content of the samples with highest enterobacterial counts and  
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5 290 detected presence of known histamine formers as *Hafnia alvei*, *Citrobacter freundii*,  
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7 291 *Enterobacter aerogenes*, *E. amnigenus* and *Raoultella* spp. was checked by analytical  
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9 292 chromatography. No dependence between the enterobacterial counts and histamine amount in  
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11 293 our samples was established, a result previously seen in other research works (Rodriguez-  
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13 294 Jerez *et al.* 1994). Neither were found in the samples analyzed any amounts surpassing the  
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15 295 legally fixed histamine levels for this type of foods.  
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19 296 Our results are in good correlation with the enterobacterial species isolated from other RTE  
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21 297 and seafood products (Yeboah-Manu *et al.* 2010; Haryani *et al.* 2008; Angelidis *et al.* 2006;  
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23 298 Thapa *et al.* 2006; Fang *et al.* 2003; Hsu *et al.* 2009; Rodriguez-Jerez *et al.* 1994). However,  
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25 299 to our knowledge there are no many studies published on the isolation and exhaustive  
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27 300 identification of the enterobacterial microflora of traditional Spanish anchovies in oil RTE  
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29 301 products.  
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33 302 In the current study we have isolated 8 different *Enterobacter* spp. isolates, identified as  
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35 303 *Enterobacter cloacae*, *E. gergoviae*, *E. amnigenus*, *E. aerogenes*, *E. cancerogenus* and *E.*  
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37 304 *hormaechei*. *Enterobacter* spp. are generally considered as the sixth most frequent cause of  
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39 305 nosocomial infections with antibiotic-resistant strains being reported in literature with  
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41 306 increasing frequency (Haryani *et al.* 2008). Due to the high incidences of antibiotic resistance  
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43 307 in *E. cloacae* it is one of the few bacteria which by official rules have to be monitored in all  
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45 308 healthcare facilities in Europe. Several reports exist on bacteremia and bloodstream infections  
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47 309 in hospitalized patients caused by *E. cloacae*, *E. aerogenes* and *Citrobacter freundii* (Deal *et*  
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49 310 *al.* 2007; Chen *et al.* 2009; Badger *et al.* 1999). *Hafnia alvei*, another representative of the  
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51 311 *Enterobacteriaceae*, that has been detected in the RTE foods analyzed in this work (2  
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53 312 isolates), has been shown to be predominantly associated with several intestinal disorders,  
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3 313 gastroenteritis, respiratory tract infections, sepsis, peritonitis, urinary infection and other  
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5 314 illnesses (Stock *et al.* 2005).  
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8 315 The medical importance and toxin production by enterohemorrhagic *Escherichia coli* strains  
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10 316 is well known and has already been widely reviewed (Kawasaki *et al.* 2009; Jothikumar *et al.*  
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12 317 2002). In our study we have reached preliminary identification for two isolates as belonging  
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14 318 to *E. coli*, one of which identified by the database of the Biolog MicroStation system as  
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16 319 pathogenic *E.coli* O157:H7. Although further confirmation of the *E. coli* identifications is  
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18 320 necessary by the application of the corresponding specific ISO procedures these results are  
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20 321 already an indication for possible food-borne illness transmission.  
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24 322 In relation to the other bacterial groups studied the staphylococcal isolates were prevailing  
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26 323 among all TVC isolates (26 %, 8 different cultures). No coagulase positive *Staphylococci*  
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28 324 were found in any of the food products analyzed. Nevertheless, some of the coagulase  
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30 325 negative staphylococcal isolates obtained on selective and non-selective media (BP-RPF,  
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32 326 PCA) represented certain potential hazard to consumer health. *Staphylococcus xylosus*,  
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34 327 isolated from one of the studied products, has been considered by some medical scientists an  
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36 328 emerging human pathogen which can cause nosocomial infections and bacteraemia in  
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38 329 hospitalized patients (Esper *et al.* 2000). Among the other mesophilic bacteria identified on  
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40 330 the TVC plates *Tsukumurella inchonensis* is a newly defined species that is related to  
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42 331 corynebacteria, *Mycobacterium*, *Nocardia* and *Rhodococcus*. It was originally isolated from  
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44 332 blood cultures of different clinical cases (Yassin *et al.* 1995) and to our knowledge has not  
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46 333 previously been reported in food samples. One isolate was identified as *Corynebacterium*  
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48 334 *nitrophilus* and one as *Rhodococcus rhodochrous*, belonging to the same group of clinically  
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50 335 significant microorganisms.  
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3 336 We have not applied any standard protocols for *Listeria monocytogenes* detection since the  
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5 337 growth of this pathogen was not supported at the low humidity conditions (35 %, water  
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7 338 activity Aw of 0.657) and high content of chlorinated compounds (12.5 %) experimentally  
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9 339 defined in the analyzed products (data not shown).  
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13 340 In summary, during the 9-month shelf life study, intended to determine the possible  
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15 341 qualitative and quantitative changes in the discovered bacterial microflora at storage  
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17 342 conditions recommended by the manufacturers of the selected RTE foods on their respective  
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19 343 packaging (approx. 1 year at temperatures between 5 and 12 °C), we have seen that most of  
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21 344 the enterobacterial species present were still viable over a three month storage period with  
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23 345 only two viable isolates obtained after 6 months of storage. This tendency was further  
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25 346 confirmed at the last point of 9 months of storage when absolutely no counts and no isolates  
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27 347 were obtained after the two-step enrichment procedure on selective media. We considered that  
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29 348 the observed decline in enterobacterial numbers and loss of viability might be due to the  
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31 349 preserving effect of the salt (high content of chlorinated compounds in the samples) and the  
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33 350 plant oil dressing. The antimicrobial effect of plant oils has been known for long and the  
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35 351 bactericidal action of some essential plant oils on enterobacterial pathogens (*E.coli* and  
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37 352 various *Salmonella* species) has previously been demonstrated (Peñalver *et al.* 2005).  
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43 353 Thus, it has been demonstrated that these traditional RTE foods, elaborated with *Engraulis* sp.  
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45 354 and preserved under cold storage in salt and oil dressing, are at high risk of being  
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47 355 contaminated by enterobacteria and staphylococci. Due to recent legislative changes  
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49 356 introduced at EU level the manufacturers need to obtain detailed information on possible  
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51 357 microbiological risks in their production lines and to design additional control actions in order  
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53 358 to assure the supply of safe and high quality products to the market.  
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3 478 **Figure 1.** Evolution of the total number of enterobacterial isolates in the products  
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5 479 analyzed during a 9-month shelf life study.  
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8 480 **Figure 2.** Evolution of the TVC isolates in the products analyzed during a 9-month  
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483 **Table 1. Identification of all enterobacterial isolates obtained from the products**  
 484 **containing *Engraulis* spp.**

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

485 †SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system.

486 Good identification by this system is normally considered for SIM index values above 0.3.

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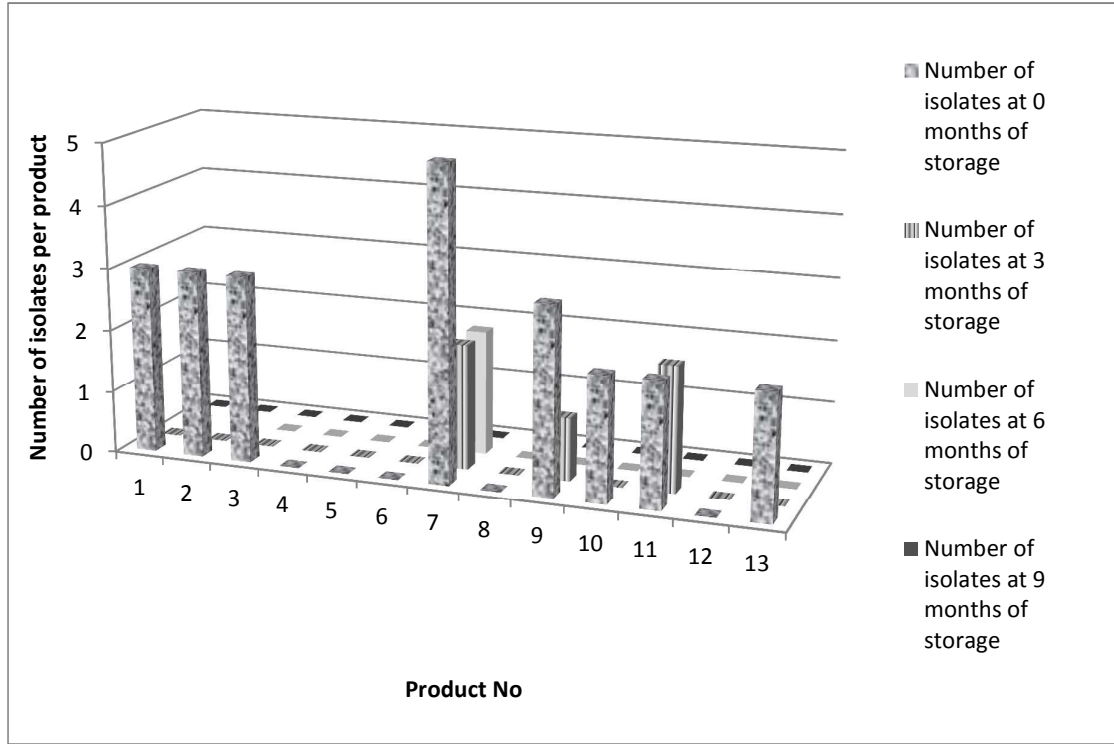
**Table 2. Identification of the total viable count (TVC) isolates from the ready-to-eat products analyzed.**

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

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3 490 †SIM – Biolog similarity index value (between 0 and 1) provided for each identification by the Biolog system.  
4 491 Good identification by this system is normally considered for SIM index values above 0.3.  
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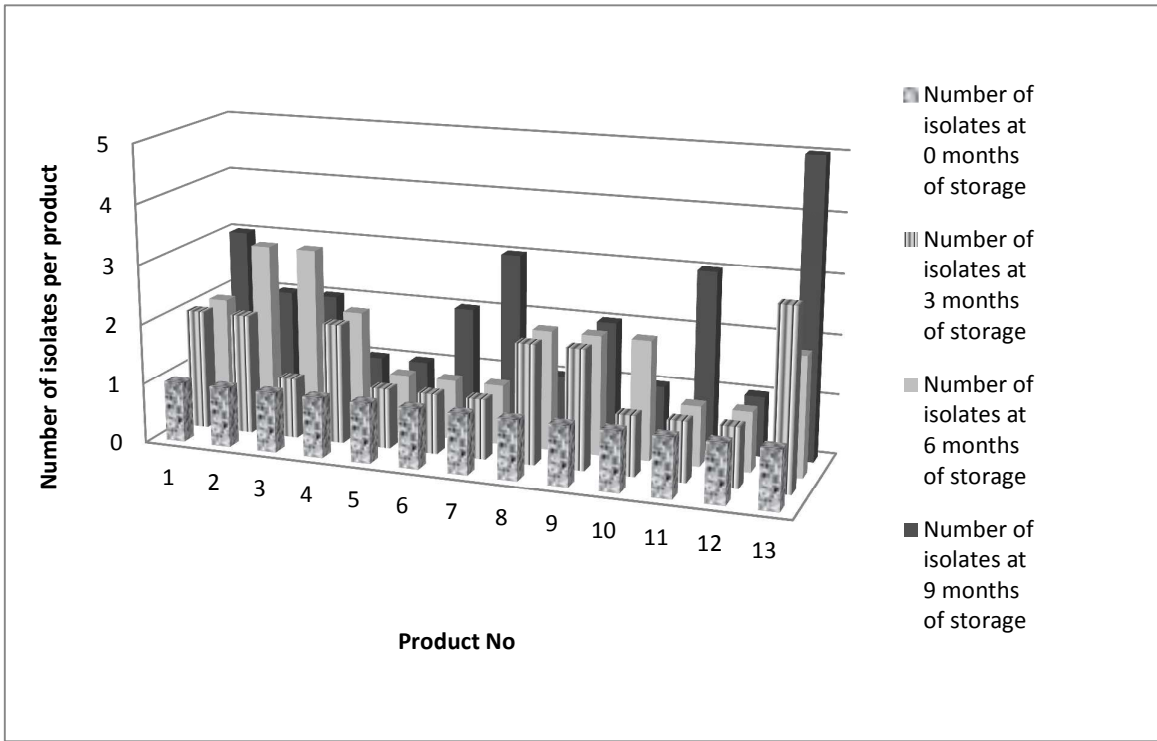
Fig. 1.



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**Fig. 2.**



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