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Shelf-life and microbiological properties of refrigerated sea urchin *(Paracentrotus lividus)* roe

Haltbarkeit und mikrobiologischen Eigenschaften von gekühltem Seeigel (Paracentrotus lividus) Rogen

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Summary

The aim of this work was to study the shelf-life and bacteriological features of *Paracentrotus lividus* fresh roe sold in the city of Palermo (Sicily, Italy). 21 glass jars, each approximately containing the fresh roe of 50 *Paracentrotus lividus,* were analysed in order to assess the shelf-life during the refrigerated storage and to evaluate the presence of bacteria belonging to genera *Vibrio, Aeromonas, Listeria, Salmonella* and *Clostridium*. The sensorial acceptability was recorded until the 72th hour, afterward off-odours and loss of firmness were observed. Sensorial characteristics were mainly related to the growth of hydrogen sulphide producing bacteria. *Listeria* spp and *Salmonella* spp. were not isolated.

Vibrio spp. were isolated from 71.43 % of samples; the species most frequently found were *V. alginolyticus* (47 %), *V. harveyi* (16 %), *V. mimicus* and *V. mediterranei* (10 %), followed by *V. hepatarius* (7 %), *V. rotiferanus* and *V. diabolicus* (5 %) and *V. ponticus* (2 %). Although the isolated *Vibrio* strains are rarely cause of foodborne diseases, the frequent raw consumption of sea urchin roe could pose some food safety issues.

Keywords: sea urchin roe; *Paracentrotus lividus;* shelf-life; microbial quality; *Vibrio* spp.

Zusammenfassung

Das Ziel dieser Arbeit war es, die Haltbarkeit und die mikrobiologischen Eigenschaften von frischem, in Palermo (Sizilien, Italien) gekauften Rogen von *Paracentrotus lividus* zu untersuchen. 21 Gläser, von denen jedes annähernd 50 g frischen Rogen von *Paracentrotus lividus* enthielt, wurden zur Beurteilung der Haltbarkeit während der gekühlten Lagerung untersucht. Die Proben wurden auf Bakterien der Gattungen *Vibrio, Aeromonas, Listeria, Salmonella* und *Clostridium* analysiert. Eine sensorische Akzeptanz wurde bis zu 72 Stunden erfasst, danach wurden unangenehme Gerüche und ein Verlust der Festigkeit beobachtet. Sensorische Besonderheiten waren hauptsächlich mit einem Wachstum von Schwefelwasserstoff produzierenden Bakterien verbunden.

In 71,4 % der untersuchten Proben konnte genotypisch und phänotypisch *Vibrio* spp. identifiziert werden. Es waren mehrere *Vibrio*-spezies wie: *V. alginolyticus* (47 %); *V. harveyi* (16 %); *V. mimicus* und *V. mediterranei* (10 %); *V. hepatarius* (7 %); *V. rotiferanus* und *V. diabolicus* (5 %) und *V. ponticus* (2 %). Obwohl die isolierten *Vibrio*-Stämme selten eine Ursache für menschliche Erkrankungen sind, könnte ein häufiger Verzehr von rohem Seeigel-Rogen einige Fragen zur Lebensmittelsicherheit darstellen. In dieser Untersuchung konnten *Listeria* spp. und *Salmonella* spp. nicht nachgewiesen werden.

Schlüsselwörter: Seeigelrogen; *Paracentrotus lividus;* Haltbarkeit; mikrobiologische Ergebnisse; *Vibrio* spp.

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Introduction

Sea urchins are members of a large group of marine invertebrates, phylum *Echinodermata,* class *Echinoidea.* Usually they live on rocky substrates and in seagrass meadows, from shallow waters down to about 20 m depth and feed mainly on sloughed or broken kelp but also dead fish, sponges, mussels, and small animals (Tortonese, 1965; Palese and Palese, 1991; Crook et al., 2000; Price, 2000). In recent decades, sea urchin fishery has developed throughout the world in response to increasing demand for their roe (Hagen, 1996) which are appreciated especially in Asian and Mediterranean cuisine. The largest fishery is in Japan, Chile and USA, while in Europe the catch is smaller, supplying domestic market (Kelly et al., 2001).

In the Mediterranean Sea, purple sea urchin *Paracentrotus lividus* that is the most valuable species, is widely distributed; it also occurs along the North-eastern Atlantic coast, from Scotland and Ireland to southern Morocco (Boudouresque and Verlaque, 2001). Its roe is particularly appreciated for the strong salty or iodic smell; the growth is considered slow and the harvestable size is reached in about 4 years (Allain, 1978). In Italy, the harvest is widely exerted in southern regions (Tortonese, 1965; Guidetti et al., 2004), especially from December through April when the development of gonad is at its maximum.

In European market, sea urchin roe is primarily sold fresh, while only a small amount is frozen. Frozen roe, that partially maintains its fresh taste and typical texture, is used prevalently for cooked dishes, as in the case of canned sea urchin. The latter, obtained from other species than *Paracentrotus lividus,* is imported especially from South America and is widely commercialised due to its long shelf-life, despite its sensorial characteristics are less appreciated.

In the Japanese markets urchin roe is also sold baked, steamed, and salted. Salting is used primarily for lower-grade roe (Kato and Schroeter, 1985).

Fresh sea urchin roe, as other fresh seafood, are highly perishable and spoilage begins as soon as the physical condition of the sea urchin begins to deteriorate (Kelly, 2004). Despite sea urchin are not filter-feeding organisms as mussels, their microflora is mainly constituted by marine bacteria. According to Unkles (1977), microrganisms most frequently isolated from *Echinus esculentus* belong to genera *Vibrio, Aeromonas* and *Pseudomonas.* With regard to *Vibrio* spp., Sawabe et al. (1995) isolated *Vibrio* spp. in 96.6 % of bacteria from *Strongylocentrotus intermedius* and *Strongylocentrotus nudus;* El-Sahn et al. (1982) found a high prevalence of *Vibrio parahaemolyticus* in *Echinus* spp. samples from the Mediterranean Sea around Alexandria (Egypt), during the summer season.

However, sea urchin can be also contaminated during processing by several kinds of spoilage microorganisms and food poisoning bacteria. Kajikazawa et al. (2007a) isolated *Bacillus cereus, Bacillus weihenstephanensis, Staphylococcus aureus, Staphylococcus equorum, Stenotrophomonas maltophila, Bukholderia cepacia* and *Serratia proteamaculans* by using 16S rDNA sequence analysis in processed fresh edible sea urchin.

In Italy, especially along the southern region shores, a large amount of sea urchin sold at retail is previously shelled and roe is packed in small plastic or glass jars. This aims to increase the consumer convenience, allowing an easier storage at domestic or restaurant level. In this regard, the aim of this work was to study the shelf-life and bacteriological features of this kind of product, sold in the city of Palermo (Sicily, Italy).

Materials and methods

Sampling plan

In this study were used 21 glass jars each approximately containing the fresh roe of 50 specimen of *Paracentrotus lividus.* Products were obtained from 7 seafood retailers, using sea urchins harvested along the shore of Palermo (Sicily, Italy). All samples, kept at 4 °C, were transported to the laboratory within three hours and analysed at time 0 and after 24, 48, 72 and 96 hours of refrigerated storage at 2±1 °C, with regard to: Total Viable Count at 30°C and 18°C (TVC-30 °C and TVC-18 °C, respectively); counts of Hydrogen Sulphide Producing Bacteria (HSPB), Total Coliforms (TCs), Sulphite Reducing Clostridia (SRC), *Aeromonas* spp. and *Vibrio* spp.; qualitative analysis of *Salmonella* spp. and *Listeria monocytogenes;* measurement of pH; sensory evaluation.

Microbiological analysis

For microbiological analysis 10 g of sea urchin roe from each jar were sampled under aseptic conditions, diluted in 90 ml of buffered peptone water (BPW; Oxoid, Italy) and homogenized in sterile plastic bags with a stomacher blender (model 400, International PBI s.p.a., Milan, Italy) for 2 min.

Tenfold dilutions were prepared in BPW and appropriate aliquots (0.1 or 1 ml) were poured or spread in duplicate on agar plates. Particularly, for TVC, the pour-overlay method on Plate Count Agar (Oxoid) was carried out and plates were incubated at 30 °C for 72 h (TVC 30 °C) and at 18 °C for 5 d (TVC 18 °C). TCs were enumerated by the pour-overlay method using Violet Red Bile Glucose agar (Oxoid). Plates were incubated at 37 °C for 24 h. Purple colonies with halo larger than 0.5 mm in diameter were enumerated and recorded as coliform bacteria. Iron Sulphite agar (Oxoid) was used for the count of HSPB. Iron sulphite agar plates were incubated aerobically at 25 °C and black colonies were enumerated after 3 days.

The count of *Aeromonas* spp. was performed by spreading 0.1 ml of each BPW dilution onto *Aeromonas* medium base (Oxoid) plus ampicillin selective supplement (Oxoid). Plates were incubated at 35 °C for 24 h, and opaque green colonies with a dark centre were picked and purified by streaking onto tubes of Trypticase Soy agar (TSA) (Oxoid). The identification was performed by Gram stain and biochemical tests (API 20E bioMérieux, Marcy l'Etoile, France).

SRC were counted by inoculating 1 ml of each dilution into Sulphite Polymixine Sulfadiazine agar (SPS) (Oxoid) plates incubated at 37 °C for 48 h using anaerobic jars with a gas-generating kit (bioMérieux); black colonies, presumptively considered as SRC, were phenotypically identified by API 20A system (bioMérieux).

For the analysis for *Listeria monocytogenes,* 5 g portion of each sample was mixed with 45 ml of Half Fraser broth (Oxoid), homogenised in a stomacher blender for 2 min. and then incubated at 30 °C for 24 h. Afterwards, an aliquot

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(0.1 ml) of pre-enriched culture was transferred into Fraser broth (Oxoid) and incubated at 37 °C for 48 h. After the incubation period, the culture was streaked onto *Listeria*-selective agar, Oxford formulation (Oxoid). The plates were examined for typical colonies of *Listeria* after 24 h and 48 h of incubation at 37 °C. Presumptive positive colonies were identified by Gram stain, catalase test and by API *Listeria* kit (bioMérieux).

For the analysis for *Salmonella* spp., 10 g of each sample was diluted in 90 ml of BPW and incubated at 35 °C for 18 h; then, 0.1 ml and 1 ml of pre-enrichments were transferred, respectively, into 10 ml of Rappaport-Vassiliadis Enrichment Broth (Oxoid) tubes and into 9 ml of Selenite Broth Base (Oxoid) tubes. These enrichments were incubated, respectively, at 42±1 °C for 24–48 h and at 35±1 °C for 18–24 h, and streaked onto plates of Xylose Lysine Desoxycholate Agar (XLD medium, Oxoid) and Desoxycholate Citrate agar (Oxoid) and incubated at 35±2 °C for 24–48 h. Presumptive colonies were subjected to serological (Sclavo, Italy) and biochemical (API 20E bioMérieux) tests.

The count of *Vibrio* spp. was carried out by spreading 0.1 ml of each BPW dilution onto Thiosulfate Citrate Salt Sucrose Agar (TCBS) (Difco, Italy) plates, incubated at 30 °C for 24 h. Suspect colonies were subcultured on TSA (Oxoid) with 2 % NaCl and, according to Ottaviani et al. (2003), examined for Gram reaction, mobility, oxidase (identification sticks, Oxidase, Oxoid), carbohydrate fermentation and the production of hydrogen sulphide on Kliger iron agar (KIA) (Oxoid) and resistance to vibriostatic agent 0/129 (10 and 150 µg disks) (Oxoid). A total of 105 strains presumptively belonging to *Vibrio* genus were subjected to a specific Polymerase Chain Reaction, based on the RNA polymerase alpha subunit *(rpoA)* gene, according to Dalmasso et al. (2009). In particular, strains were inoculated in Tryptone Soya Broth (Oxoid) overnight at 30 °C and DNA was extracted using the following protocol: 1 mL of broth culture was centrifuged at 12,000 rpm for 5 min; the pellet was resuspended in 200 µl of sterile deionized water, boiled for 5 min, and centrifuged again at 4 °C. The lysate supernatant was stored at –20 °C until use. DNA was quantified by means of a spectrophotometer (SmartSpectm Plus, Bio-Rad, Milan, Italy).

The PCR for *rpoA* gene was performed according to Dalmasso et al. (2009) by using the primers 5'-AAATCAGGCTCGGGCCCT-3' (sense) and 5'-GCAATTTT(A/G)TC(A/G/T)AC(C/T)GG-3' (antisense), corresponding, respectively, to positions 294 to 311 and 519 to 535 of *V. parahaemolyticus,* in a PCR Sprint thermal cycler (Hybad, UK). The PCR products were resolved by electrophoresis on a 2 % agarose gel (Sigma-Aldrich, Italy), run in Tris acetate EDTA buffer (Eppendorf, Italy) for 45 min at 100 V, stained with ethidium bromide (Sigma-Aldrich) for 20 min and observed and recorded using a gel documentation system (Bio-Rad).

Finally, species identification was performed on the basis of API 20E system (bioMérieux) and biochemical analyses as proposed by Noguerola and Blanch (2008).

Sensory analysis and pH measurement

The sensory evaluation was performed, at each time interval, by a trained sensory panel of three persons, considering odour, appearance and taste. The pH value was recorded by a pH meter (mod. WTW pH330i.) by dipping the glass electrode into the jar containing the samples.

Results and discussion

Microbial counts, sensory analysis and pH measurement

Microbial counts and their trends during the storage period at 2±1 °C, are shown in Figs. 1–3. In particular, at 0 hours, TVC at 30 °C ranged from lg 2.95 to lg 5.14 cfu/g with a mean of lg 4.21±0.69 cfu/g while TVC at 18 °C ranged from lg 2.60 to lg 3.98 cfu/g with a mean of lg 3.50±0.46 cfu/g. These parameters tended to slightly increase during the storage reaching lg 4.60±0.19 and 4.05±0.36 cfu/g at 96 hours, respectively (Fig. 1). TCs and HSPB, at 0 hours, showed similar mean loads (respectively, lg 2.82±1.03 cfu/g and lg 3.15±1.09 cfu/g); however TCs slightly decreased during the storage to lg 2.52±0.59 while HSPB reached lg 4.37±0.58 at 96 hours (Fig. 2). *Aeromonas* spp. and *Vibrio* spp. counts at 0 hours averaged lg 2.66±1.01 and lg 3.38±1.42 cfu/g, respectively, decreasing during the storage to lg 1.99±0.53 cfu/g and lg 1.32±0.72 cfu/g, respectively (Fig. 3). Finally, SRC were counted in 9 samples only with a concentration between lg 1 cfu/g and lg 2 cfu/g.

With regard to sensory evaluation, samples maintained a salty smell and taste as well as a high degree of firmness until the 24th hour. After 48–72 hours samples had a iodic odour or, in some cases, smelled sour; furthermore they were beginning to be soft and with a bitter taste. At the 96th hour smell and taste were ammoniacal and roe appeared widely devoid of firmness afterwards products showed a full sensorial decay despite the mean bacterial counts did not exceed lg 5 cfu/g.

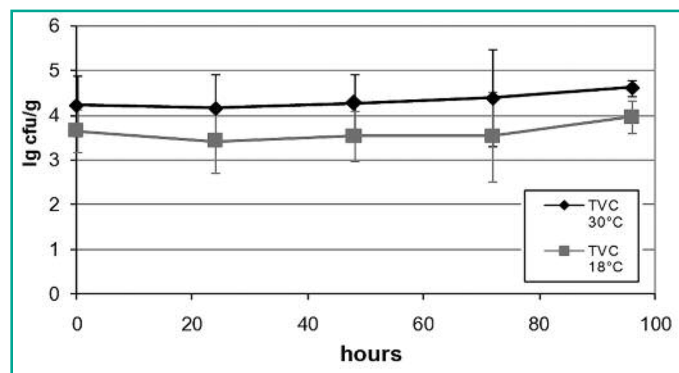

FIGURE 1: *Growth of Total Viable Count (TVC) at 30 °C (◆) and at 18 °C (■) in refrigerated sea urchin roe during the storage (Error bars indicate the standard deviation of 21 samples).*

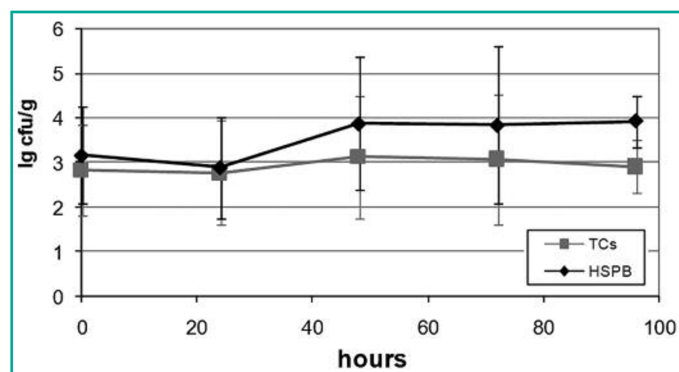

FIGURE 2: *Growth of Hydrogen Sulphide Producing Bacteria (HSPB) (◆) and Total Coliforms (TCs) (■) in refrigerated sea urchin roe during the storage (Error bars indicate the standard deviation of 21 samples).*

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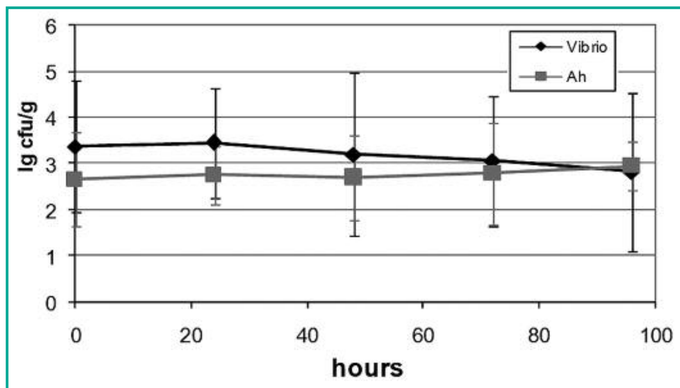

FIGURE 3: *Growth of Vibrio spp. (◆) and Aeromonas spp. (Ah) (■) in refrigerated sea urchin roe during the storage (Error bars indicate the standard deviation of 21 samples).*

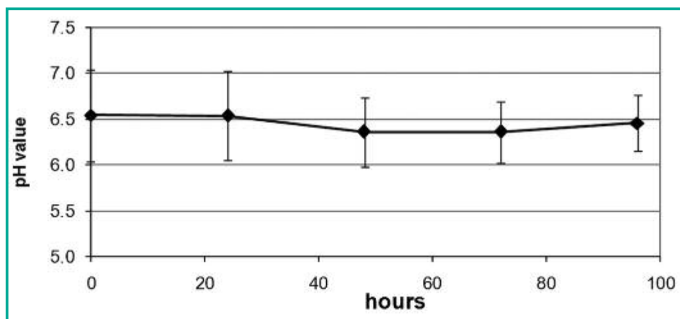

FIGURE 4: *pH value of sea urchin roe during the storage (Error bars indicate the standard deviation of 21 samples).*

FIGURE 5: *Agarose gel electrophoresis of the rpoA amplification products of thirteen strains. Lanes 1–3: V. alginolyticus, Lanes 4–6: V. harveyi, Lane 7: V. mimicus, Lane 8: V. mediterranei, Lane 9: V. hepatarius, Lane 10: V. rotiferanus, Lane 11: V. diabolicus, Lane 12: V. ponticus, Lane 13: positive control strain. M 100 pb ladder (Roche Applied Science, Italy), W: control reagents.*

This aspect agrees with Kajikazawa et al. (2007b) who found a mean bacterial load of lg 4.59 and a mean bacterial increase of lg 1.11 cfu/g from the first day of storage, in refrigerated sea urchin roe after nine days of storage.

These results show that sea urchin roe are greatly perishable, with a shelf life within 48–72 hours.

In this regard, HSPB were the only population with significant growth during the storage while the other bacterial populations did remain more o less constant or, as in the case of TCs, *Aeromonas* spp. and *Vibrio* spp., decreased. It is well known that hydrogen sulphide producing bacteria include several psychrotropic spoilage agents such as *Shewanella* spp. and some *Pseudomonas* or *Photobacterium* strains (Dalgaard, 1995; Gram and Dalgaard, 2002). All these species are recognized as Specific Spoilage Organisms (SSO) since they are able to produce spoilage metabolites (trimethylamine oxide, ammonia, biogenic amines, organic acids and sulphur compounds, hypoxanthine, etc.) in several seafood, and consequently, off-odours. Our results show a low SSO load can induce a high degree of sensorial decay of sea urchin roe during refrigerated storage; the related minimal spoilage level (MSL) could be identified in lg 4.5 cfu/g but it is also important to take into account the potential spoilage activity of native enzymes. In any case, other studies on novel packaging techniques such as modified atmosphere packaging, have to be carried out in order to increase the shelf life of sea urchin fresh roe.

Mean pH values, shown in fig. 4, were always around 6.5 with a slight decrease after 48 hours of storage.

Qualitative microbial analyses and identifications

Listeria monocytogenes and *Salmonella* spp. were not detected throughout storage time. Concerning SRC colonies from SPS agar, all strains were phenotypically belonging to *Clostridium beijerinckii.*

Overall, bacteria belonging to *Vibrio* genus were isolated in 71.43 % of samples. All colonies from TCBS agar, belonging to the *Vibrio* genus phenotypically (Gram-negative, oxidase-positive, mobiles, sensivity to vibriostatic agent 0/129, carbohydrate fermentation and no hydrogen sulphide production in KIA) led to a positive result for the rpoA gene (Fig. 5). The further biochemical identification, according to Nogeruola and Blanch (2008) allowed to identity different *Vibrio* species: *V. alginolyticus* (47 %), *V. harveyi* (16 %); *V. mimicus* and *V. mediterranei* (10 %); *V. hepatarius* (7 %); *V. rotiferanus* and *V. diabolicus* (5 %) and *V. ponticus* (2 %).

Although the isolated *Vibrio* strains are rarely cause of foodboorne diseases (Austin, 2010), the frequent raw consumption of sea urchin roe could pose some food safety issues.

It is well known that *Vibrio* infections are generally divided into three well recognized clinical syndromes: gastroenteritis, primary septicaemia and wound infection (Tantillo et al., 2004). Gastroenteritis and primary septicaemia are acquired through ingestion of contaminated raw or undercooked seafood while wound infection is related to the handling of seafood, harvesting of shellfish, shucking of oysters, etc. (Lefkowitz et al., 1992; Fiore et al., 1996). *V. alginolyticus* has been implicated with ear, soft tissue and wound infections (Horii et al., 2005) and is rarely associated with primary septicaemia while *V. mimicus* could be rarely associated to gastroenteritis and wound infection.

Conclusion

This study confirms the high perishability of shelled sea urchin which reaches its time of rejection within 48–72 hours. This short shelf life could not allow a wide commercialisation unless a novel packaging techniques was applied. In this regard, the application of modified atmosphere packaging could not appear effective due to the activity of some psycrothrophic spoilage bacteria such as *Photobacterium phosphoreum* which is able to grow under anaerobic or semi-anaerobic conditions, as shown for other packed seafood (Dalgaard et al., 1997; Gram and Dalgaard, 2002).

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With regard to food safety concerns, the frequent isolation *V. alginolyticus, V. harveyi* and *V. mimicus* which are rarely causes of gastroenteritis (Austin, 2010), does not involve a high risk for consumers. However, these bacteria are associated to wound infection which appears to be especially linked with occupational activities around seawater. In particular, some studies have focused on the biohazards posed by *Vibrio* spp. in the shellfish industry (Lefkowitz et al., 1992; Fiore et al., 1996). Since the manual processing (handling and shucking) of sea urchin can easily produce skin lesions, we believe that an efficient surveillance system on *Vibrio* infections have to take into account also in this segment of seafood industry.

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