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RESEARCH ARTICLE – Food Microbiology

Molecular characterization of *Shewanella* and *Aeromonas* isolates associated with spoilage of Common carp (*Cyprinus carpio*)

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ABSTRACT

Storage in ice is a common way of preserving commercial fish species but some microorganisms can still contaminate and participate in the spoilage of the product; therefore, identification of potential harmful microbes is important. Thirteen colonies were isolated from common carp (*Cyprinus carpio*) that had been stored in ice, whose phenotypic identification revealed that they belonged to the genera *Aeromonas* ($n = 5$) and *Shewanella* ($n = 8$). Molecular genotyping with ERIC-PCR showed clonality only among two of the five *Aeromonas* isolates and for two groups ($n = 3$; $n = 2$) of the eight *Shewanella* isolates. Sequencing the *rpoD* gene showed that four *Aeromonas* isolates belonged to the species *Aeromonas salmonicida* and one to *A. sobria*. Of the eight *Shewanella*, seven isolates cluster with *Shewanella putrefaciens* and one with *Shewanella profunda* in the 16S rRNA phylogenetic tree. However, analysis of the *gyrB* gene showed that these eight isolates could constitute a new species closely related to *S. baltica*. The *Shewanella* and *A. salmonicida* isolates produce off-odours and reduce trimethylamine oxide, indicating that they might contribute to the spoilage of the fish.

Key words: fish spoilage; *Aeromonas*; *Shewanella*; *rpoD*; *gyrB*

INTRODUCTION

Bacteria present in skin, guts and gills of newly caught fish can change through the stages of food processing and storage and may contribute to the spoilage of fish before consumption. Iced fresh fish is most commonly spoiled by Gram-negative psychrotrophic rods such as members of the genera *Shewanella*,

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Pseudomonas and *Aeromonas* (Gram and Dalgaard 2002; Vogel *et al.*, 2005; Parlapani *et al.*, 2013; Janda and Abbott 2014).

Aeromonas species are autochthonous inhabitants of aquatic environments and can be isolated from clinical and environmental samples, including water, healthy or diseased fish, food products or from infections of humans and animals (Beaz-Hidalgo *et al.*, 2010; Janda and Abbott 2010; Beaz-Hidalgo and Figueras 2013; Figueras and Beaz-Hidalgo 2014). The psychrotrophic species *Aeromonas salmonicida* is responsible for producing furunculosis, an important fish infection (Beaz-Hidalgo and Figueras 2013) and has also been identified as part of spoilage microbiota in ice-stored fish (Parlapani *et al.*, 2013).

The genus *Shewanella* is an ubiquitous group of bacteria that embraces psychrophilic and mesophilic species that are commonly isolated from a wide range of habitats, terrestrial sites and marine environments including seawater, marine sediments, invertebrates and fish (Bowman 2005; Vogel *et al.*, 2005; Sung, Yoon and Ghim 2012; Janda and Abbott 2014). *Shewanella* species are commonly linked to the spoilage of proteinaceous chilled foods and are opportunistic pathogens of humans and aquatic animals (Bowman 2005; Sung *et al.*, 2012; Janda and Abbott 2014). The species *Shewanella putrefaciens* is a bacteria that is well known for spoiling iced fish such as cod or mackerel that produce off-odours (described as 'sulphur', 'dish rag' or 'wet dog' odours), off-flavour and slime (Parlapani *et al.*, 2013; Janda and Abbott 2014). This species was first isolated from common carp by Kozińska and Pękala (2004) and was described as pathogenic to fish. *Shewanella baltica* was found to be the dominant species producing spoilage in ice-stored fish caught in the Danish Baltic Sea, despite other psychrotolerant H_2S-producing species have also been isolated like *S. algae, S. glacialipiscicola* and *S. algidipiscicola* (Vogel *et al.* 2005; Satomi *et al.*, 2007).

Commercial phenotypic systems and conventional biochemical assays are able to identify *Aeromonas* and *Shewanella* strains to genus level but confusing and incorrect identifications to species level are common because of their variable response to many phenotypic tests (Figueras *et al.*, 2011). Nowadays, molecular methods like sequencing housekeeping genes allow more reliable identification to species level (Castro-Escarpulli *et al.*, 2003; Satomi *et al.*, 2007; Beaz-Hidalgo *et al.*, 2010; Figueras *et al.*, 2011; Beaz-Hidalgo and Figueras 2013; Figueras and Beaz-Hidalgo 2014; Janda and Abbott 2014), but these methods are not commonly used in food control laboratories.

Common carp (*Cyprinus carpio*) is an important commercial food product in Argentina. However, there is scarce information on the microbiological spoilage of this freshwater fish in Argentina and there are few studies that have investigated the incidence of spoilage microorganisms in iced fish using molecular methods (Castro-Escarpulli *et al.*, 2003; Parlapani *et al.*, 2013). Therefore, the aims of the present study were to phenotypically and genetically characterize and identify bacteria that have the potential to spoil common carp stored in ice and to determine their spoilage potential.

MATERIALS AND METHODS

Sources of the isolates

Sampling was performed for the development of a quality index method for common carp mainly based on changes in sensory properties (skin, texture, appearance of eyes and gills). At the time of sampling, the approximate temperature of the water was 17°C. The catching, handling and preservation of the carp was performed according to current practices undertaken by fishermen and adjusted according to the guidelines established for good manufacturing practices.

Isolates were obtained from six carp specimens captured from a lagoon located in Buenos Aires, Argentina. The fish were slaughtered, eviscerated and stored in ice in a chilled chamber at 2 ± 1°C. The sensory rejection point based on the evaluation of sensory attributes of the raw fish (based on 'gill smell' and 'odour' of cooked carp) occurred at day 18 of storage in ice. At this time, 10 g of dorsal muscle were aseptically removed, diluted in sterile peptone water (90 mL) and homogenized in a stomacher blender (Stomacher 400-Labsystem) for 2 min. Then, 10-fold serial dilutions were made up to 10^{-6} and were inoculated on iron agar (Gram, Trolle and Huss 1987) and incubated at 25°C for 3 days to recover bacteria that produce hydrogen sulphide (H_2S), as previously described (Hozbor *et al.*, 2006).

Phenotypic characterization

For the phenotypic characterization and identification, a total of 27 phenotypic tests (Table S1, Supporting Information) were carried out as described by the International Commission on Microbiological Specifications for Foods (1983), Cowan and Steel's Manual (Barrow and Feltham 1999) and in Bergey's Manual of Systematic Bacteriology (Bowman 2005; Martin-Carnahan and Joseph 2005). In parallel, the commercial identification system API 20NE (bioMérieux, Marcy I´Etolle, France) was used.

Molecular typing and identification

DNA was extracted from single colonies after growth in trypticase soya agar at 30°C for 24 h using the InstaGene Matrix (Bio-Rad). In order to determine the presence of clones, isolates were genotyped by enterobacterial repetitive intergenic consensus (ERIC)-PCR using the primers and conditions described by Versalovic, Koeuth and Lupski (1991).

Sequences of the *rpoD* gene were obtained to genetically identify the isolates presumed to be *Aeromonas* based on phenotypic tests as previously described (Soler *et al.*, 2004). The phenotypically identified *Shewanella* isolates were genetically identified by sequencing the 16S rRNA and the *gyrB* genes as previously described (Martínez-Murcia, Benlloch and Collins 1992; Yamamoto and Harayama 1995).

The *rpoD* sequences obtained were aligned with sequences of type strains of members of the genus *Aeromonas* taken from our in-house database (Martínez-Murcia *et al.*, 2011), and the 16S rDNA and *gyrB* sequences were aligned with those of the type strains of members of the genus *Shewanella* that were available at the GenBank. Genetic distances and clustering were obtained using Kimura´s two-parameter model, phylogenetic trees were constructed using the neighbour-joining method and pairwise similarities of the 16S rRNA and *gyrB* genes were calculated with the MEGA5 program (Tamura *et al.*, 2011).

Spoilage evaluation

The fish isolates were inoculated in sterile carp broth (Gram *et al.*, 1987) and incubated at chill temperature (5°C) for 3–4 weeks in order to determine spoilage. Six panellists experienced in sensory assessment evaluated the developing odours by ranking them as 'fishy', 'sulphidy', 'sour', 'sweet', 'wet dog', etc. (Gram, Wedell-Neergaard and Huss 1990) and categorizing the strength of odour into three scores: I (strains producing no off-odours), II (strains producing light off-odours) and III (strains producing strong off-odours). The production of an off-odour

was considered positive with scores greater than II (Dalgaard 1995).

Trimethylamine oxide (TMAO) medium was also used for testing the ability of the strains to reduce TMAO to trimethylamine (TMA), which is usually responsible for the odours associated with bacterial food spoilage (Gram *et al.*, 1987). The test was carried out under anaerobic conditions at 25°C for 3 days.

RESULTS

Phenotypic identification

Thirteen black H_2S-producing colonies were isolated from iron agar. All the isolates were Gram-negative rods, aerobic/anaerobic facultative, motile and oxidase positive. Other phenotypic tests are outlined in Table S1 (Supporting Information). Eight of the 13 isolates were not able to ferment glucose and were presumptively identified as *Shewanella* sp. The remaining five isolates were able to ferment glucose and were identified as members of the genus *Aeromonas*, of which four isolates (A3, A11, A12, A13) were identified as *A. hydrophila/A. caviae* with the API 20NE. The other *Aeromonas* (A8) and all *Shewanella* isolates could not be assigned to a known species in the API 20NE database.

Molecular and genotypic identification

Identical ERIC-PCR profiles were observed for two (A11 and A12) of the five presumptive *Aeromonas* isolates and for two (A1 and A7) and three (A6, A9 and A10) of the eight presumptive *Shewanella* isolates (data not shown).

The *rpoD* phylogenetic tree showed that isolates A3, A11, A12 and A13 belong to the species *A. salmonicida* as they clustered with its type strain with a 100% bootstrap value. Isolates A11 and A12 showed an identical *rpoD* sequence and this confirmed the clonal relationship obtained with the ERIC-PCR. Strain A8 clustered with the species *A. sobria* and also with a 100% bootstrap value (Fig. 1).

The other eight isolates presumptively identified as belonging to the genus *Shewanella* were confirmed as such from their 16S rRNA and *gyrB* genes sequences. As expected, clonal isolates showed identical sequences of both genes. Of the eight *Shewanella* isolates, seven cluster with *S. putrefaciens* (56% bootstrap) and one with *S. profunda* (49% bootstrap) in the 16S rRNA phylogenetic tree (Fig. 2). In the *gyrB* phylogenetic analysis, all the eight isolates grouped together in a separate cluster supported with a 98% bootstrap having as the closest neighbour the species *S. baltica* (Fig. 3). Pairwise sequence similarities of both genes among the fish strains and with the closely related species are shown in Tables S2 and S3 (Supporting Information).

Spoilage potential

All the *Shewanella* isolates were able to produce very strong off-odours (III) in carp broth and were described as 'wet dog' odours, and all except strain A2 reduced TMAO after 48 h. The four *A. salmonicida* isolates produced weak odours (II) and were characterized as 'cheese' and 'sour' and all also showed the ability to reduce TMAO. However, the strain of the species *A. sobria* was unable to either produce off-odours or reduce TMAO.

DISCUSSION

The classic phenotypic tests carried out for identifying the isolates were only able to identify all *Shewanella* and *Aeromonas* isolates up to the genus level and the API 20NE system erroneously identified two strains as *A. hydrophila/A. caviae*. Results for some phenotypic tests obtained with *A. sobria* and *A. salmonicida* isolates differed from previously published data (Table S1, Supporting Information; indicated in brackets) (Abbott, Cheung and Janda 2003; Martin-Carnahan and Joseph 2005; Beaz-Hidalgo *et al.*, 2010). According to Austin and Austin (2007), the only subspecies of *A. salmonicida* that are H_2S producers are *salmonicida*, *pectinolytica* and *smithia*; in addition, both species produce a diffusible pigment and are non-motile. The *A. salmonicida* isolates obtained in this study produced H_2S, but did not produce pigment and were motile so they could not be assigned to any subspecies and therefore were considered as 'atypical' *A. salmonicida*. The inability to discriminate the subspecies of *A. salmonicida* due to the number of variable and atypical reactions has been previously described (Martínez-Murcia *et al.*, 2005).

For the *Shewanella* isolates, the negative response to the ornithine decarboxylase was a differential character from the closely related species *S. putrefaciens*, *S. baltica* and *S. profunda* (Toffin *et al.*, 2004; Bowman 2005). Furthermore, the isolates (except isolate A2) could be differentiated from *S. profunda* by their inability to grow at 6% NaCl (Toffin *et al.*, 2004).

Phenotypic identification systems can give misleading results because of the great variability of the responses and the discordant results using different methodologies; reliable molecular methods are required for a correct identification to species level (Beaz-Hidalgo *et al.*, 2010; Figueras *et al.*, 2011; Figueras and Beaz-Hidalgo 2014). For certain genera, the 16S rRNA gene sometimes lacks sufficient specificity for differentiating closely related species, as in *Aeromonas* and *Shewanella* (Figueras *et al.*, 2011; Janda and Abbott 2014). The interspecies similarity range of the 16S rRNA gene sequences for the genus *Shewanella* is from 93 to 99.9% (Janda and Abbott 2014) and for the genus *Aeromonas* is from 96.8 to 100% (Figueras *et al.*, 2011). Closely related species, such as *A. salmonicida, A. bestiarum* and *A. piscicola*, share 99.8–100% 16S rRNA gene similarity and therefore less conserved and more rapidly evolving housekeeping genes (like *rpoD* or *gyrB*) are required for the correct identification of them (Beaz-Hidalgo *et al.*, 2010; Figueras *et al.*, 2011; Figueras and Beaz-Hidalgo 2014). In the present study, *rpoD* sequencing enabled one *A. sobria* and four *A. salmonicida* isolates to be identified from ice-stored carp (Fig. 1). These species have been found previously in association with fish disease (Beaz-Hidalgo *et al.*, 2010; Vega-Sánchez *et al.*, 2014) and in frozen tilapia (*Oreochromis niloticus*) intended for human consumption (Castro-Escarpulli *et al.*, 2003). Most *A. salmonicida* environmental strains are psychrotrophic, increasing the probability of contaminating refrigerated food, including freshwater fish (Gram *et al.*, 1990; Castro-Escarpulli *et al.*, 2003). In fact, the *A. salmonicida* strains recovered in this study showed the atypical motile, mesophilic behaviour as other atypical *A. salmonicida* strains confusingly referred to as 'A. hydrophila' and classically represented by the reference strain Popoff 316 (Martínez-Murcia *et al.*, 2005; Beaz-Hidalgo *et al.*, 2010).

In a recent work, Parlapani *et al.* (2013) reported *Aeromonas* as part of the dominant microbiota at the end of the shelf life of farmed sea bream (*Sparus aurata*) stored in ice when detected by sequencing the 16S rRNA gene from DNA extracted directly from the fish flesh (culture-independent technique). These authors identified *A. salmonicida* as a co-dominating microorganism with *Pseudomonas fluorescens* and *S. putrefaciens*. However, the phylotypes of *A. salmonicida* identified by Parlapani *et al.* (2013) could in fact also belong to *A. bestiarum* or *A. piscicola* as these three species share an almost identical 16S rRNA gene

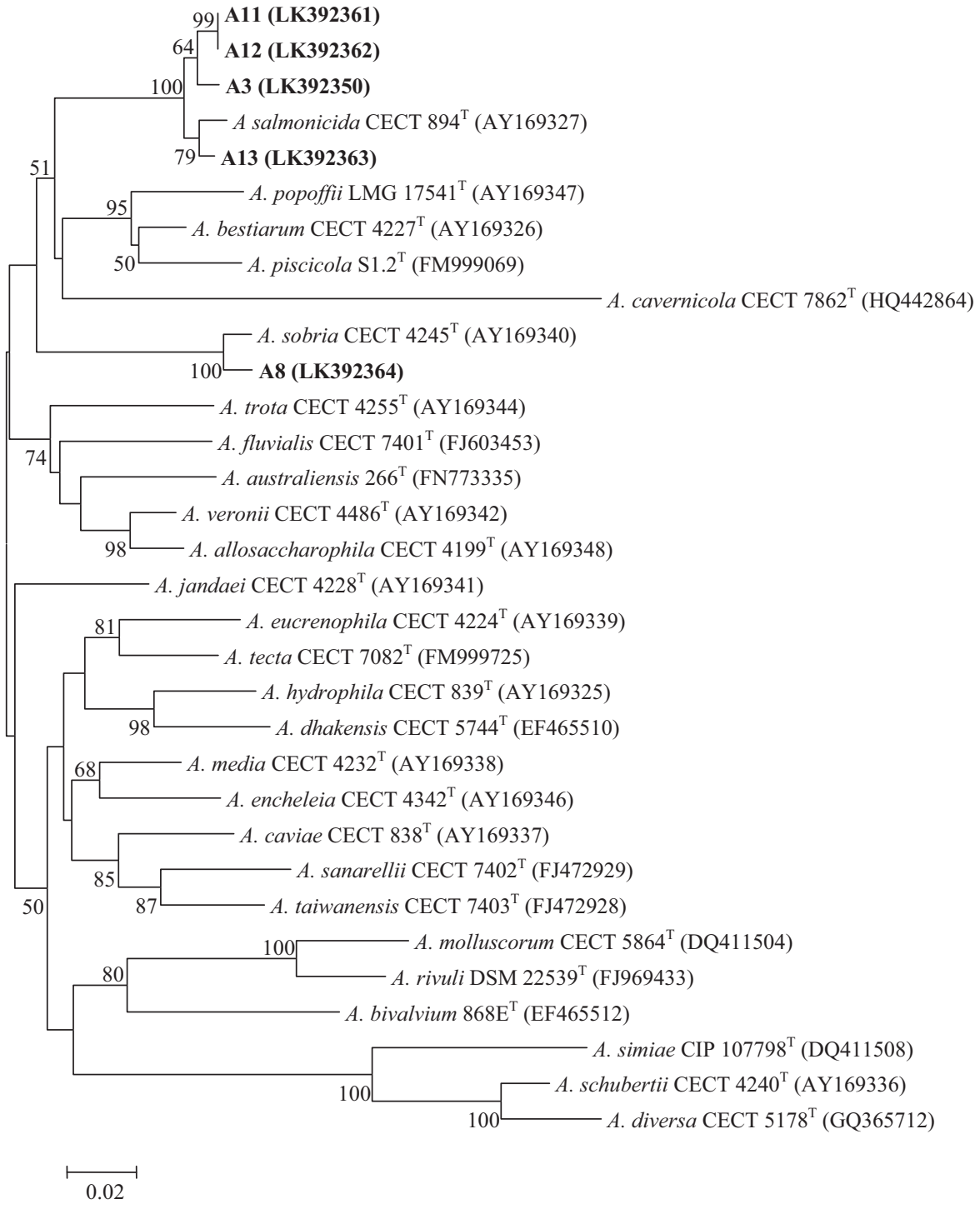

Figure 1. Unrooted neighbour-joining phylogenetic tree derived from *rpoD* gene (510 bp) sequences showing the relationships of *Aeromonas* fish isolates to the type strains of all other currently known species of *Aeromonas*. Numbers at nodes indicate bootstrap values (percentages of 1000 replicates, >50%). Bar, 0.02 substitutions per nucleotide position.

sequences. These authors also reported that *Aeromonas* could not be detected when cultivation-based techniques were used; however, they did not perform an enrichment step which could have increased the sensitivity of the culturing method. They also highlighted the importance of using molecular methods for detecting fish spoilage microbiota. However, one must also bear in mind that DNA detection by PCR does not imply that the bacteria is alive, so culturing techniques are useful for demonstrating the bacteria's viability. Other authors have also found that *Aeromonas* spp. represent a significant part of the spoilage microbiota in the Nile perch (*Lates niloticus*) stored at ambient temperature (Gram *et al.*, 1990), sea salmon (*Pseudopercis semifasciata*) stored in ice (Hozbor *et al.*, 2006) and in cold-smoked salmon (*Salmo salar*) (Stohr *et al.*, 2001).

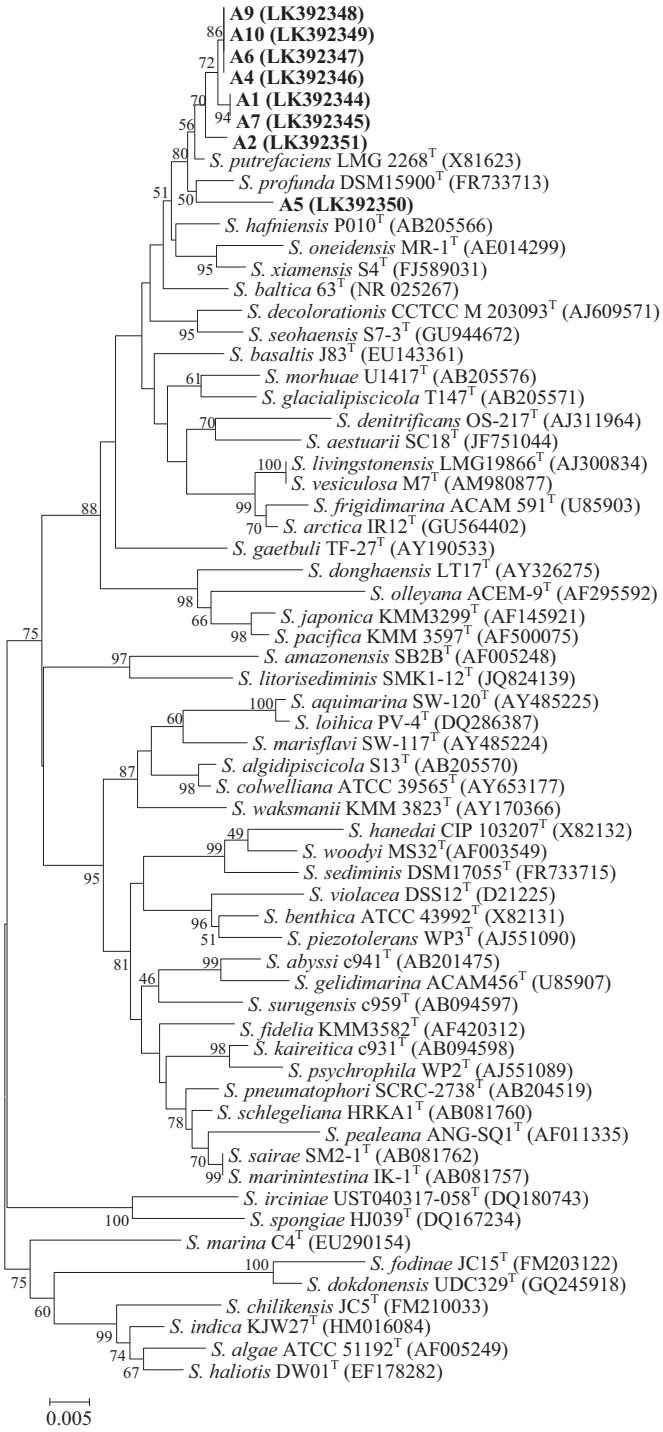

Figure 2. Unrooted neighbour-joining phylogenetic tree derived from the 16S rRNA gene sequences (1344 bp) showing the relationship between the carp isolates and members of the genus *Shewanella*. Numbers at the nodes indicate bootstrap values (percentage of 1000 replicates, >50%). Bar, 0.005 substitutions per nucleotide position.

The analysis of the 16S rRNA gene sequences showed that all *Shewanella* isolates except A5 shared the highest sequence similarity (99.4–99.8%) with the type strain of *S. putrefaciens* and in the phylogenetic tree clustered with the latter species with a low bootstrap of 56% (Table S2, Supporting Information; Fig. 2). However, we found that two sequences of the type strain of *S. putrefaciens* available in the GenBank from two different culture collections (accession numbers U91550 from strain ATCC 8071^T and X81623 from strain LMG 2268^T) differed by four nucleotides. The sequence from strain LMG 2268^T (X81623) was selected for analysis because the other sequence had an indetermination. This is a problem that to our knowledge has not been reported so far despite authors are using one or the other sequence indistinctly (Satomi *et al.*, 2007; Lee and Yoon 2012;

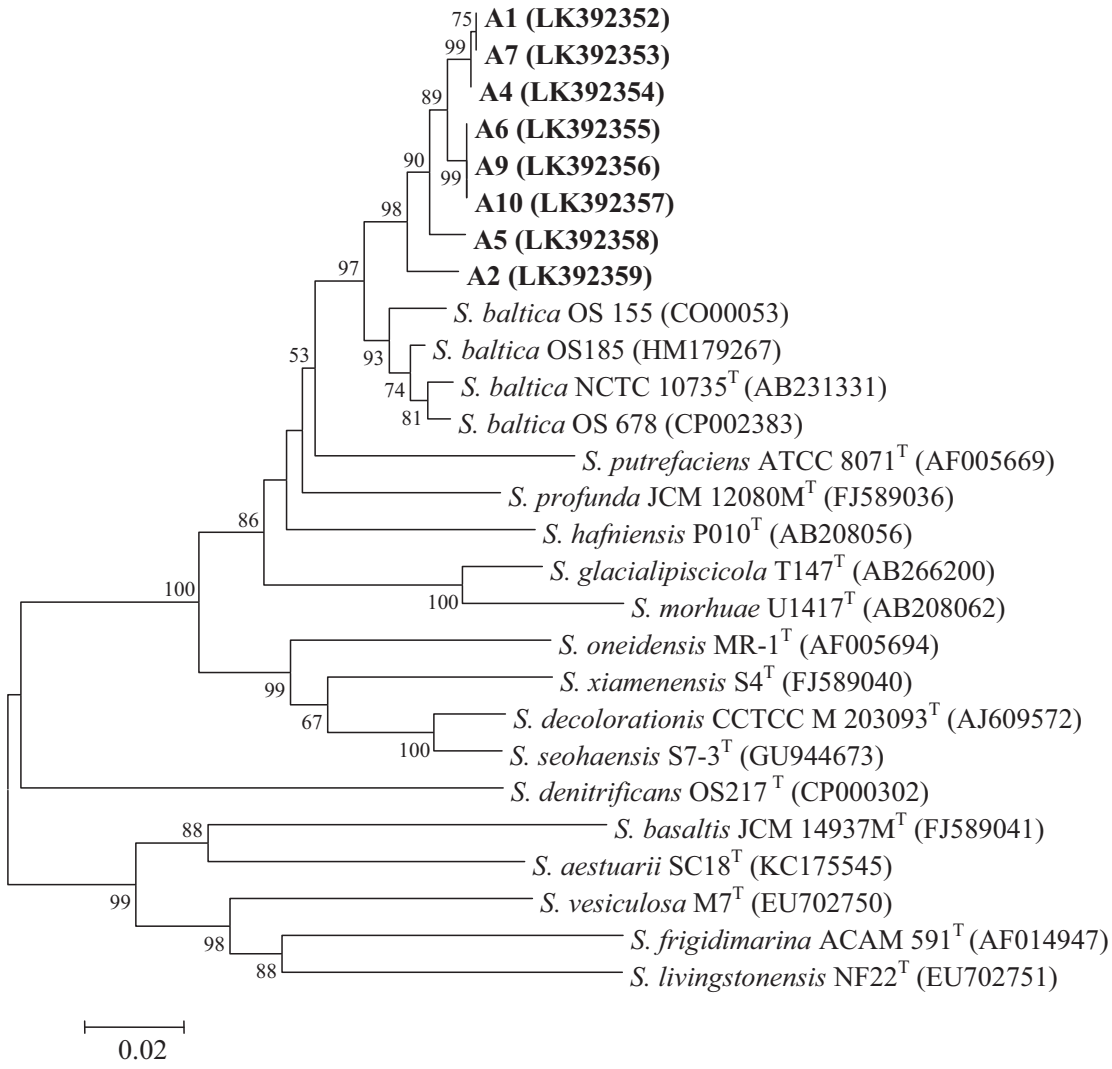

Figure 3. Unrooted neighbour-joining phylogenetic tree derived from the *gyrB* gene sequences (940 bp) showing the relationship between the fish strains and the most closely related members of the genus *Shewanella*. Numbers at the nodes indicate bootstrap values (percentage of 1000 replicates, >50%). Bar, 0.02 substitutions per nucleotide position.

Sung *et al.*, 2012; Yoon *et al.*, 2012; Park and Jean 2013), which could lead to different interpretations. Strain A5 formed in the 16S rRNA tree a long-independent branch that clusters with *S. profunda*, even though it shared practically the same similarity (98.6–98.7%) with *S. putrefaciens* and *S. profunda* (Table S2, Supporting Information).

Considering that previous studies have reported that the resolution of the 16S rRNA gene is not always enough to determine the precise phylogenetic positions for some *Shewanella* species (Satomi, Oikawa and Yano 2003; Satomi *et al.*, 2007; Janda and Abbott 2014), the *gyrB* gene was also sequenced to confirm the results. The type strains of the closest species based on the 16S rRNA gene phylogeny were included in the *gyrB* analysis except the species *S. artica* because its sequence could not be found. In contrast to the 16S rRNA results, all the isolates shared the highest *gyrB* similarity (96.3–96.8%) with *S. baltica* (Table S3, Supporting Information). Therefore, three additional *gyrB* sequences from strains of the latter species, used in a study performed by Deng *et al.* (2014), were added to the phylogenetic tree to balance the cluster formation (Fig. 3). Interestingly, the eight *Shewanella* isolates formed an independent cluster supported by a bootstrap of 98% indicating that they represent a different and unknown phylogenetic line close to *S. baltica*. Deng *et al.* (2014) also observed phenotypic and genetic heterogeneity among the 46 *S. baltica* strains analysed in their study and concluded that they might represent unnamed species. Based on the evidence presented, the eight fish isolates represent an unknown species within the genus *Shewanella* closely related to the halotolerant, psychrotolerant and non-halophilic species *S. baltica*, although further studies are required to confirm this. The species *S. baltica* together with *S. putrefaciens* are among the most common bacteria that spoil refrigerated food and are frequently isolated from dairy products, poultry, beef and seafood (Bowman 2005; Vogel *et al.*, 2005; Parlapani *et al.*, 2013). In fact, Vogel *et al.* (2005) found that the main H_2S-producing organism in ice-stored marine fish caught in the Danish Baltic Sea was *S. baltica* as they recognized that 76% (394/518) of the recovered strains belonged to this species. So far, *S. putrefaciens* has been considered the predominating microorganism that spoils marine species stored in ice (Gram and Huss 1996; Gram and Dalgaard 2002; Hozbor *et al.*, 2006; Parlapani *et al.*, 2013) and has also been found in common carp (Kozińska and Pękala 2004) and in other

freshwater fish, such as Nile perch (Gram *et al.*, 1990), trout (Chytiri *et al.*, 2004; Kozińska and Pękala 2004) and tilapia (Lu and Levin 2010). *Shewanella* spp. are opportunistic fish pathogens that can kill stressed and/or immunocompromised fish (Kozińska and Pękala 2004; Bowman 2005). Human infections such as bacteremia, soft tissue and eye infections, pneumonia, arthritis, peritonitis and epyema have been attributed to *S. putrefaciens* (Bowman 2005; Janda and Abbott 2014), highlighting the importance of its detection and identification in food, especially in goods that are sometimes consumed raw or poorly cooked, like fish.

Off-odours and -flavours developed in aerobically stored fish depend on the climatic and storage conditions, the type of fish and even its origin (Hozbor *et al.*, 2006). The TMAO (a naturally occurring substance found in many fish species) is a terminal electron acceptor present among *Shewanella* species and its reduction to TMA under anaerobic or microaerophilic conditions is responsible for the odours and 'ammonia-like' and 'fishy' off-flavours associated with fish spoilage (Gram and Dalgaard 2002; Hozbor *et al.*, 2006). Identifying specific spoilage bacteria relies on detecting sensory odours and chemical compounds of spoiled products, like TMA and H_2S (Gram and Dalgaard 2002). In the present study, the ability of all *Shewanella* strains to produce H_2S and to reduce TMAO indicates that they are active spoilers in the deterioration of eviscerated and chilled carp. The *A. salmonicida* isolates recovered from fish in this study were positive for the TMAO reduction test, indicating their contribution in the deterioration of the fish. It has been reported that *Aeromonas* is able to grow and spoil freshwater fish stored in ice or at room temperature (Gram *et al.*, 1990; Castro-Escarpulli *et al.*, 2003). Furthermore, Stohr *et al.* (2001) have linked bacteria of this genus to the production of off-odours in cold smoked salmon, using other specific sensory descriptors such as 'cheese-like', 'sour' and 'neutral. However, Gram *et al.* (1990) described the odours produced by 15 *Aeromonas* isolates present in the Nile perch as 'fishy', 'rotten' and 'sulphidy'.

In conclusion, the use of molecular tools in this study enabled to recognize the presence of *A. salmonicida, A. sobria* and of *Shewanella* strains belonging to an unknown phylogenetic lineage that is closely related to *S. baltica* in common carp stored in ice at the time of sensory rejection. The spoilage ability of the species *A. salmonicida* and of the potential new *Shewanella* sp. in chilled stored carp has also been proven.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSLE online.

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Conflict of interest statement. None declared.

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