Food Sci. Biotechnol. 22(1): 87-92 (2013)
DOI 10.1007/s10068-013-0012-1

RESEARCH ARTICLE

Identification of Extracellular DNase-producing Bacterial Populations on Catfish Fillets during Refrigerated Storage

Michael E. Hickey, Samuel A. Besong, Venu Kalavacharla, and Jung-Lim Lee

Received: 6 June 2012 / Revised: 24 August 2012 / Accepted: 18 September 2012 / Published Online: 28 February 2013

Abstract Food spoilage is a major problem faced by consumers across the globe. As an enzyme that degrades DNA, DNase production on fish tissue seemed likely to aid in fish spoilage. Based on physical characteristics, bacteria producing extracellular DNase were isolated on selective media. 16S rDNA sequences were obtained identifying isolates as bacteria belonging to *Aeromonas* spp., *Serratia* spp., *Shewanella* spp., and *Rahnella* spp. *Aeromonas* spp. were the predominant bacteria isolated in this study; this statistically suggests that *Aeromonas* spp. are dominant in DNase-producing bacterial populations on catfish tissue. Results obtained in this study suggest that extracellular DNase-producing bacteria play a large role in catfish spoilage and support the need for further research on the role of *Aeromonas* spp. in fish spoilage. *Rahnella* spp. was isolated from catfish fillets in this study and identified, for the first time, as DNase producing bacteria.

Keywords: DNase-producing bacteria, fish spoilage, catfish, *Aeromonas* spp., *Serratia* spp.

Introduction

Microbial spoilage of fish tissue begins immediately after death due to the absence of normal body regulatory mechanisms. Bacteria freely enter postmortem fish through body cavities, intestines, and vascular tissue until microbial growth changes the sensory properties of the tissue enough that it is no longer suitable as a food source for human consumption (1-6). With the modern competition for organic foods, preservative containing products are difficult to market. Microbial degradation of fish is often the cause of food poisoning such as salmonellosis, listeriosis, and histamine fish poisoning (HFP); a foodborne, chemical intoxication caused by consuming spoiled or bacterially contaminated fish (7-12). Microbial spoilage limits the shelf life of fish products (9,13,14). It has been estimated that microbial activity is responsible for the spoilage of up to 25% of all postharvest food with fish being the largest proportion loss of any food source in the world (2,15-17). The preservation of fish is entirely reliant on the understanding of microbial activity and its spoilage effects on fish tissue. Therefore, a better understanding of microbial activity on fish is crucial for the health of fish consumers and the overall economic and commercial viability of fish and fish-related products (18-23).

To better understand bacterial spoilage and the role of bacterial communities on fishery products, the present study was carried out to analyze bacteria producing extracellular deoxyribonuclease (DNase) on catfish spoilage. For the most part, studies like these focus only on the ability of specific bacterial spp. to produce DNase (24-27). To the best of our knowledge, this was the first attempt to study DNase-producing bacteria on catfish spoilage. Distinct bacterial colonies producing extracellular DNase were isolated and PCR was used to screen for *Pseudomonas* spp. which are widely recognized as one of the most responsible bacteria for the spoilage of fish fillets due to their exceptional growth rate at psychrotrophic temperatures (22,28-36). DNase-producing bacteria from catfish fillets

Michael E. Hickey, Samuel A. Besong, Jung-Lim Lee (✉)
Department of Human Ecology, College of Agriculture and Related Sciences, Delaware State University, Dover, DE 19901, USA
Tel: +1-302-857-6448; Fax: +1-302-857-6441
E-mail: jlee@desu.edu

Michael E. Hickey
Department of Biology, Wesley College, Dover, DE 19901, USA

Venu Kalavacharla
Department of Agriculture and Natural Resources and Center for Integrated Biological and Environmental Research, Delaware State University, Dover, DE 19901, USA

were examined during refrigerated storage and isolates were identified using 16S rDNA sequencing. In this study, the contribution of extracellular DNase-producing bacteria on the spoilage of catfish fillets was evaluated.

Materials and Methods

Fish sample Ten North American catfish (*Ictalurus punctatus*) fillets were purchased from a retail source in Dover, Delaware, USA. The fillets were kept on ice immediately following purchase and then refrigerated at 4°C for the duration of the study. Bacteria were isolated from the fillets immediately upon laboratory arrival and every 2 days for 16 days.

Bacterial isolation Catfish fillets were used for isolation of bacteria every 2 days for 9 trials. Bacteria were isolated from 25 g of catfish tissue in 75 mL of 0.85% saline solution using a stomacher (Stomacher 3500; Seward Inc., Bohemia, NY, USA). One mL inoculated saline solution was used for decimal dilutions in tryptic soy broth (TSB) (Carolina Biological Supply, Burlington, NC, USA). Based on the age of the fish fillets and previous results, 6 dilutions were plated in triplicate/trial.

DNase testing Each dilution was spread on DNase test agar with methyl green (25,37) (BD, Sparks, MD, USA) and tryptic soy agar (TSA) plates for each trial. The specified bacterial growth plates were incubated for 3 days at 21°C to ensure maximum bacterial growth. Total bacteria and extracellular DNase-producing bacteria were enumerated. Specific extracellular DNase-producing bacterial colonies were isolated based on distinct physical characteristics, such as size, color, DNase-production, texture, or shape, and inoculated in 10 mL of TSB. Bacteria inoculated into TSB were incubated at 21°C for 3 days.

DNA extraction Bacteria producing extracellular DNase were pelleted by centrifuging 500 μL of each sample at 13,000×*g* for 4 min at 14°C. Supernatants were immediately removed from pellets. Pellets were suspended in 200 μL of 2x tetrazolium (TZ) solution (4) and 200 μL of molecular grade water for DNA extraction. Samples were boiled at 100°C for 10 min and centrifuged at 13,000×*g* for 4 min at 14°C. The DNA-containing supernatant from each sample was transferred to fresh tubes for DNA amplification.

PCR screening for *Pseudomonas* spp. PCR amplification screening for *Pseudomonas* spp. was performed using a Bio-Rad MyCycler (Bio-Rad Lab., Hercules, CA, USA). Each PCR reaction contained: 2 μL of extracted DNA template; 10 μL of PCR water (Bioline, Taunton, MA, USA); 1 μL of forward primer; 1 μL of reverse primer; and 15 μL of 2x OneTaq (New England BioLabs, Ipswich, MA, USA). PCR was performed using the following parameters: initial DNA denaturation at 95°C for 4 min, 34 cycles consisting of DNA denaturation at 95°C for 35 s, annealing at 54°C for 30 s, extension at 72°C for 50 s, and final extension of DNA at 72°C for 4 min. Primers used, to identify *Pseudomonas* spp., were 10 μM universal primers PSEG30F and PSEG790R consisting of sequences 5'-ATY-GAA-ATC-GCC-AAR-CG-3' and 5'-CGG-TTG-ATK-TCC-TTG-A-3' (Sigma Genosys, Woodlands, TX, USA) respectively. They produced a 736-bp amplicons of the *rpo*D target gene (36).

Gel electrophoresis The presence of PCR products were confirmed by 0.5% agarose gel electrophoresis (Amresco Inc., Solon, OH, USA) in Tris acetate-EDTA (TAE) buffer (Amresco) and stained with ethidium bromide. PCR bands were photographed (Syngene G: Box, Frederick, MD, USA) for visual analysis.

Partial 16S rRNA gene analysis Based on unique growth characteristics, 20 DNase-producing bacterial samples isolated from catfish were partial sequenced for the 16S rRNA gene fragment using protocols by MIDI Laboratories (Newark, DE, USA). By use of universal 16S primers, specific to the 500 bp sequence 0005F-0531R and 500 bp sequence 0005F-0531R, 16S rDNA sequences of isolated genomic DNA were obtained, purified, and verified for quality on an agarose gel. Genomic DNA sequence analysis was performed using Sherlock microbial analysis software. Final sequence data were compared to Sherlock DNA data base as well as GenBank for sequence differences and bacterial identification.

Real-time PCR assay Samples were rescreened using real-time analysis of PCR amplicons based on the 16S target gene using a thermal cycler (Bio-RadMy Cycler™; Bio-Rad Lab.). Each PCR reaction contained: 2 μL of extracted DNA template, 10 μL of PCR water (Bioline), 1 μL of forward primer, 1 μL of reverse primer, and 15 μL of 2x SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). PCR was performed using the following parameters: initial DNA denaturation at 95°C for 4 min, followed by 35 cycles of DNA denaturation at 95°C for 35 s, annealing at 60°C for 25 s, and extension at 72°C for 50 s. The melt curve of DNA was determined by subjecting the amplicons to 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s at 0.3°C increments. Primers used, to verify results, were 10 μM universal Pseudomonas primers PSEG30F and PSEG790R (as described above) and 10 μM universal primers PS-F and PS-R, consisting of sequences 5'-GGT-CTG-AGA-GGA-TGA-TCA-GT-3' and 5'-TTA-

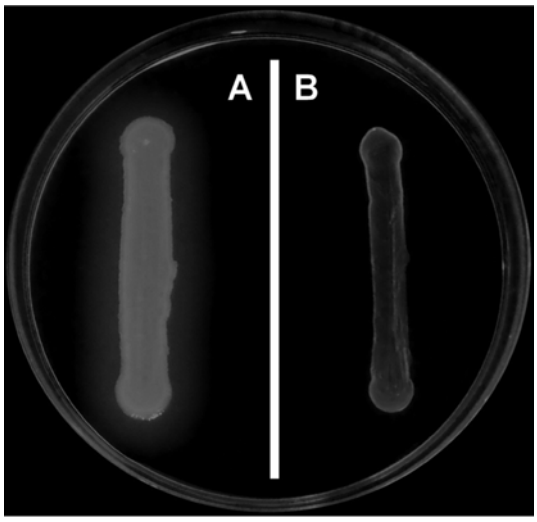

Fig. 1. Differential identification of extracellular DNase-producing (A) and non-DNase-producing (B) bacteria on methyl green test agar. Bacterial isolate D36, *Aeromonas sobria,* was plated next to ATCC 12598, *Salmonella typhi*. Combination of hydrolyzed DNA with methyl green results in a clear region surrounding the bacterial isolates.

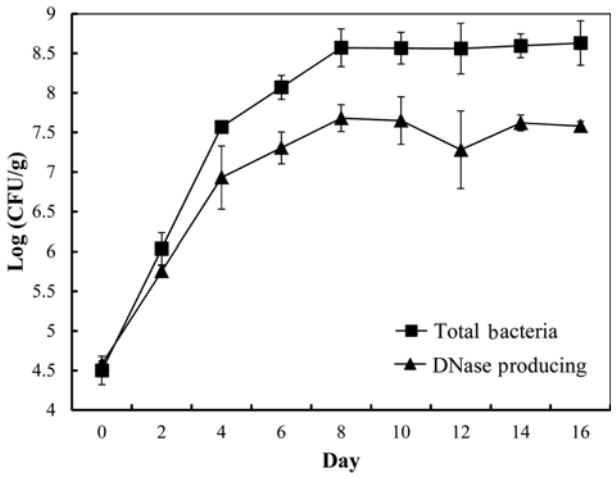

Fig. 2. Total growth of isolated bacteria from catfish fillets on DNase test agar with methyl green during storage at 4°C.

GCT-CCA-CCT-CGC-GGC-3' (Sigma Genosys) that amplified 989 bp of the 16S rRNA target gene (38).

Results and Discussion

Bacterial enumeration DNase-producing isolates were identified by clear zones around colonies or streaks of colonies on DNase test agar with methyl green (Fig. 1). Total bacterial growth and DNase-producing bacterial populations are shown in Fig. 2 (39,40). Bacteria steadily increased for the first 8 days, at which point, bacteria present on the fish fillets began stationary growth. Of all culturable bacteria isolated from the catfish fillets, 16% were extracellular DNase-producing bacteria. However, 65% of all DNase-producing bacteria isolated from the catfish fillets were enumerated within the first 6 day spoilage period (data not shown) suggesting that the majority of spoilage effects from DNase are present in the exponential phase of bacterial growth. Only 5.6% of isolated bacteria were DNase-producing bacteria present in the stationary phase of bacterial growth. CFU/g present on catfish fillets over 2-day intervals obtained in this study were consistent with data obtained by Lee and Levin (41).

PCR screening for *Pseudomonas* spp. A total of 54 extracellular DNase-producing bacterial samples were isolated and PCR screened for *Pseudomonas* spp.; a bacterial strain that causes spoilage of fish muscle (22,28-36). Gel electrophoresis of PCR amplicons of the *rpo*D gene specific to *Pseudomonas* spp. resulted in identification of 31 isolates as *Pseudomonas* spp., however, further identification of isolates based on 16S rDNA sequencing concluded that no DNase producing pseudomonads were isolated from the catfish fillets in this study. Due to conflicting identification data, PCR data was rescreened in real-time using 16S primers specific to *Pseudomonas* spp. One-hundred % of 54 PCR-screened samples were negative for *Pseudomonas* spp. This was consistent with data obtained by MIDI Laboratories indicating that none of the sequenced isolates were pseudomonads based on 16S rDNA sequencing. The data obtained using primers specific to the *rpo*D gene of *Pseudomonas* spp. proved inaccurate in identifying pseudomonads; supporting data obtained by Mulet *et al.* (38). Based on data obtained in this study, it was concluded that the *rpo*D region of *Pseudomonas* spp. is a poor basis for identification and questions the relationship between *Aeromonas* spp., *Shewanella* spp., and *Pseudomonas* spp. as the current known phylogeny of *Gammaproteobacteria* shows a distant relation between *Aeromonas* and *Shewanella* spp. with *Pseudomonas* spp. (42).

16S rDNA sequencing The identity of isolates based on 16S rDNA sequencing is shown in Table 1; microbial identification was very specific as genomic differences matched database records of at least 98.5%. Fifty-four total isolated samples were separated into 20 distinct selections based on in-depth record of morphology. Ten of 20 sequenced samples belonged to *Aeromonas* spp., as shown in Table 1. A study performed by Lerke *et al.* (33) classified 100% *Aeromonas* spp. isolates present in English sole (*Parophrys vetulus*) fillets as strong spoilers (5). Four of 20 samples sequenced were identified as *Shewanella baltica*. As shown in Fig. 3, *S. baltica* is a close relative of *Shewanella putrefaciens* which has been considered the main cause of

Table 1. Distinct DNase-producing bacterial isolates identified by 16S rDNA sequencing

Catfish isolate	Bacterial identification 16S rDNA sequencing result	Homology (%)[1)]	Length (Base pairs)	GenBank accession	Marine type origin	Sample identification[2)]
D1	*Serratia-proteamaculans-quinovora*	1.52	528	AJ279053.1	Fresh/Brackish	C63339
D3	*Aeromonas-bestiarum/salmonicida*	0.00	530	HM007582.1	Fresh/Brackish	C63330
D4	*Aeromonas-bestiarum/salmonicida*	0.00	530	HM007582.1	Fresh/Brackish	C63341
D5	*Shewanella-baltica*	0.00	528	AY158034	Brackish	C63342
D8	*Aeromonas-bestiarum/salmonicida*	0.00	530	HM007582.1	Fresh/Brackish	C63343
D10	*Shewanella-baltica*	0.00	528	AY158034	Brackish	C63344
D11	*Serratia-proteamaculans-quinovora*	1.52	528	AJ279053.1	Fresh/Brackish	C63345
D12	*Shewanella-baltica*	0.00	528	AY158034	Brackish	C63346
D13	*Serratia-proteamaculans-quinovora*	1.52	528	AJ279053.1	Fresh/Brackish	C63347
D14	*Aeromonas-bestiarum/salmonicida*	0.09	530	HM007582.1	Fresh/Brackish	C63348
D15	*Rahnella* spp.	1.00	526	HQ694787	Fresh	C63349
D16	*Shewanella-baltica*	1.00	528	CP000753.1	Brackish	C63350
D17	*Aeromonas-bestiarum/salmonicida*	0.00	530	HM007582.1	Fresh/Brackish	C63351
D18	*Aeromonas-bestiarum/salmonicida*	0.28	530	HM007582.1	Fresh/Brackish	C63352
D19	*Aeromonas-bestiarum/salmonicida*	0.00	530	HM007582.1	Fresh/Brackish	C63353
D27	*Aeromonas-bestiarum/salmonicida*	0.00	530	HM007582.1	Fresh/Brackish	C63354
D36	*Aeromonas-sobria*	0.00	530	X60412.2	Fresh/Brackish	C63355
D41	*Serratia-proteamaculans-quinovora*	1.14	528	AJ279053.1	Fresh/Brackish	C63356
D45	*Serratia-proteamaculans-quinovora*	0.95	528	AJ279053.1	Fresh/Brackish	C63357
D48	*Aeromonas-bestiarum/salmonicida*	0.00	530	HM007582.1	Fresh/Brackish	C63358

[1)]Homology of 16S rDNA region between catfish isolated DNase producing samples and closest relative found in Sherlock® DNA or GenBank database.
[2)]Sample identification number represented in phylogenic analysis.

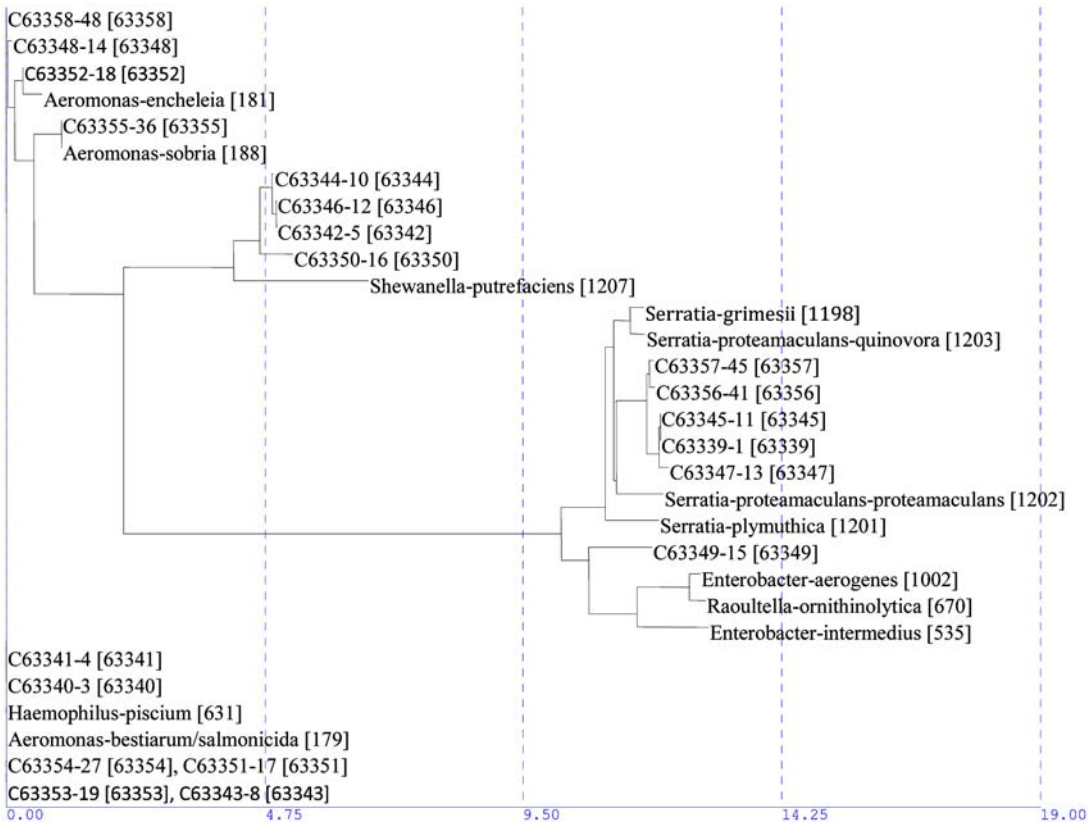

Fig. 3. Phylogenic tree based on partial 16S rDNA identification of extracellular producing DNase-producing bacteria. All DNase-producing isolates identified during this study belong to the γ-proteobacterial class.

seafood spoilage at low-temperature storage. *S. baltica* was classified as a stronger spoilage organism of seafood than *S. putrefaciens* as the organism produces high levels of hydrogen sulfide (43). *Serratia proteamaculans* accounted for 25% of DNase producing isolates in this study. In related research, by Gram *et al.* (15) and Bruhn *et al.* (44), *S. proteamaculans* was studied to determine its effects on food quality at psychrotrophic storage temperatures. The spoilage effects of *S. proteamaculans* were not classified due to inconsistent results; however, the production of exoenzymes by *S. proteamaculans* seemed to be a determining factor of food quality alteration by the organism (15,44). Research concerning *S. proteamaculans* and *S. baltica* on catfish spoilage has not been performed previously. Further study of these bacteria is necessary to further our understanding of their spoilage mechanisms on catfish. As discussed in one study, *Rahnella* spp. is associated with unsafe levels of histamine production on fish tissue as well as likely allergens (45); supporting the data obtained in this study. To the best of our knowledge, *Rahnella* spp. has not been known to produce DNase prior to this study. Because our *Rahnella* spp. sample was identified only to genus level, a further study of this sample is necessary. *Aeromonas sobria* produced the most DNase in this study, but only one isolate was identified (Table 1). The phenotype of this isolate was recognizably small and bright yellow while DNase production clearly outsized neighboring colonies on differential media.

In conclusion, data obtained in this study suggests that extracellular production of DNase by bacteria present on catfish tissue plays a role in catfish spoilage. Because no DNase-producing *Pseudomonas* spp. or *Achromobacter* spp. were isolated, it appears that other bacteria, namely *Serratia proteamaculans/quinovora*, *Shewanella baltica*, *Aeromonas bestiarum/salmonicida*, and *Rahnella* spp. may also play a significant role in fish spoilage by virtue of their strong DNase-production during refrigerated storage. Data obtained in this study pertain to catfish samples derived from North Carolina, USA. Geographically, it is important that data obtained in further studies be compared to data obtained in the present study as there is a lack of publicized data on DNase-producing bacterial activity and their effects on fish tissue.

Acknowledgments We thank NSF's EPS 0814251 to Delaware EPSCoR and NSF's REU grant 1003971 for support to this study.

References

1. Fraser OP, Sumar S. Compositional changes and spoilage in fish - Microbiological induced deterioration. Nutr. Food Sci. 98: 325-329 (1998)
2. Gram L, Dalgaard P. Fish spoilage bacteria - Problems and solutions. Curr. Opin. Biotechnol. 13: 262-266 (2002)
3. Harewood P. Detection of fish spoilage by colorimetry. U.S. Patent 5,744,321 (1998)
4. Lee JL, Levin RE. Use of ethidium bromide monoazide for quantification of viable and dead mixed bacterial flora from fish fillets by polymerase chain reaction. J. Microbiol. Meth. 67: 456-462 (2006)
5. Lerke P, Farber L, Adams R. Bacteriology of spoilage of fish muscle - IV. Role of protein. J. Appl. Microbiol. 15: 770-776 (1967)
6. Shangong W, Gao T, Zheng Y, Wang W, Cheng Y, Wang G. Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*). Aquaculture 303: 1-7 (2010)
7. Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Appl. Environ. Microb. 65: 969-973 (1999)
8. Kanki M, Yoda T, Ishibashi M, Tsukamoto T. *Photobacterium phosphoreum* caused a histamine fish poisoning incident. Int. J. Food Microbiol. 92: 79-87 (2004)
9. Lee JL, Levin RE. Selection of universal primers for PCR quantification of total bacteria associated with fish fillets. Food Biotechnol. 20: 275-285 (2006)
10. Lehane L, Olley J. Histamine fish poisoning revisited. Int. J. Food Microbiol. 58: 1-37 (2000)
11. Suhalim RR, Huang Y-W, Chen J. Interaction of *Escherichia coli* O157:H7 E318 cells with the mucus of harvested channel catfish (*Ictalurus punctatus*). Food Sci. Technol. -LEB 40: 1266-1270 (2007)
12. Suhalim RR, Huang Y-W, Burtle GJ. Survival of *Escherichia coli* O157:H7 in channel catfish pond and holding tank water. Food Sci. Technol. -LEB 41: 1116-1121 (2008)
13. Dalgaard P. Modeling of microbial activity and prediction of shelf life for packed fresh fish. Int. J. Food Microbiol. 26: 305-317 (1995)
14. Lee JL, Levin RE. Quantification of total viable bacteria on fish fillets by using ethidium bromide monoazide real-time polymerase chain reaction. Int. J. Food Microbiol. 118: 312-317 (2007)
15. Gram L, Ravn L, Rasch M, Bruhn JB, Christensen AB, Givskov M. Food spoilage - Interactions between food spoilage bacteria. Int. J. Food Microbiol. 78: 79-97 (2002)
16. Nellemann C. The Environmental Food Crisis: The Environment's Role in Averting Future Food Crises. UNEP, Arendal, Norway. pp. 23-30 (2009)
17. Park J-M, Shin J-H, Lee D-W, Song J-C, Suh H-J, Chang U-J, Kim J-M. Identification of the lactic acid bacteria in kimchi according to initial and over-ripened fermentation using PCR and 16S rRNA gene sequence analysis. Food Sci. Biotechnol. 19: 541-546 (2010)
18. Da Silva LVA, Prinyawiwatkul W, King JM, No HK, Bankston JD, Ge B. Effect of preservatives on microbial safety and quality of smoked blue catfish (*Ictalurus furcatus*) steaks during room-temperature storage. Food Microbiol. 25: 958-963 (2008)
19. Diep CN, Cam PM, Vung NH, Lai TT, My NT. Isolation of *Pseudomonas stutzeri* in wastewater of catfish fish-ponds in the Mekong delta and its application for wastewater treatment. Bioresource Technol. 100: 3787-3791 (2009)
20. Manju S, Jose L, Srinivasagopal TK, Ravishankar CN, Lalitha KV. Effects of sodium acetate dip treatment and vacuum-packaging on chemical, microbiological, textural, and sensory changes of pearlspot (*Etroplus suratensis*) during chill storage. Food Chem. 102: 27-35 (2007)
21. Nawaz M, Khan AA, Khan S, Sung K, Steele R. Isolation and characterization of tetracycline-resistant *Citrobacter* spp. from catfish. Food Microbiol. 25: 85-91 (2008)
22. Sarter S, Khanguyen H, Hung L, Lazard J, Montet D. Antibiotic resistance in Gram-negative bacteria isolated from farmed catfish. Food Control 18: 1391-1396 (2007)
23. Ye S, Li H, Qiao G, Li Z. First case of *Edwardsiella ictaluri* infection in china farmed yellow catfish *Pelteobagrus fulvidraco*. Aquaculture 292: 6-10 (2009)

24. Janda JM, Bottone EJ. *Pseudomonas aeruginosa* enzyme profiling: Predictor of potential invasiveness and use as an epidemiological tool. J. Clin. Microbiol. 14: 55-60 (1981)
25. Porschen RK, Sonntag S. Extracellular deoxyribonuclease production by anaerobic bacteria. J. Appl. Microbiol. 27: 1031-1033 (1974)
26. Sadovski AY, Levin RE. Extracellular nuclease activity of fish spoilage bacteria, fish pathogens, and related species. J. Appl. Microbiol. 17: 787-789 (1969)
27. Castro-Escarpulli G, Figueras MJ, Aguilera-Arreola G, Soler L, Fernandez-Rendon, Aparicia GO, Guarro J, Chacon MR. Characterization of *Aeromonas* spp. isolated from frozen fish intended for human consumption in Mexico. Int. J. Food Microbiol. 84: 41-49 (2003)
28. Adams R, Farber L, Lerke P. Bacteriology of spoilage of fish muscle - II. Incidence of spoilers during spoilage. J. Appl. Microbiol. 12: 277-279 (1964)
29. Chai T, Chen C, Rosen A, Levin RE. Detection and incidence of specific species of spoilage bacteria on fish. J. Appl. Microbiol. 16: 1738-1741 (1968)
30. Gram L. Inhibitory effect against pathogenic and spoilage bacteria of *Pseudomonas* strains isolated from spoiled and fresh fish. Appl. Environ. Microb. 59: 2197-2203 (1993)
31. Gram L, Huss HH. Microbial spoilage of fish and fish products. Int. J. Food Microbiol. 33: 121-137 (1996)
32. Lerke P, Adams R, Farber L. Bacteriology of spoilage of fish muscle - I. Sterile press juice as a suitable experimental medium. J. Appl. Microbiol. 11: 458-462 (1963)
33. Lerke P, Adams R, Farber L. Bacteriology of spoilage of fish muscle - III. Characterization of spoilers. J. Appl. Microbiol. 13: 625-630 (1965)
34. Levin RE. Correlation of DNA base composition and metabolism of *Pseudomonas putrefaciens* isolates from food, human clinical specimens, and other sources. Anton. Leeuw. Int. J. G. 38: 121-127 (1972)
35. Levin RE. Detection and incidence of specific species of spoilage bacteria on fish - I. Methodology. J. Appl. Microbiol. 16: 1734-1737 (1968)
36. Lobben JC, Lee JS. Roles of microorganisms in the deterioration of rockfish. J. Appl. Microbiol. 16: 1320-1325 (1968)
37. Smith PB, Hancock GA, Rhoden DL. Improved medium for detecting deoxyribonuclease-producing bacteria. J. Appl. Microbiol. 18: 991-993 (1969)
38. Mulet M, Bennasar A, Lalucat J, Garcia-Valdes E. An *rpo*D-based PCR procedure for the identification of *Pseudomonas* spp. and for their detection in environmental samples. Mol. Cell. Probe 23: 140-147 (2009)
39. Zwietering MH, Jongenburger I, Rombouts FM, Van't Riet K. Modeling of the bacterial growth curve. Appl. Environ. Microb. 56: 1875-1881(1990)
40. Oliver JD. The viable but nonculturable state in bacteria. J. Microbiol. 43: 93-100 (2005)
41. Lee J-L, Levin RE. Direct application of the polymerase chain reaction for quantification of total bacteria on fish fillets. Food Biotechnol. 20: 287-298 (2006)
42. Williams KP, Gillespie JJ, Sobral BWS, Nordberg EK, Snyder EE, Shallom JM, Dickerman AW. Phylogeny of gammaproteobacteria. J. Bacteriol. 192: 2305-2314 (2010)
43. Vogel BF, Venkateswaran K, Satomi M, Gram L. Identification of *Shewanella baltica* as the most important H_2S-producing species during iced storage of Danish marine fish. Appl. Environ. Microb. 71: 6689-6697 (2005)
44. Bruhn JB, Christensen AB, Flodgaard LR, Nielsen KF, Larsen TO, Givskov M, Gram L. Presence of acylated homoserine lactones (AHLs) and AHL-producing bacteria in meat and potential role of AHL in spoilage of meat. Appl. Environ. Microb. 70: 4293-4302 (2004)
45. Tash K. *Rahnella aquatilis* bacteremia from a suspected urinary source. J. Clin. Microbiol. 43: 2526-2528 (2005)