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Research Note

Bacterial Flora and Antimicrobial Resistance in Raw Frozen Cultured Seafood Imported to Denmark

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ABSTRACT

Intensified aquaculture includes the use of antimicrobials for disease control. In contrast to the situation in livestock, *Escherichia coli* and enterococci are not part of the normal gastrointestinal flora of fish and shrimp and therefore not suitable indicators of antimicrobial resistance in seafood. In this study, the diversity and phenotypic characteristics of the bacterial flora in raw frozen cultured and wild-caught shrimp and fish were evaluated to identify potential indicators of antimicrobial resistance. The bacterial flora cultured on various agar media at different temperatures yielded total viable counts of 4.0×10^4 to 3.0×10^5 CFU g^{-1}. Bacterial diversity was indicated by 16S rRNA sequence analysis of 84 isolates representing different colony types; 24 genera and 51 species were identified. *Pseudomonas* spp. (23% of isolates), *Psychrobacter* spp. (17%), *Serratia* spp. (13%), *Exiguobacterium* spp. (7%), *Staphylococcus* spp. (6%), and *Micrococcus* spp. (6%) dominated. Disk susceptibility testing of 39 bacterial isolates to 11 antimicrobials revealed resistance to ampicillin, amoxicillin–clavulanic acid, erythromycin, and third generation cephalosporins. Resistance to third generation cephalosporins was found in *Pseudomonas,* a genus naturally resistant to most β-lactam antibiotics, and in *Staphylococcus hominis*. Half of the isolates were susceptible to all antimicrobials tested. Results indicate that identification of a single bacterial resistance indicator naturally present in seafood at point of harvest is unlikely. The bacterial flora found likely represents a processing rather than a raw fish flora because of repeated exposure of raw material to water during processing. Methods and appropriate indicators, such as quantitative PCR of resistance genes, are needed to determine how antimicrobials used in aquaculture affect resistance of bacteria in retailed products.

Fish and shellfish products are sources of high-quality protein with a low fat content and are therefore widely promoted as a healthy protein source. Aquaculture production has increased almost exponentially in Asia to serve increased demands from overseas and domestic markets. In particular, Asian shrimp and pangasius (catfish) have gained popularity among consumers in Europe, the United States, and elsewhere. According to the Food and Agriculture Organization of the United Nations, aquaculture production has increased nearly 9% annually in recent years *(14),* and half of the global seafood consumption will originate from aquaculture by 2020 *(13).*

Human pathogens, mainly bacterial and parasites, may be associated with fish and shellfish products. Bacterial pathogens of fecal origin can be transmitted to aquacultured products in the pond or during subsequent handling and processing as a result of inadequate hygienic conditions. Other human pathogens, e.g., *Vibrio* spp. and *Listeria monocytogenes,* are naturally occurring and originate from the pond or processing environment *(32).* Improper storage and handling of seafood products may also lead to increased growth of spoilage bacteria such as *Lactobacillus* spp., *Proteus* spp., *Shewanella putrefaciens,* and *Pseudomonas* spp. *(4, 27, 39, 43).*

Occurrence of disease is a major obstacle in commercial aquaculture production. Antimicrobials are effective for preventing and controlling diseases in livestock and are widely used for similar purposes in aquaculture, although less information is available about effective and prudent use practices in this industry *(36).* In contrast to the research done on animals and humans, limited information is available about antimicrobial use in aquaculture, in particular in countries without national surveillance and registration of antimicrobial usage. In general, the positive correlation between the type and amounts of antimicrobials used and the antimicrobial resistance found in the intestinal bacterial flora of human and animals is well documented *(12, 15).* Types and levels of antimicrobial resistance in the bacterial microflora of cultured fish and shellfish also can be expected to be closely correlated with antimicrobial use patterns in aquaculture *(6, 37).*

Limited information is available about the antimicrobial resistance of the natural bacterial flora in fish and shellfish, mainly because in contrast to other animals and humans, good bacterial indicators of antimicrobial resistance have not been identified for seafood. *Escherichia coli* and enterococci are widely used as indicators to monitor antimicrobial resistance in animals, animal food products,

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TABLE 1. *Total viable bacterial growth in subtropical fish and shellfish*[a]

Incubation temp (°C)	Bacterial counts (CFU g^{-1}) on: MHA	PCA	LHA
15	4.0×10^4 (6.5×10^3–1.0×10^5)	5.4×10^4 (2.3×10^3–1.6×10^5)	4.3×10^4 (6.2×10^3–8.0×10^4)
20	7.0×10^4 (5.4×10^3–2.0×10^5)	1.0×10^5 (5.8×10^3–3.0×10^5)	5.7×10^4 (5.7×10^3–2.2×10^5)
37	5.8×10^4 (9.0×10^2–2.0×10^5)	7.5×10^4 (1.2×10^4–2.0×10^5)	5.8×10^4 (2.2×10^3–1.3×10^5)

[a] Samples were cultured on Mueller-Hinton agar (MHA), plate count agar (PCA), and Long and Hammer agar (LHA) for 48 h. Values are the mean (range) for two shrimp and fish samples.

and humans because these bacteria are part of the normal intestinal flora and develop resistance in response to antimicrobial exposure *(6, 37)*. However, these two fecal indicators are not part of the normal bacterial flora of fish and shellfish, and their presence is rather a sign of animal and/or human fecal pollution of the aquaculture environment *(12)*. Thus, *E. coli* and enterococci are inappropriate for monitoring antimicrobial resistance in fish and shellfish to determine the impact of preventive and therapeutic antimicrobial usage at the pond level *(12, 20)*.

Considering the increased consumption of imported fish and shellfish products in developed countries and the lack of regulations on antimicrobial use in some producing countries, indicator bacteria that represent the natural flora of fish and shellfish at the point of harvest should be identified and tested for their usefulness for monitoring antimicrobial resistance in retailed products. The objective of this study was to evaluate the diversity of the bacterial flora in raw frozen fish and shrimp products and to identify potential indicators of antimicrobial resistance. We characterized the culturable bacterial flora for a variety of local and imported fish and shrimp products available in the Danish market and determined the antimicrobial susceptibility patterns of the most common bacterial genera found in these products.

MATERIALS AND METHODS

Fish and shrimp samples. Raw frozen fish and shrimp imported into Denmark from Asian countries were used in this study: pangasius (*Pangasius hypopthalamus*) fillets, tilapia (*Oreochromis niloticus*) whole fish, white shrimp (*Penaeus vannamei*), tiger shrimp (*Penaeus monodon*). For comparisons, wild caught cod (*Gadus macrocephalus*), salmon (*Oncorhynchus keta*), trout (*Oncorhynchus mykiss*), and halibut (*Reinhardtius hippoglossoides*) originating from temperate areas such as Denmark and Alaska were purchased from Danish supermarkets and stored at −20°C before laboratory analysis. Samples were thawed for 24 h in a refrigerator (4 to 5°C) before bacteriological analysis.

Isolation of bacteria. Ten grams of fish fillet or shrimp (flesh, skin, and shrimp shell) was aseptically blended in 90 ml of 0.1% (wt/vol) peptone water for 1 min at 260 rpm in a Stomacher 400 lab blender (Seward Medical, London, UK). A series of 10-fold dilutions of the homogenate were prepared in 0.1% peptone water. Total viable bacteria counts (TVC) were obtained for two subtropical fish and shrimp samples by surface plating of 100-μl sample dilutions on Mueller-Hinton agar (MHA; CM0337, Oxoid, Basingstoke, UK), plate count agar (PCA; CM0325, Oxoid), and Long and Hammer agar (LHA) prepared as described by van Spreekens *(40)* (Table 1). For all agar media, NaCl was added when needed to obtain a concentration of 0.5% NaCl, and cultures were incubated at 15, 20, and 37°C for 48 h before colonies were enumerated.

Three fish and two shrimp samples from subtropical areas and four fish samples from temperate areas were analyzed for potential spoilage bacteria on iron agar (CM0964, Oxoid) incubated at 15 and 20°C for 48 h. TVC and colony morphology of these samples were determined by bacterial enumeration on MHA incubated at 15, 20, and 37°C for 48 h (Tables 2 and 3). The bacterial colonies on MHA were divided into different types and enumerated according to their colony characteristics: color, opacity, surface structure, and diameter size. Three to five representatives of each colony type from each sample were subcultured on Blood agar (CM0055, Oxoid) to obtain pure cultures. The isolates were characterized by the Gram reaction using 3% (wt/vol) potassium hydroxide (Bie and Berntsen, Herlev, Denmark), motility, cytochrome oxidase test (*N*,*N*-dimethyl-*p*-phenylene-diamine dihydrochloride, Remel Europe Ltd., Dartford, UK), and catalase test (Merck, Darmstadt, Germany) according to procedures recommended by Cowan *(10)*. All strains were cultured in Mueller-Hinton broth (CM0405, Oxoid) supplemented with 0.5% NaCl and stored at −80°C with 30% (vol/vol) glycerol.

TABLE 2. *Total viable bacterial counts for subtropical raw frozen fish and shrimp and temperate fish*[a]

Sample origin	Bacterial counts (CFU g^{-1}) on: Mueller Hinton agar 15°C	20°C	37°C	Iron agar 15°C	20°C	37°C
Subtropical (aquaculture)	1.8×10^6 (1.8×10^5 to $>3.0 \times 10^6$)	1.9×10^6 (1.1×10^5 to $>3.0 \times 10^6$)	2.1×10^6 (6.4×10^5 to $>3.0 \times 10^6$)	1.8×10^6 (4.0×10^4 to $>3 \times 10^6$)	1.8×10^6 (3.0×10^4 to $>3.0 \times 10^6$)	No growth
Temperate (wild caught)	9.1×10^4 (1.0×10^4–2.5×10^5)	8.6×10^4 (1.0×10^4–2×10^5)	5.8×10^3 (9.0×10^2–1.1×10^4)	8.2×10^4 (3.0×10^3–3×10^5)	2.1×10^4 (5.0×10^3–4.0×10^4)	No growth

[a] Samples were cultured on Mueller-Hinton agar and iron agar at 15, 20, and 37°C for 48 h. Values are the mean (range) for three fish and two shrimp samples from subtropical aquaculture sources and four fish samples wild caught in temperate areas.

Bacterial identification by 16S rRNA sequence analysis. A total of 23, 22, and 39 bacterial isolates obtained on MHA incubated for 48 h at 15, 20, and 37°C, respectively, were selected to represent the different colony morphology types (three to five isolates selected for each colony morphology type) found in fish and shrimp samples. The isolates were identified by 16S rRNA gene sequence analysis. Colonies were suspended in 100 μl of sterilized distilled water, the suspension was boiled and centrifuged, and the supernatant was used as template DNA for PCR with 17 isolates. Because of incomplete sequence data obtained with the boiled lysate method, total DNA was extracted from the remaining 67 isolates using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for gram-positive and gram-negative bacteria. The 16S rRNA genes were amplified by PCR with the universal primer sets described by Weisberg et al. *(41)*: 8-27F (5′-AGA GTT TGA TCC TGG CTC AG-3′), 1390-1408 (5′-TGA CGG GCG GTG TGT ACA A-3′), 786F (5′-GAT TAG ATA CCC TGG TAG-3′), 344R (5′-ACT GCT GCC TCC CGT-3′), 786R (5′-CTA CCA GGG ATAT CTA ATC-3′), 344F (5′-ACG GGA GGC AGC AGT-3′), 785 805 F (5′-GGA TTA GAT ACC CNG GTA GTC-3′), 37F (5′-GGC TCA GRW YGA ACG C-3′), and 785 805R (5′-GAC TAC CNG GGT ATC TAA TCC-3′). The PCR amplicons were visualized by electrophoresis in 1% (wt/vol) agarose gels stained with ethidium bromide. *Exiguobacterium artemiae* 9AN was used as a positive control, and water was used as a negative control. Sequencing of the amplified DNA fragments was done by Macrogen, Inc (Seoul, Korea). The 16S rRNA sequences were compared with available sequence data in the GenBank and EZ-taxon databases using the BLAST algorithm *(7)* (Table 3).

Antimicrobial susceptibility testing. Because the distribution of bacterial species isolated on MHA was not significantly influenced by incubation temperature, antimicrobial susceptibility testing was limited to all the 39 bacterial isolates obtained on MHA incubated at 37°C, which is the standard temperature used for antimicrobial susceptibility testing *(9)*. Susceptibility to 11 antimicrobial agents (Table 4) was determined by the disk diffusion method *(8)* according to procedures of the Clinical and Laboratory Standard Institute (CLSI) *(9)* on MHA supplemented with 0.5% NaCl *(24)*. Bacterial isolates were cultured on blood agar to obtain a fresh culture, and one loopful of colony material was mixed with 5 ml of phosphate-buffered saline (pH 7.4) and vortexed well to obtain a turbidity of 0.5 McFarland opacity standard (bioMérieux, Marcy 1'Etoile, France). The bacterial suspensions were streaked on MHA plates with a cotton swab. With an antibiotic disc dispenser, the discs were placed on the agar surface, plates were incubated at 37°C for 24 h, and the diameter of the inhibition zones was measured. The isolates were classified as sensitive, intermediate, and resistant based on the diameter of the clearing zone according to CLSI *(9)* guidelines. The following antimicrobials (Oxoid) were tested: ampicillin (AMP, 10 μg), amoxicillin–clavulanic acid (AMC, 30 μg), ceftiofur (CFF, 30 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 10 μg), enrofloxacin (ENR, 5 μg), erythromycin (ERY, 15 μg), gentamicin (GEN, 120 μg), rifampin (RIF, 5 μg), sulfamethoxazole-trimethoprim (SXT, 25 μg), and tetracycline (TET, 30 μg). Gram-positive *Staphylococcus* spp. were also tested against oxacillin (OXA, 1 μg).

RESULTS AND DISCUSSION

Microflora of raw frozen fish and shrimp products. TVC on MHA, PCA, and LHA revealed that type of growth medium and incubation temperature (15, 20, and 37°C) did not significantly influence the bacterial counts for subtropical seafood (Table 1), with counts of 4.0×10^4 to 3.0×10^5 CFU g^{-1}. Thus, MHA was used for subsequent TVC.

Similar colony morphology types were found on MHA and iron agar (Table 2). On iron agar, 3.0×10^4 to $>3.0 \times 10^6$ CFU g^{-1} were obtained regardless of incubation temperature. Means for subtropical products (1.8×10^6 CFU g^{-1}) were markedly higher than those for wild-caught temperate fish (8.2×10^4 CFU g^{-1}). A similar result found for MHA cultures; means for subtropical products were 10^5 to 10^6 CFU g^{-1} and those for temperate fish were 10^2 to 10^4 CFU g^{-1} (Table 2). The higher bacterial counts in products from subtropical aquaculture may be due to higher bacterial levels in both the water and the processing environments in such areas compared with the levels in the environments where the wild-caught fish originated and were processed *(2, 19)*.

The colonies isolated on MHA differed in size (0.5 to 5 mm diameter) and color (creamy, white, black, yellow, light yellow, deep orange, and grayish white), shape (convex, flat, and ''fried egg''), and surface structure (rough, mucoid, and smooth surface), and more variation was found after incubation at 37°C (Table 3). No clear visual difference in the colony morphology characteristics was found between the colonies isolated from subtropical aquaculture samples and those from wild-caught temperate samples.

16S rRNA sequence analysis of 84 gram-positive and gram-negative bacteria from fish and shrimp resulted in identification of 24 genera and 51 species (Table 3). The dominant genera were *Pseudomonas* (23%), *Psychrobacter* (17%), *Serratia* (13%), *Exiguobacterium* (7%), *Staphylococcus* (6%), *Micrococcus* (6%), and *Microbacterium* (6%). The remaining genera (including *Acinetobacter*) together made up less than 5% of the isolates, which agrees with findings in other studies *(5, 26, 29)*. *Pseudomonas* spp. were recovered from all samples regardless of incubation temperature. Other spoilage bacteria such as *S. putrefaciens* and *Brochothrix thermosphacta (31)* were less common (Table 3). *Serratia, Citrobacter,* and *Acinetobacter,* which affect safety and shelf life of seafood products because of their production of histamine and H_2S *(17, 23, 27, 33),* also were found. Some of the species were found only in cultures incubated at 15°C, which is in accordance with the psychrophilic nature of these bacteria (Table 3).

It was not clear whether the bacterial species found originated from the aquaculture environment (subtropical fish and shrimp) and waters where the temperate fish were caught or from the processing environments. However, several of the bacterial species have previously been found in processing environments, e.g., *Exiguobacterium* spp. was identified in a fish processing plant in Japan *(43)* and *Staphylococcus* spp. in foods often are associated with human contact (ubiquitous on skin). However, because of the halophilic nature of *Staphylococcus* spp. *(35)* they can survive and grow in aquaculture environments and have been isolated from whole catfish and catfish fillets in the United States *(30)*. *Acinetobacter* spp. have previously been reported as ubiquitous in various aquatic environments and

TABLE 3. *Bacterial species isolated from raw frozen seafood products after incubation on Mueller-Hinton agar for 48 h and subsequent identification by 16S rRNA sequence analysis*

Type of seafood		Bacterial species identified					
		Culture at 15°C (n = 23)		Culture at 20°C (n = 22)		Culture at 37°C (n = 39)	
Common name (no. of samples)	Latin name, country of origin	Species (colony morphology)	Accession no. (% nucleotide similarity)	Species (colony morphology)	Accession no. (% nucleotide similarity)	Species (colony morphology)	Accession no. (% nucleotide similarity)
Pangasius (2)	*Pangasius hypopthalamus*, Vietnam	*Psychrobacter alimentarius* (creamy, convex, 2.5 mm)	AY 513645 (100)[a]	*Psychrobacter pulmonis* (black, rough surface, 5 mm)	AJ 437696 (95)[a]	*Micrococcus yunnanensis* (white, convex, 2 mm)	FJ 214355 (92)[a]
		Rothia mucilaginosa (yellow, convex, 2 mm)	DQ 870701 (99)[b]	*P. alimentarius* (creamy, convex, 4 mm)	AY 513645 (98)[a]	*Microbacterium paraoxydans* (creamy, convex, 0.5 mm)	EU 714351 (98)[b]
		Citrobacter freundii (black, rough surface, 3.5 mm)	DQ 294285 (100)[b]	*P. pulmonis* (black, rough surface, 6 mm)	AJ 437696 (99)[a]	*Exiguobacterium artemiae* (yellow, convex, 3 mm)	AM 072763 (100)[b]
		Microbacterium oxydans (yellow, ''fried egg,'' 4 mm)	Y 17227 (100)[a]	*Macrococcus caseolyticus* (black, rough surface, 5 mm)	AP 009484 (99)[a]	*P. pulmonis* (white, convex, 3 mm)	AJ 437696 (100)[a]
		Serratia quinivorans (light orange, convex, 5 mm)	AJ 279045 (96)[a]	*Serratia marcescens* (red, convex, 5 mm)	EU 221361 (99)[bc]	*Pseudomonas trivialis* (creamy, convex, 4 mm)	AJ 492831 (100)[a]
		S. nematodiphila (red, convex, 1 mm)	EU 036987 (98)[a]	*Pseudomonas simiae* (creamy, flat, 5 mm)	AJ 936933 (98)[a]	*P. libanensis* (creamy, convex, 1.5 mm)	AF 057645 (100)[a]
				Microbacterium maritypicum (yellow, convex, 2 mm)	AJ 853910 (100)[a]	*Serratia grimesii* (creamy, convex, 3 mm)	AJ 23343 (100)[a]
				Chryseobacterium indologenes (deep orange, convex, 2 mm)	AY 050493 (98)[b]	*P. libanensis* (creamy, convex, 2.5 mm)	AF 057645 (100)[a]
						Exiguobacterium undae (yellow, convex, 3 mm)	DQ 019165 (100)[a]
						Kocuria rhizophila (yellow, convex, 5 mm)	Y 16264 (100)[a]
						M. yunnanensis (white, convex, 2.5 mm)	FJ 214355 (100)[a]

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TABLE 3. *Continued*

Type of seafood		Bacterial species identified					
		Culture at 15°C (n = 23)		Culture at 20°C (n = 22)		Culture at 37°C (n = 39)	
Common name (no. of samples)	Latin name, country of origin	Species (colony morphology)	Accession no. (% nucleotide similarity)	Species (colony morphology)	Accession no. (% nucleotide similarity)	Species (colony morphology)	Accession no. (% nucleotide similarity)
Tilapia (1)	*Oreochromis niloticus*, China	*Pseudomonas lurida* (creamy, flat, 2.5 mm)	AJ 581999 (99)[a]	*Pseudomonas azotoformans* (creamy, flat, 2.5 mm)	D 84009 (100)[a]	*Pseudomonas extremorientalis* (creamy, convex, 3.5 mm)	AF 405328 (100)[a]
		P. simiae (creamy, convex, 1 mm)	AJ 936933 (99)[a]	*P. synxantha* (creamy, convex, 1.5 mm)	AY 486386 (100)[b]	*Staphylococcus epidermidis* (white, convex, 1 mm)	L 37605 (100)[ac]
		Rothia nasimurium (deep orange, convex, 1.5 mm)	AJ 131121 (98)[a]			*P. libanensis* (yellow, convex, 1.5 mm)	AF 057645 (100)[a]
						Micrococcus luteus (yellow, convex, 3 mm)	AJ 717368 (100)[b]
						Dermabacter hominis (grayish white, convex, 0.5 mm)	X 91034 (82)[a]
						P. pulmonis (creamy, transparent, flat, 2 mm)	AJ 437696 (99)[a]
						P. trivialis (creamy, convex, 3.5 mm)	AJ 492831 (100)[a]
						E. artemiae (yellow, ''fried egg,'' 3.5 mm)	AM 072763 (100)[a]
						S. grimesii (white, convex, 1 mm)	AJ 233430 (100)[a]
						M. yunnanensis (yellow, convex, 2.5 mm)	FJ 214355 (99)[a]

TABLE 3. *Continued*

Type of seafood		Bacterial species identified					
		Culture at 15°C (n = 23)		Culture at 20°C (n = 22)		Culture at 37°C (n = 39)	
Common name (no. of samples)	Latin name, country of origin	Species (colony morphology)	Accession no. (% nucleotide similarity)	Species (colony morphology)	Accession no. (% nucleotide similarity)	Species (colony morphology)	Accession no. (% nucleotide similarity)
White shrimp (1)	*Penaeus vannamei*, Vietnam	*P. alimentarius* (creamy, convex, 1 mm)	AY 513645 (99)[a]	*P. libanensis* (yellow, convex, 1.5 mm)	AF 057645 (99)[a]	*E. artemiae* (yellow, convex, 2 mm)	MA 072763 (99)[a]
						Psychrobacter fecalis (creamy, convex, 1 mm)	AJ 421528 (100)[a]
						P. azotoformans (yellow, convex, 0.5 mm)	D 84009 (100)[a]
						S. grimesii (white, convex, 1.5 mm)	AJ 233430 (100)[a]
						Serratia proteamaculans (transparent, convex, 1.5 mm)	AJ 233434 (99)[a]
Tiger shrimp (1)	*Penaeus monodon*, Vietnam	*Psychrobacter cryohalolentis* (creamy, convex, 1.5 mm)	AY 660685 (98)[b]	*Psychrobacter cibarius* (creamy, convex, 1.5 mm)	AY 639872 (100)[b]	*S. grimesii* (white, convex, 2 mm)	AJ 233430 (100)[a]
		Brochothrix thermosphacta (creamy, flat, 3 mm)	AY 543023 (99)[a]	*Carnobacterium divergens* (light white, convex, 2 mm)	AY 543016 (100)[b]	*P. azotoformans* (creamy, convex, 0.5 mm)	D 84009 (100)[a]
		Shewanella putrefaciens (black, rough surface, 3 mm)	X 81623 (99)[b]			*Staphylococcus hominis* (creamy, convex, 1.5 mm)	X 66101 (100)[a]
		Lactococcus garviae (deep orange, convex, 2 mm)	HM 573320 (88)[b]			*E. undae* (yellow, convex, 1.5 mm)	DQ 019165 (100)[a]
						Acinetobacter johnsonii (creamy, convex, 0.5 mm)	X 81663 (100)[a]
Cod fish (1)	*Gadus macrocephalus*, Denmark	*Pseudomonas gessardi* (creamy, convex, mucoid surface, 3 mm)	AF 074384 (100)[a]	*Zooshikella ganghwensis* (creamy, convex, 2 mm)	AY 130994 (99)[a]	*P. pulmonis* (creamy, convex, 2 mm)	AJ 437696 (100)[a]
				M. oxydans (yellow, convex, 2 mm)	AJ 421528 (100)[a]	*Staphylococcus warneri* (white, convex, 2 mm)	AY 275500 (99)[b]

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TABLE 3. *Continued*

Type of seafood		Bacterial species identified					
		Culture at 15°C (n = 23)		Culture at 20°C (n = 22)		Culture at 37°C (n = 39)	
Common name (no. of samples)	Latin name, country of origin	Species (colony morphology)	Accession no. (% nucleotide similarity)	Species (colony morphology)	Accession no. (% nucleotide similarity)	Species (colony morphology)	Accession no. (% nucleotide similarity)
Trout (1)	*Oncorhynchus mykiss*, Denmark	*P. pulmonis* (white, convex, 2 mm)	AJ 437696 (98)[a]	*Pseudomonas lundensis* (light orange, convex, 2.5 mm)	AB 021395 (99)[a]	*M. maritypicum* (creamy, convex, 2 mm)	AJ 853910 (91)[a]
		Vagococcus salmoninarum (black, rough surface, 1.5 mm)	Y 18097 (93)[a]	*S. proteamaculans* (white, convex, 2 mm)	AJ 233434 (99)[a]	*S. proteamaculans* (white, convex, 2.5 mm)	AJ 233434 (100)[a]
		P. alimentarius (creamy, convex, 2 mm)	AY 513646 (100)[b]	*Buttiauxella gaviniae* (creamy, convex, 2 mm)	AJ 233403 (98)[a]		
Salmon (1)	*Oncorhynchus keta*, Alaska	*Wautersiella falsenii* (light orange, convex, 4 mm)	AM 084341 (98)[a]	*Pseudomonas putida* (yellow, convex, 3 mm)	FM 211694 (99)[b]	*S. grimesii* (white, convex, 3 mm)	AJ 233430 (100)[a]
		P. azotoformans (creamy convex, 4.5 mm)	D 84009 (100)[a]	*Acinetobacter guillouiae* (creamy, convex, 2 mm)	X 81659 (99)[a]	*E. artemiae* (creamy, convex, 2 mm)	AM 072763 (99)[a]
Halibut (1)	*Reinhardtius hippoglossoides,* Denmark	*Arthrobacter psychrolactophilus* (yellow, convex, 1.5 mm)	AB 097842 (99)[b]	*Rahnella aquatilis* (light orange, convex, 5 mm)	FJ 811860 (98)[b]	*S. warneri* (white, convex, 2 mm)	FR 682748 (100)[b]
		Psychrobacter aquimaris (creamy flat, rough surface, 3 mm)	AY 722804 (100)[b]	*M. yunnanensis* (yellow, convex, 2 mm)	FJ 214355 (98)[a]	*S. warneri* (white, convex, 1.5 mm)	FR 682748 (100)[b]
		Pseudomonas migulae (creamy, convex, mucoid surface, 2.5 mm)	NR 024927 (100)[b]				

[a] Accession number for the Ez-Taxon database.
[b] Accession number for the GenBank database.
[c] Unpublished reference.

TABLE 4. *Antimicrobial resistance for 39 isolates from raw frozen seafood*

Bacteria[a]	No. of isolates	Antimicrobial resistance[b]							
		Penaeus monodon (tiger shrimp)	*P. vannamei* (white shrimp)	*Pangasius hypopthalamus* (pangasius)	*Oreochromis niloticus* (tilapia)	*Oncorhynchus mykiss* (trout)	*O. keta* (salmon)	*Gadus macrocephalus* (cod)	*Reinhardtius hippoglossoides* (halibut)
Staphylococcus	5	AMP, ERY, AMC, CAZ (*S. hominis*)			AMC, AMP, ERY, SXT (*S. epidermidis*)			AMP, ERY (*S. warneri*)	ERY (*S. warneri*); AMP, ERY, TET (*S. warneri*)
Micrococcus	4				ERY (*M. luteus*, *M. yunnanensis*)				
Pseudomonas	8	AMP, CFF, CTX (*P. azotoformans*)	AMC, AMP (*P. azotoformans*)	AMC, AMP, CFF, CTX (*P. trivialis*); AMC, AMP, CTX (*P. libanensis*); AMC, AMP; CTX, CFF (*P. libanensis*)	AMC, AMP (*P. trivialis*); AMC, AMP, CFF, CTX (*P. extremorientalis*)				
Serratia	7	AMP (*S. grimesii*)			AMC, AMP, TET (*S. grimesii*)	AMC (*S. proteamaculans*)	AMP (*S. grimesii*)		
Microbacterium	2			AMP (*M. paraoxydans*)		AMP (*M. maritypicum*)			
Acinetobacter	1	*A. johnsonii*							
Exiguobacterium	6	*E. undae*	*E. artemiae*	*E. undae*, *E. artemiae*	*E. artemiae*		*E. artemiae*		
Kocuria	1			*K. rhizophila*					
Dermabacter	1				*D. hominis*				
Psychrobacter	4		*P. fecalis*	*P. pulmonis*	*P. pulmonis*			*P. pulmonis*	

[a] Genera listed are those recovered on Mueller-Hinton agar after incubation at 37°C for 48 h (Table 2).

[b] Species with no antimicrobials listed were susceptible to all 11 antimicrobials. Resistance was determined by the disc method following CLSI guidelines *(9).* AMP, ampicillin; AMC, amoxicillin–clavulanic acid; CAZ, ceftazidime; CFF, ceftiofur; CTX, cefotaxime; ERY, erythromycin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline.

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have been used as an indicator to monitor bacterial antimicrobial resistance in aquaculture systems, streams, and sewage systems *(18)*. On only two occasions did we isolate *Acinetobacter*: *A. guillouiae* from salmon and *A. johnsonii* from tiger shrimp. Before excluding *Acinetobacter* as an indicator of antimicrobial resistance in seafood, further studies using a selective preenrichment procedure are needed to determine whether this genus is common in cultured seafood products. Studies comparing the bacterial species composition from point of harvest to processed product, e.g., using tracer bacteria with particular characteristics (e.g., antimicrobial resistance markers), also are needed to determine to what extent the bacterial flora of cultured fish and shrimp changes during processing.

Antimicrobial susceptibility. A total of 39 isolates were tested for antimicrobial susceptibility; 19 were gram positive (*Microbacterium* [2 isolates], *Exiguobacterium* [6], *Micrococcus* [4], *Kocuria* [1], *Staphylococcus* [5], and *Dermabacter* [1]), and 20 were gram negative (*Acinetobacter* [1], *Serratia* [7], *Pseudomonas* [8], and *Psychrobacter* [4]), for a total of 10 genera and 20 species. A few isolates were resistant to AMP, AMC, ERY, TET, and SXT (Table 4). Resistance to third generation cephalosporins (CFF, CTX and CAZ) was found in *Pseudomonas,* a genus that is known to be naturally resistant to the majority of β-lactam antibiotics *(22),* and *Staphylococcus hominis*. All 39 isolates were susceptible to gentamicin, rifampin, and enrofloxacin, and oxacillin (methicillin) resistance was not observed for any *Staphylococcus* isolates. Half of the isolates (19 of 39) were susceptible to all antimicrobials tested (Table 4).

Previous studies on antimicrobial resistance in seafood have almost entirely determined resistance for other, mainly zoonotic, bacterial pathogens, including *Vibrio (1, 3, 11, 16, 24, 32, 38), Aeromonas (1, 3, 25, 28, 42), Salmonella (16, 32),* and the fecal indicator *E. coli (1, 16, 21, 34)*. Little is known about the normal bacterial flora in cultured shrimp and fish at the point of harvest or in the final processed products. Both *Vibrio* spp. and *Aeromonas* spp. are ubiquitous in aquatic environments; *Vibrio* is most often found in brackish and marine water and *Aeromonas* is more common in fresh water. However, although the tiger and white shrimp tested were cultured in brackish water and the pangasius and tilapia were cultured in fresh water, we did not isolate any *Vibrio* or *Aeromonas* from shrimp and fish products, respectively (Table 3). Although antimicrobial resistance of *Salmonella* and *E. coli* strains found in seafood may be of food safety relevance, these bacteria are not part of the normal flora in aquaculture environments but should be considered indicators of fecal pollution. Any resistance among such fecal bacteria would be an expected outcome of selective antimicrobial pressure in warm-blooded animals rather than associated with antimicrobial usage in aquaculture.

In conclusion, the results of this study indicate no major differences in the distribution of bacterial species between products originating from the Asian aquaculture industry and from wild-caught or cultured fish from temperate areas. The bacterial microflora recovered from retailed products probably represents the microflora that fish fillets, whole fish, and shrimp are exposed to during processing rather than the indigenous microflora of the fish or shrimp or the environmental microflora of the aquaculture pond of origin. This processing contamination might be due to repeated handling and exposure to contaminated surfaces and water during processing. Future studies could be designed to test this hypothesis using a farm-to-fork approach and molecular methods (rather than culture methods) for accurate assessment of the changes in microbial diversity along the production line. Based on current knowledge, the risk that antimicrobial resistance present in aquaculture facilities will be transmitted to consumers through consumption of raw frozen seafood products imported from subtropical areas appears to be low. Our results suggest that even if high levels of antimicrobial resistance were present in bacteria in fish and shellfish at the point of harvest, native resistant bacteria may be absent or present in low numbers in processed and frozen produce because of substantial changes in microflora composition caused by processing, in particular repeated exposures to water and variations in temperature.

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