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Foodborne and Indicator Bacteria in Farmed Molluscan Shellfish before and after Depuration

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ABSTRACT

Galicia's coast (northwestern Spain) is a major producer of bivalve molluscs. Over an 18-month period, the presence of *Salmonella, Aeromonas, Plesiomonas shigelloides, Vibrio parahaemolyticus,* and *Clostridium botulinum* was determined by PCR methods in mussels (22 batches) and infaunal bivalves (31 batches of clams and cockles) before and after depuration. All batches were harvested from Galician class B harvesting areas where bivalve molluscs must not exceed 4,600 *Escherichia coli* per 100 g of flesh and liquor in 90% of the samples. Virulence-associated genes of *Salmonella* (*invA*), *Aeromonas* (*aerA, hlyA, alt, ast,* and *laf*), *P. shigelloides* (*hugA*), *V. parahaemolyticus* (*tdh* and *trh*), and *C. botulinum* (*BoNT*) were not detected. The pR72H chromosomal DNA fragment, which is conservative in *V. parahaemolyticus* strains, was detected in five (4.7%) samples. A number of 192 suspect isolates did not fit the description of clinical *Aeromonas* phenospecies, pathogenic *Vibrio* spp., or *P. shigelloides.* The effectiveness of commercial depuration in reducing bacterial indicators was also examined. *E. coli* was reduced to ≤230/100 g of flesh and liquor in 90.9% of mussel lots but in only 70.9% of infaunal bivalve lots. For total coliform elimination, mussels were also more effective. Total counts significantly ($P < 0.005$) correlated with numbers of *Pseudomonas, Aeromonas,* and *Vibrio.* Our data indicate that *Salmonella* and pathogenic bacteria indigenous to estuarine environments do not appear to be significant hazards in Galician molluscan shellfish. A reason for concern, however, is that clearance of *E. coli* to acceptable levels was not always achieved especially in infaunal bivalves.

Spanish aquaculture production reached over 372,344 tons in 2006, of which 308,682.3 tons corresponded to bivalve molluscs. Bivalve cultivation is dominated by mussels with 301,865.9 tons *(30)*; thus, Spain is becoming the largest mussel producer in Europe *(14).* Approximately 70% of the mussels produced is destined for domestic consumption, and the remaining 30% is exported to European markets. The Spanish mussel industry is heavily concentrated in Galicia (northwestern Spain), and mussel farming is based on suspended raft culture, with activity centered on boundary estuaries called rías. In addition to mussels, Galicia is also an important producer of infaunal bivalve molluscs such as clams and cockles *(30).*

Marine bivalves carry a natural population of bacteria dominated by species of *Vibrio, Pseudomonas,* and other gram-negative bacteria colonizing the gut, body surfaces, hemolymph, or tissues and being prominent in postmortem spoilage *(7, 21, 32).* In the process of filter feeding, bivalve shellfish may also concentrate and retain human pathogens naturally occurring in aquatic environments and/or derived from sewage-polluted waters. The hazards posed by bioaccumulation are compounded by the traditional consumption of molluscan shellfish raw or after minimal heat treatment and by consumption of the entire animal, including the viscera *(21).* Foodborne bacteria members of the normal microflora of the estuarine environment include some motile *Aeromonas* spp., *Plesiomonas shigelloides,* pathogenic strains of *Vibrio parahaemolyticus,* and psychrotrophic types of *Clostridium botulinum (21, 41).*

Currently, *Escherichia coli* or fecal coliforms are used to monitor the sanitary quality of shellfish-growing waters and bivalve molluscs. Additional control measures test growing areas for total coliforms and harvested shellfish for aerobic mesophilic counts *(21, 39).* In the European Union (EU), requirements for the production and marketing of bivalve molluscs are laid down in Regulation (EC) No. 853/2004 *(11).* To reduce contamination and render safe for human consumption, live bivalve molluscs from class B (≤4,600 *E. coli* per 100 g of flesh and liquor) or class C (≤46,000 *E. coli* per 100 g of flesh and liquor) production areas should undergo purification or relaying. Purification, also known as depuration, involves the transfer of the molluscs from the harvesting area to enclosed systems with flowing clean water with the appropriate salinity and temperature. Here, the molluscs continue filtration and normal digestive activity and over a period of about 48 h, they purge themselves of most bacterial contamination present, although under certain conditions, longer depuration periods may be required for adequate purification *(8).*

One aim of this study was to determine the incidence of human pathogenic bacteria, especially those naturally occurring in estuarine environments in nondepurated and depurated farmed bivalve molluscs from Galicia class B harvesting areas. We also wanted to establish whether commercial depuration was effective in reducing bacterial indicators to acceptable levels and the effect of depuration in the numbers of some genera of bivalves' resident bacteria.

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MATERIALS AND METHODS

Bivalve molluscs and depuration. Over an 18-month period, 53 batches of bivalve molluscs were studied. At approximately 2-week intervals, 1.5 kg of live animals from each batch were obtained before and after depuration from a commercial purification center in the Province of Pontevedra (Galicia). Each batch was a quantity of bivalve molluscs harvested on the same day from a production area and subsequently depurated through the same cycle of the same depuration system.

Bivalve mollusc batches were harvested from different locations along the Ría de Arousa coast, where the surface water temperatures varied from ca. 20°C in summer to 12°C during winter, and salinity ranged from ca. 35 practical salinity units (PSU) to ca. 30 PSU in summer and winter, respectively (Spanish Institute of Oceanography; http://indamar.ieo.es). Ría de Arousa is located between the coordinates 42°41′ to 42°28′N and 9°01′ to 8°44′W and is the most extensive (230 km^2) of the four Galicia's Rías Baixas. It is wedge-shaped, with a longitudinal axis of 28 km and a maximum width of 10 km. Its depth varies from 70 m at its mouth to 5 to 10 m at its innermost part. At the entrance is the Isle of Sálvora, which provides some level of protection against the Atlantic weather, particularly in the winter months.

The studied molluscs were farmed mussels (*Mytilus galloprovincialis,* 22 batches) and two groups of farmed infaunal edible bivalves: clams (pullet carpet shell [*Venerupis pullastra*], 15 batches; Japanese carpet shell [*Ruditapes philippinarum*], 4 batches; and rayed Artemis [*Dosinia exoleta*], 3 batches) and common edible cockles (*Cerastoderma edule,* 9 batches).

Galicia mussel culture is divided into five stages: seeding or procuring the seed, attaching seed to the ropes, thinning, rearing, and harvesting (http://www.fao.org/fishery/culturedspecies/Mytilus_edulis/en). Culturing of cockles is carried out in protected areas with clean, fine sand, called parks. The seed is collected from natural beds, taken to the parks, and distributed at densities up to 400 individuals per square meter. For clams, farmers obtain seed from their own parks or from the natural clam populations in the spring. They dig the clam seed with sand by using a small shovel, pass it through a sieve to retain the seed, take it to their parks, and spread it in densities of about 800 individuals per square meter. Periodically, they have to clean their parks of predators and mud.

Depuration was carried out in vertical stack purification systems using filtered natural seawater disinfected by UV. Briefly, batches of live harvested animals accompanied by the registration document (gatherer's identity and address, date of harvesting, location of the production area, health status of the production area, shellfish species and quantity, and batch destination) were transported by boats or trucks to the purification center, washed, debyssed (mussels), inspected, and sorted before being placed in containers for depuration. The steps after 42 to 48 h of depuration were unloading, washing, packaging, and storage under 2 to 10°C, depending on the species. The registration document after depuration also included address of the purification center, the duration of purification, and the dates on which the batch entered and left the purification center. Temperature, dissolved oxygen, salinity, turbidity, and pH of the seawater was maintained within acceptable levels, recommended conditions being 14 to 18°C, more than 5.5 mg/liter dissolved oxygen, salinity over 30 PSU, and turbidity less than 15 nephelometric turbidity units.

From each batch, nondepurated and depurated shellfish samples were placed in insulated shipping containers with plastic-encased ice packs and shipped to the laboratory. Bacteriological analysis was performed within 12 to 24 h after harvesting or depuration. Upon receipt, shellfish temperature measurement ensured that temperature in the shipping container had remained below 10°C.

Sample preparation. A minimum of 15 mussels were taken from each sample. For infaunal bivalve species, the number of specimens per sample was that needed to produce at least 150 g of flesh and liquor *(13).* Specimens were randomly chosen, the dead or broken being discarded.

Cleaning of the shell and removal of the shell contents were performed according to procedures recommended by the American Public Health Association *(8).* Tissue and shell liquid samples were weighed, mixed with an equal amount by weight of phosphate-buffered saline (Oxoid, Ltd., Basingstoke, UK) and homogenized for 60 to 90 s in a Omni-Mixer (Sorvall-DuPont Co., Newtown, CT) at 14,000 rpm. For bacterial counts, decimal dilutions were prepared in 0.1% peptone (Oxoid, Ltd.) water containing 0.85% NaCl.

Single qualitative PCR assays for detection of foodborne pathogens in flesh and liquor homogenates. The target genes and the *V. parahaemolyticus* DNA fragment investigated in this study, the sequence of each primer, the predicted product length for each primer pair, and their references are listed in Table 1. For the *alt* and *ast* genes, primers were designed based on published nucleotide sequence data (GenBank nucleotide sequence accession nos. L77573 and AF419157, respectively) with the aid of the Vector NTI software (Invitrogen Corp., Carlsbad, CA), and their suitability analyzed by using the BLAST program (http://www.ncbi.nim.nih.gov/BLAST/). The primers specificity was verified by using total DNA prepared from *Aeromonas hydrophila* strain AH3 (alt^+ast^+) *(15),* isolates of various *Aeromonas* spp. (alt^+ast^-, alt^-ast^+, and alt^-ast^-), and 15 strains of bacterial species common in water and/or foods (*E. coli, Lactobacillus delbrueckii, Salmonella* Aboni, *Listeria monocytogenes, Pseudomonas fluorescens, Pseudomonas putida, Yersinia enterocolitica, Vibrio parahaemolyticus, Enterobacter aerogenes, Shewanella putrefaciens,* and *Plesiomonas shigelloides*—all from our own collection).

Sample preparation, enrichment, and DNA extraction were performed as previously described *(16),* taking into account International Organization for Standardization (ISO) 22174 and 20837 principles and criteria *(22, 24).* Except for *C. botulinum,* homogenates were enriched at 30°C overnight, with constant shaking (150 rev min^{-1}) in Trypticase soy broth (Difco, Becton Dickinson, Sparks, MD) plus 0.6% yeast extract. For *C. botulinum,* enrichment was carried out in cooked meat medium (Oxoid, Ltd.) at 35°C for 5 days. One milliliter of each enrichment culture was frozen with 40% (wt/vol) glycerol and stored at −40°C for further testing if needed.

PCR amplification and detection of PCR products in 0.5-ml enrichment cultures were performed, taking into account the general requirements described in ISO 22174 and ISO 20838 *(22, 25).* The PCR mixture contained 5 μl of 10× PCR buffer, 0.2 mM deoxynucleoside triphosphate mix, 0.5 μM each primer, 0.8 U of DNA polymerase (all reagents from Biotools, Madrid, Spain), the template DNA (5 μl of the DNA extract), and deionized water, for a final volume of 50 μl. PCR was performed in a Mastercycler Personal apparatus (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). PCR was run according to the following temperature time profiles: initial denaturation at 94°C for 3 min, 30 cycles of denaturation (92°C for 30 s), primer annealing for 30 s (annealing temperatures are indicated in Table 1), elongation at 72°C for 1 min, and a final extension at 72°C for 1.5 min. The amplified mixtures (5 μl) were analyzed by 2% agarose gel electrophoresis and viewed, after ethidium bromide staining,

TABLE 1. PCR *primers, annealing temperatures, and expected PCR products for virulence-associated genes or DNA fragments*

Bacteria	Target	Primer sequences (5′ → 3′)	Annealing temp (°C)	Amplicon length (bp)	Reference
Salmonella	*invA*	TATCGCCACGTTCGGGCAA TCGCACCGTCAAAGGAACC	60	284	*34*
Motile *Aeromonas*	*hlyA*	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGG	62	597	*40*
Motile *Aeromonas*	*aerA*	GC(A/T)GA(A/G)CCC(A/G)TCTATCC(A/T)G TTTCTCCGGTAACAGGATTG	55	252	*38*
Motile *Aeromonas*	*alt*	CCATCCCCAGCCTTTACGCCAT TTTCACCGAGGTGACGCCGT	63	338	This study
Motile *Aeromonas*	*ast*	ATGCACGCACGTACCGCCAT ATCCGGTCGTCGCTCTTGGT	66	260	This study
Motile *Aeromonas*	*laf*	GGTCTGCGCATCCAACTC GCTCCAGACGGTTGATG	60	550	*15*
Plesiomonas shigelloides	*hugA*	GCGAGCGGGAAGGGAAGAACC GTCGCCCCAAACGCTAACTCATCA	63	435	*17*
Vibrio parahaemolyticus	pR72H	CGAATCCTTGAACATACGCAGC TGCGAATTCGATAGGGTGTAAACC	60	387	*29*
V. parahaemolyticus	*tdh*	TTTCATGATTATTCAGTTT TTTGTTGGATATACACAT	52	623	*36*
V. parahaemolyticus	*trh*	TTGGCTTCGATATTTTCAGTATCT CATAACAAACATATGCCCATTTCCG	58	500	*4*
C. botulinum	*BoNT*	TAT(A/G)TAGGATC(T/C)TGCTTTAAATATA(G/A)(G/T)(A/T)A(A/T) TGATTAGT(T/A)ATAGTTACAAAAATCCA(T/C)(T/C)T(A/G)TTTATATA	45	1,100	*5*

under UV light. All PCR assays were performed in two independent experiments.

Bacteriological analysis. Aerobic mesophilic counts (APC) and psychrotrophic counts were determined on Trypticase soy agar plus 0.5% NaCl, incubated at 30 and 4.5°C for 2 and 14 days, respectively. *Pseudomonas* numbers were determined, after 2 days of incubation at 25°C, on *Pseudomonas* agar base (Oxoid, Ltd.) to which cetrimide, fucidin, and cephaloridine (Oxoid, Ltd.) as a supplement was added. Presumptive counts of *Vibrio* spp. were obtained after 2 days of incubation on thiosulfate-citrate-bile-sucrose agar (Difco, Becton Dickinson) at 30°C. Presumptive *Aeromonas* spp. were determined on starch ampicillin agar *(33)* incubated at 28°C for 2 days. Lactic acid bacteria (LAB) were enumerated on overlaid plates of deMan Rogosa Sharpe (Oxoid, Ltd.) agar after 3 days of incubation at 25°C. Total coliforms and *E. coli* were obtained on 3M Petrifilm *E. coli*/Coliform Count plates (3M Microbiology Products, St. Paul, MN). Colonies were counted according to the manufacturer's instructions after 24 and 48 h incubation at 35 ± 1°C (AOAC International Official Method 991.14 *(1)*). For *E. coli,* the five-tube, three-dilution most-probable-number (MPN) reference method specified in ISO 16649-3 was also used *(23).*

Five colonies from each set of counts on selective media were randomly selected, streaked to purity on plate count agar (Oxoid, Ltd.) and examined for Gram reaction, cell morphology, motility, oxidase and catalase reactions, and for the oxidation-fermentation test. They were then grouped together according to the criteria of Mossel et al. *(31)* and tested for the most relevant characteristics of each group. Presumptive *Pseudomonas, Aeromonas,* and *Vibrio* isolates were identified to the genus level by the simple key schemes of the above authors. A total of 168 isolates assigned to the genera *Aeromonas* (96) and *Vibrio* (72) were further tested for a number of characters needed to identify seven clinical *Aeromonas* phenospecies *(28)* and *V. parahaemolyticus, V. cholerae,* and *V. vulnificus (26).*

For detection of *P. shigelloides,* a conventional two-step procedure was used. Briefly, 10-g homogenate samples were blended with 90 ml of tetrathionate broth without iodine (Oxoid, Ltd.). One half of the enrichment broth was incubated at 35°C and the other half at 40°C. After 24 h of incubation, the enrichment cultures were streaked onto inositol–brilliant green–bile salts agar and the plates incubated at 35°C for 24 h. Twenty-four suspect pinkish colonies were purified, Gram stained, and picked to triple sugar iron slants (Oxoid, Ltd.) and inositol gelatin medium (deep stab). Isolates that were alkaline over acid without gas or H_2S in triple sugar iron, produced acid but no gas from inositol, and did not hydrolyze gelatin were tested for oxidase production *(17).*

Statistical analysis. The number of microorganisms per gram was transformed and expressed as log CFU per gram. Results are presented as means ± standard deviations (SD). Statistical correlations and significance of differences between means of bacteriological counts were determined with the STATISTICA for Windows, release 6.0, software (StatSoft Inc., Tulsa, OK).

RESULTS

All molluscan samples tested negative for the *Salmonella* invasion gene *invA*. Five genes (*aerA, hlyA, laf, ast,* and *alt*), which may contribute to diarrhea-related virulence in various *Aeromonas* spp., were not detected in any of the analyzed shellfish samples, nor was the *hugA* gene, which encodes an outer membrane receptor, HugA, required by *P.*

TABLE 2. *Bacterial numbers in 53 paired batches of farmed bivalve molluscs before and after commercial depuration*

Counts	Before depuration[a]	After depuration[a]	% reduction
APC	3.523 ± 0.820	**3.237 ± 0.985**[b]	48.2
Psychrotrophic bacteria	3.990 ± 0.725	**3.643 ± 0.806**	55.0
Pseudomonas spp.	3.354 ± 0.662	3.078 ± 0.620	47.0
LAB	1.747 ± 0.971	1.635 ± 0.845	22.7
Aeromonas spp.	2.853 ± 1.017	**2.498 ± 1.224**	55.8
Vibrio spp.	2.802 ± 1.205	2.712 ± 1.279	18.7
Total coliforms	1.751 ± 0.843	**1.023 ± 0.985**	81.3
Escherichia coli[c]	1.437 ± 1.072	**0.835 ± 0.694**	75.0
E. coli[d]	2,735 ± 1,180	683 ± 494	

[a] Values are means ± SD of log CFU per gram.
[b] Significant ($P < 0.05$) differences are in boldface.
[c] Values here are log MPN per gram, using ISO Method 16649-3.
[d] Values here are MPN per 100 g.

shigelloides for heme iron utilization *(9),* nor the neurotoxin *BoNT* gene of *C. botulinum (5).* The presence of the pR72H DNA fragment, which is conserved in all *V. parahaemolyticus* strains *(29),* was detected in five lots: one mussel lot before depuration, one mussel lot after depuration, two depurated clam lots, and one lot of depurated cockles. The hemolytic genes *tdh* and *trh* associated with *V. parahaemolyticus* pathogenic strains were not detected in any sample.

Based on phenotypic characterization, *Vibrio* isolates did not belong to the species *V. cholerae, V. parahaemolyticus,* or *V. vulnificus. Aeromonas* isolates did not fit the description of seven recognized clinical phenospecies (*A. hydrophila, A. caviae, A. veronii* biovar *sobria, A. veronii* biovar *veronii, A. jandaei, A. schubertii,* and *A. trota*). Phenotypic characterization of the presumptive *P. shigelloides* isolates did not allow establishing their identity at the genus level.

Table 2 compares the overall microbiological status of nondepurated and depurated molluscs. Depuration resulted in significant ($P < 0.05$) reductions in the levels of APC and psychrotrophic bacteria, *Aeromonas* spp., total coliforms, and *E. coli.* For mussels, depuration resulted in significant ($P < 0.05$) reductions in numbers of *Aeromonas* spp., total coliforms, and *E. coli* while for infaunal bivalves, depuration resulted in significant ($P < 0.05$) reductions of APC and total coliforms (Table 3).

Before and after depuration there were significant ($P < 0.005$) correlations between APC levels and numbers of *Aeromonas* spp., *Vibrio* spp., and *Pseudomonas.* LAB numbers increased after depuration in infaunal bivalves and significantly ($P < 0.05$) correlated with APC. After depuration, a significant correlation was also found between total coliforms and *E. coli* ($r = 0.717$, $P < 0.005$), although it was higher for mussels ($r = 0.921$, $P < 0.005$) than it was for the remaining molluscs ($r = 0.611$, $P < 0.05$).

DISCUSSION

Although seafood, particularly bivalve molluscs, has the potential to carry food-poisoning organisms, all molluscan shellfish samples in this study were negative for the presence of genes encoding virulence factors in *Salmonella,* motile *Aeromonas* of clinical significance, *P. shigelloides, V. parahaemolyticus,* and *C. botulinum.* Conventional phenotypic identification of randomly selected and suspect isolates was also negative for *P. shigelloides,* seven clinical phenospecies of *Aeromonas,* and pathogenic *Vibrio* spp., although the pR72H DNA fragment of *V. parahaemolyticus* was detected in five (4.7%) samples. Lee et al. *(29)* demonstrated that PCR amplification of the pR72H DNA fragment is able to detect the presence of viable but noncultivable cells of *V. parahaemolyticus.* In France and Italy, several surveys of pathogenic vibrios in shellfish showed that the prevalence of *V. parahaemolyticus* ranged between 1.6 and 7.8%, with less than 5% of the isolates harboring the virulence associated hemolytic genes *tdh* and *trh (2, 18, 37).* Recently, Beaz Hidalgo et al. *(3)* reported that *V. cy-*

TABLE 3. *Bacterial numbers in 22 paired batches of farmed mussels (*Mytilus galloprovincialis*) and 31 paired batches of infaunal bivalve molluscs (*Vibrio pullastra, Cerastoderma edule, Ruditapes philippinarum, *and* Dosinia exoleta*) before and after commercial depuration*

	Mussels[a]			Infaunal edible bivalve molluscs[a]		
Counts	Before depuration	After depuration	% reduction	Before depuration	After depuration	% reduction
APC	3.454 ± 0.885	3.257 ± 1.113	36.5	3.574 ± 0.782	**3.221 ± 0.902**[b]	55.6
Psychrotrophs	3.863 ± 0.633	3.391 ± 0.800	66.3	4.084 ± 0.785	3.829 ± 0.773	44.4
Pseudomonas spp.	3.080 ± 0.671	2.964 ± 0.817	23.4	3.554 ± 0.591	3.228 ± 0.571	52.8
LAB	1.500 ± 1.026	1.178 ± 0.894	52.4	1.928 ± 0.907	1.968 ± 0.638	−9.6
Aeromonas spp.	3.108 ± 1.162	**2.670 ± 1.274**	63.5	2.818 ± 1.189	2.443 ± 1.236	57.8
Vibrio spp.	2.839 ± 1.067	2.422 ± 1.094	61.7	2.930 ± 1.530	2.954 ± 1.361	−5.7
Total coliforms	1.919 ± 0.769	**0.731 ± 0.451**	93.5	1.658 ± 0.887	**1.183 ± 0.852**	66.5
Escherichia coli[c]	1.585 ± 0.782	**0.519 ± 0.379**	91.4	1.209 ± 0.968	0.841 ± 0.709	57.1
E. coli[d]	3,845 ± 605	331 ± 239		1,618 ± 928	693 ± 511	

[a] Values are means ± SD of log CFU per gram.
[b] Significant ($P < 0.05$) differences are in boldface.
[c] Values here are log MPN per gram, using ISO Method 16649-3.
[d] Values here are MPN per 100 g.

clitrophicus, V. splendidus, and *V. alginolyticus* were the most abundantly represented species among vibrios isolated from cultured clams in Galicia, although only 57 of 145 strains could be identified at the species level.

In Spain, seafood ranked third (5.7%) on the list of food items implicated in 1,221 foodborne outbreaks reported in 2003 *(6).* Unknown agents caused 34 of the 70 seafood-related outbreaks. The remaining resulted mainly from pathogenic bacteria, which are not naturally found in estuarine environments (*Salmonella* with 14 outbreaks, and *Campylobacter* and *Staphylococcus aureus* with 1 outbreak each) and histamine (9 outbreaks). Other causative agents were noroviruses and other viruses, *V. parahaemolyticus* (4 outbreaks each) and marine toxins (3 outbreaks). Thus, naturally occurring bacterial pathogens contributed to only 5.7% of the seafood-related outbreaks. Surveillance data did not provide information on the type of seafood product involved in outbreaks but did on the places where incriminated foods were eaten or acquired and the contributing factors. The most common settings implicated in outbreaks were restaurants, mass catering for especial groups, and private homes, with the top contributing factors being cross-contamination and/or poor temperature control. These data suggest that the bacteriological risk associated with fish and shellfish in Spain appear to result most from cross-contamination of cooked products by allochthonous bacteria, mainly *Salmonella,* or from contamination during preparation, which is then followed by time-temperature abuse. This occurs mainly at the food service level and private homes, which is common to all foods and not specific for seafood products. In France, available surveillance data *(42)* reported that *Salmonella, S. aureus,* and *C. perfringens* were major sources of illness attributed to seafood in 1999 and 2000.

E. coli is widely utilized as an indicator of the sanitary quality of bivalve molluscs and their growing areas because it is directly associated with the feces of humans and warm-blooded animals such as domestic and farm animals, land-based wildlife, marine mammals, and birds. *E. coli* meets the requirements of a good indicator organism, is easy to determine, and consistently present in large numbers in sewage. In member states of the EU, production areas are categorized by the level of *E. coli* contamination found in molluscan shellfish sampled from a site. These areas are classified as class A ($\leq$230 *E. coli* per 100 g of flesh and liquor), from which live bivalve molluscs may be collected for direct human consumption; class B ($\leq$4,600 *E. coli* per 100 g of flesh and liquor), with shellfish requiring treatment in a purification center or relaying; and class C ($\leq$46,000 *E. coli* per 100 g of flesh and liquor), requiring relaying over a long period *(12).* Shellfish derived from classes B or C areas may be placed on the market once they comply with the microbiological standard of $\leq$230 *E. coli* per 100 g of flesh and liquor and absence of *Salmonella* in 25 g of flesh *(13).* In this study, *E. coli* levels before depuration confirmed that all batches were collected from class B harvesting sites, *Salmonella* not being detected in any flesh sample. For *E. coli,* statistical analysis of composite data (Table 2) showed that depuration under commercial conditions resulted in a significant ($P < 0.05$) decrease in *E. coli* numbers, although the average elimination was 75%, which is much lower than most achieved in controlled purification studies of naturally contaminated bivalve molluscs *(8, 10).* It should be noted, however, that there was a marked difference in elimination rates between mussels and infaunal bivalves. Thus, although the initial mean *E. coli* numbers (MPN per 100 g) observed in mussels was over twofold higher than that found in clams and cockles, mussels eliminated *E. coli* more efficiently (91.4 versus 57.1%), the process being effective in reducing the *E. coli* levels to below 230/100 g in 90.9% of mussel lots, but only in 70.9% of infaunal bivalve lots. Under EU legislation *(11),* bivalve molluscs from class B production areas must be continuously purified for a period sufficient to meet EU microbiological criteria, but our data indicate that bivalves did not always reduce *E. coli* numbers to acceptable levels within the commercial depuration period. Failure to achieve clearance to this level may indicate a problem with the purification system and raises the question of whether the length of the depuration period was adequate, especially for infaunal bivalves. Research has demonstrated that the success of *E. coli* reduction in depurating shellfish depends on many factors, some of them being peculiar to the species of shellfish and their physiological conditions *(35).*

Total coliforms do not necessarily indicate fecal contamination, but they had been used in the EU until 2006 for assessing the general quality of coastal seawater (Directive 1976/160/EC) and in some non-EU countries for assessment of shellfish harvesting areas *(39).* This bacterial group was significantly reduced by depuration, although mussels were again more effective (93.5% reduction, $P < 0.005$) than were infaunal bivalves (66.5% reduction, $P < 0.05$). The high correlation found between these bacteria and *E. coli* after depuration suggests that at least in mussels, *E. coli* was eliminated at a rate slower than non–*E. coli* coliform species.

Aerobic and psychrotrophic plate counts may also indicate the microbiological quality of shellfish *(27).* For fresh bivalve molluscs, the International Commission on Microbiological Specifications for Foods *(20)* recommends APC as indicative of general quality and to a lesser extent, of handling and storage procedures. Bivalve molluscs are considered to be satisfactory if the APC does not exceed 5×10^5 CFU/g ($\leq$5.70 log CFU/g), which is 2 log greater than the mean numbers found by us before and after depuration. Although there were differences between mussels and infaunal bivalves (Table 3), overall mean APC and psychrotrophic counts were significantly ($P < 0.05$) reduced after depuration (Table 2) but seldom to less than 10^3 CFU/g. The failure of total counts to decrease below 10^3 to 10^4 CFU/g has been related to the maintenance of the gram-negative bacteria predominant in the endogenous microbiota *(7, 21).* Olafsen et al. *(32)* demonstrated that numbers in the range 10^2 to 10^3 CFU/ml or g were present in the hemolymph and soft tissues of both Pacific oysters (*Crassostrea gigas*) and horse mussels (*Modiolus modiolus*), and identified *Pseudomonas, Aeromonas,* and *Vibrio* as the major genera present. In this study, the highly sig-

nificant ($P < 0.005$) correlations between the three genera and APC suggest that *Pseudomonas, Vibrio,* and *Aeromonas* were responsible for a large fraction of the total flora. This is of practical significance because the three genera are particularly involved in the proteolytic spoilage of shucked bivalve mollusc. Spoilage of some molluscan shellfish also involves the breakdown of glycogen by LAB, although it may not occur consistently *(7, 19, 21).*

In conclusion, *Salmonella* and foodborne bacteria occurring naturally in estuarine waters do not appear to be significant hazards in molluscan shellfish from Galicia class B harvesting areas. Although current depuration procedures are unable to guarantee the production of bivalve molluscs free from all microbiological contaminants, purification plays a significant role in eliminating pathogenic bacteria and reducing the viral content of shellfish. The microbiological criteria that determine the acceptability of a product or process based on the microbiological levels should be applied within a food safety management system. This is intended to be proportional to risk and not place unnecessary burdens on business. Therefore, it is of concern that clearance of *E. coli* to EU shellfish end-product standard levels (≤230/100 g of flesh and liquor) are not always achieved, especially in infaunal species. Under EU regulations, both food business operators and the competent authority are responsible for ensuring compliance with the relevant legislation. Finally, this study confirms that *Vibrio, Pseudomonas,* and *Aeromonas* constitute a significant part of the resident molluscan microbiota.

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