Research Note

Evaluation of the microbiological quality of jacopevers and plaices in Korea, 2015-2016

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Abstract In Southeast Asian countries, including Korea, China, and Japan, the considerable amounts of raw fish have been annually consumed in the manner of live fish fillets without minimally thermal processing, increasing the risks of causing food-borne diseases. This study investigated the occurrence of total aerobic bacteria (TAB), coliform, *Vibrio parahaemolyticus*, *Salmonella enterica* serovar spp., *Listeria monocytogenes*, and *Staphylococcus aureus* in jacopevers and plaices. Total 200 live fishes were collected from randomly selected restaurants located in Anseong-si, and then they were microbiologically monitored. TAB ranged from 3.09 to 3.21 Log₁₀ CFU/g in jacopever and plaice. Coliform in the levels of 1.54 Log₁₀ CFU/g were detected in samples. Out of 100 jacopevers, a single jacopever (1%) exhibited the prevalence of *S. aureus* in the edible part, though none of pathogenic bacteria were detected. These results will be useful for understanding the microbial prevalence in the domestic living jacopevers and plaices.

Keywords: direct plating count, live fish fillet, microbiological quality

Introduction

Recently, the annual dietary intake of seafood such as jacopever, plaice, gray mullet, and red seabream has been increasing in Korea (1,2). Overall productions of the fish farming have risen by 14% from the previous year and the production value has exceeded 1.7 billion won. It was reported that the output of live fish fillets accounted for 64% of the gross production of the fish farming industry (3). Now, consumers are taking it into consideration that the consumption of seafood was implicated with some health benefits, including decreasing risks of cardiovascular diseases, decreasing progression of atherosclerosis in coronary patients, and a mild decline in blood pressures of humans with hypertension (4-6). However, previously published literatures have concerned the immoderate consumption of seafood contaminated with either pathogenic bacteria or toxic materials through a wide variety of channels (7,8). Until now, food-borne outbreaks associated with seafood have displayed increasing frequencies. The isolation of pathogenic bacteria such as *Vibrio parahaemolyticus*, *Salmonella enterica* serovar spp., *Listeria monocytogenes*, and *Staphylococcus aureus* from a wide variety of seafood were also reported (9,10). Raw fish, clam, mussel, and other seafood are recognized as the major sources of bacterial contaminations caused by *L. monocytogenes*, and *S. aureus* (11-14). These results implied that seafood become contaminated with microbiological pollutants such as bacteria and yeast/mold easily if good hygienic

practice is not implemented during the entire processing. Some Asian countries might prefer to consume a number of marine organisms in the form of either live fish fillets or sushi that are not thermally processed, posing a severe risk of causing the food-borne disease outbreaks. Then, there is a broad consensus on conducting monitoring strategies for the detection of pathogenic bacteria in seafood. Therefore, the microbiological quality of jacopevers and plaices, which occupy a majority of the production value in the domestic fishery industry, was investigated either quantitatively or qualitatively in response to the seasonal difference. In this study, the prevalence of *V. parahaemolyticus*, *Salmonella* spp., *L. monocytogenes*, and *S. aureus* in fish samples harvested during spring, summer, autumn, and winter was examined for isolating these pathogenic bacteria in seafood by way of plato count method seasonally.

Materials and Methods

Sampling Each of 25 jacopevers and 25 plaices alive was purchased quarterly from randomly selected seafood restaurants located in Anseong-si, Korea. These live fishes were transferred onto the laboratory within the day, and then they were separated into the edible and intestine/gill part with a sterile knife. Samples weighing 25 g were transferred into a stomacher bag (Difco Laboratories,

Detroit, MI, USA) containing 225 mL of sterile buffered peptone water (BPW, Difco), alkaline peptone water (APW, Oxoid Inc., Basingstoke, UK), tryptic soy broth (TSB, Difco) supplemented with 10% sodium chloride, and *Listeria* enrichment broth (LEB, Difco), respectively. Individual sample bags were stomached (Colworth Stomacher 100; Seward Ltd., London, UK) for 2 min.

Direct plating-count In order to investigate the microbial prevalence in jacopecters and plaices quantitatively, this following process was carried out according to the standard manual represented in the Korean Food Code (15). One mL of the sample suspensions was serially diluted with 9 mL of sterile BPW for enumerating TAB, coliform, *Salmonella* spp., and *L. monocytogenes*, TSB for *S. aureus*, and APW for *V. parahaemolyticus*. Then, 0.1 mL of the aliquot was spread-plated onto each of the selective media. Thiosulphate-citrate-bile salts-sucrose agar (TCBS, Difco), Xylose-lysine-deoxycholate agar (XLD, Difco), Baird-parker agar (BP, Difco) supplemented with egg-yolk tellurite (Oxoid), and Oxford agar (OAB, Difco) supplemented with *Listeria* antimicrobial substance (Difco) were prepared according to the instructions provided with the manufacturer. In particular, for the dilution in APW and tryptic soy broth (TSB, Difco) to be plated, 1 mL of the sample suspension was transferred into 3 agar plates of TCBS and BP, distributing 1 mL of the inoculums equitably to the 3 plates (for an instance, 0.4, 0.3, and 0.3 mL). Different types of the petrifilms (Petrifilm™-aerobic count plate and coliform, 3M Microbiology Company, St. Paul, MN, USA) were used for enumerating the number of TAB and coliform, respectively. All agar media were incubated at 37°C for 24 h, and then colonies developed on media were enumerated, followed by the further identification.

Enrichment Sample bags filled with each of the buffered suspensions were incubated at 37°C for 24 h (15). An aliquot of the bacterial suspensions was spread-plated onto each of the selective media, using a sterile loopful (1.0 µL). All agar media were incubated at 37°C for 24 h, and then colonies developed on media were identified by API kits (Difco), followed by the confirmation with 16S rRNA sequencing.

Confirmation The colonies developed on each of selective media was streaked on the same medium as well as a non-selective medium such as tryptic soy agar (TSA, Difco), and then they were incubated at 37°C 24 h. These activated cells were transferred into 5 mL of sterile deionized water, vortexed for 2 min. Then, 100 µL of the aliquots was inoculated into the individual cubes developed on the API 20E, API STAPH, and API LISTERIA kits, respectively. Colonies which appeared to be different from those of typical *V. parahaemolyticus*, *Salmonella* spp., *S. aureus*, and *L. monocytogenes* on selective media were excluded from the following 16S rRNA sequencing. Briefly, a loopful (1.0 µL) of colonies, presumably recognized as one of the pathogenic bacteria, was inoculated into 5 mL of TSB and incubated at 37°C overnight. These bacterial suspensions were centrifugated at 10,000×g for 3 min, washed twice, and re-suspended in 1 mL of sterile phosphate

buffered saline (PBS). Then, they were centrifugated at 10,000×g for 3 min and the supernatant was removed to collect the bacterial pellet. These cells were assigned the 16S rRNA sequencing to MacroGen Inc. (Seoul, Korea).

Results and Discussion

Quantitative monitoring Microbial risk assessment of jacopecters harvested quarterly from randomly selected seafood restaurants located in Anseong-si, South Korea has been represented in Table 1. TAB were detected in 95 out of 100 jacopecters in the edible part, showing the bacterial number of 3.15 Log₁₀ CFU/g on average. TAB in the levels of 3.09 Log₁₀ CFU/g were observed in 94 out of 100 jacopecters in the intestine/gill part. It has been also shown that the average number of coliform ranged from 1.41 to 1.51 Log₁₀ CFU/g in jacopecters. Regardless of the separated parts, the prevalence of TAB and coliform in 100 plaices ranged from 3.15 to 3.21 Log₁₀ CFU/g and from 1.21 to 1.30 Log₁₀ CFU/g, respectively (Table 2). Although it has been shown that seemingly positive colonies as the pathogenic bacteria were detected on individual selective media, all of these cells were further identified as the false positives, followed by the biochemical identification assay (data not shown). It has been well known that bacterial behaviors were significantly different, depending on the climate change. Amongst a wide variety of stressful factors, temperature might play a key role for monitoring the microbial quality in jacopecters and plaices. As shown in Fig. 1, the average temperature of seawater during spring, summer, autumn, and winter ranged from 11-12, 21-23, 9-1, and 6-7°C, respectively. Although jacopecters and plaices harvested during summer (July-August) exhibited the highest number of TAB, none of pathogenic bacteria was detected in 100 jacopecters and 100 plaices.

Qualitative monitoring As mentioned above, fish samples that experienced the enrichment procedure at 37°C for 24 h were spreading-plated on selective media for determining the qualitative abundance of pathogenic bacteria (Table 1 and 2). As a consequence, several colonies were developed on each of selective media; however, their phenotypic properties greatly differed from those of typical *V. parahaemolyticus*, *Salmonella* spp., *S. aureus*, and *L. monocytogenes* on media. Obviously, these cells were further identified as the false positives based on the biochemical identification assay. It was also revealed that the microbial prevalence in jacopecters and plaices consisted of a wide variety of bacteria, including *Aeromonas* spp., *Enterobacter cloacae*, *Escherichia fergusonii*, *Ewingella Americana*, *Listeria ivanovii*, *Pantosa* spp., *Serratia odorifera* 1, and *Staphylococcus lentus*. Nevertheless, a colony developed on BP was confirmed as *S. aureus* as a consequence of 16S rRNA sequencing. This pathogen came from the edible part of a single jacopecter harvested during winter, indicating that the prevalence of human pathogenic bacteria in jacopecters and plaices were in the levels of at least 1%. According

Table 1. Monitoring microbiological qualities in jacopevers

Category	Region	Season	Period	Bacterial population (Log ₁₀ CFU/g) or the number of a positive sample					
				TAB	Coliform	<i>V. parahaemolyticus</i>	<i>Salmonella</i> spp.	<i>S. aureus</i>	<i>L. monocytogenes</i>
Quantitative approach	Edible part	Spring (n=25)	April-May	3.14	1.34	<1.43 ¹⁾	<1.43	<1.43	<1.43
		Summer (n=25)	July-August	3.90	1.39	<1.43	<1.43	<1.43	<1.43
		Fall (n=25)	December-January	3.25	1.40	<1.43	<1.43	<1.43	<1.43
		Winter (n=25)	February-March	2.29	1.51	<1.43	<1.43	<1.43	<1.43
		Average		3.15	1.41	<1.43	<1.43	<1.43	<1.43
	Intestine/ gill part	Spring (n=25)	April-May	3.95	1.24	<1.43	<1.43	<1.43	<1.43
		Summer (n=25)	July-August	4.04	1.44	<1.43	<1.43	<1.43	<1.43
		Fall (n=25)	December-January	2.03	1.44	<1.43	<1.43	<1.43	<1.43
		Winter (n=25)	February-March	2.32	1.12	<1.43	<1.43	<1.43	<1.43
		Average		3.09	1.31	<1.43	<1.43	<1.43	<1.43
Qualitative approach	Edible part	Spring (n=25)	April-May	ND ²⁾	ND	-	-	-	-
		Summer (n=25)	July-August	ND	ND	-	-	-	-
		Fall (n=25)	December-January	ND	ND	-	-	-	-
		Winter (n=25)	February-March	ND	ND	-	-	1	-
		Total		ND	ND	0	0	1	0
	Intestine/ gill part	Spring (n=25)	April-May	ND	ND	-	-	-	-
		Summer (n=25)	July-August	ND	ND	-	-	-	-
		Fall (n=25)	December-January	ND	ND	-	-	-	-
		Winter (n=25)	February-March	ND	ND	-	-	-	-
		Total		ND	ND	0	0	0	0

¹⁾Below the detectable levels.²⁾ND, not determined.**Table 2.** Monitoring microbiological qualities in plaices

Category	Region	Season	Period	Bacterial population (Log ₁₀ CFU/g) or the number of a positive sample					
				TAB	Coliform	<i>V. parahaemolyticus</i>	<i>Salmonella</i> spp.	<i>S. aureus</i>	<i>L. monocytogenes</i>
Quantitative approach	Edible part	Spring (n=25)	April-May	3.70	1.34	<1.43 ¹⁾	<1.43	<1.43	<1.43
		Summer (n=25)	July-August	3.75	1.05	<1.43	<1.43	<1.43	<1.43
		Fall (n=25)	December-January	2.78	1.21	<1.43	<1.43	<1.43	<1.43
		Winter (n=25)	February-March	2.35	1.23	<1.43	<1.43	<1.43	<1.43
		Average		3.15	1.21	<1.43	<1.43	<1.43	<1.43
	Intestine/ gill part	Spring (n=25)	April-May	3.65	1.54	<1.43	<1.43	<1.43	<1.43
		Summer (n=25)	July-August	3.86	1.11	<1.43	<1.43	<1.43	<1.43
		Fall (n=25)	December-January	2.43	1.18	<1.43	<1.43	<1.43	<1.43
		Winter (n=25)	February-March	2.90	1.37	<1.43	<1.43	<1.43	<1.43
		Average		3.21	1.30	<1.43	<1.43	<1.43	<1.43
Qualitative approach	Edible part	Spring (n=25)	April-May	ND ²⁾	ND	-	-	-	-
		Summer (n=25)	July-August	ND	ND	-	-	-	-
		Fall (n=25)	December-January	ND	ND	-	-	-	-
		Winter (n=25)	February-March	ND	ND	-	-	-	-
		Total		ND	ND	0	0	0	0
	Intestine/ gill part	Spring (n=25)	April-May	ND	ND	-	-	-	-
		Summer (n=25)	July-August	ND	ND	-	-	-	-
		Fall (n=25)	December-January	ND	ND	-	-	-	-
		Winter (n=25)	February-March	ND	ND	-	-	-	-
		Total		ND	ND	0	0	0	0

¹⁾Below the detectable levels.²⁾ND, not determined.

Fig. 1. The average seawater temperature in Korea, 2015-2016 (24).

to a study conducted by Cañigral *et al.* (16), two oysters and two mussels (10%) out of 40 natural seafood samples were positive for *V. vulnificus* as a consequence of the biomolecular analysis. Lee (17) stated that none of *V. parahaemolyticus* and *V. vulnificus* were detected in oysters purchased from the Korean wholesale seafood market during May. In addition, the overall occurrence of *Salmonella enterica* serovar spp. in shellfish was in the levels of 0.74% in a domestic field (18), implying that the prevalence of pathogenic bacteria was very rare in seafood. Survivals of the pathogens were significantly dependent on various environmental challenges, including temperature, osmotic stress, salinity, and others. Various bacteria, including *Vibrio* spp., *L. monocytogenes*, *Shigella flexneri* or *Yersinia* spp. were very sensitive to these stressful factors, thereby resulting in the loss of culturabilities on a solid agar medium (19). It has been reported that these bacteria failed to grow on media on which it would typically grow and develop into their own colonies when subjected to the cold starvation of less than 15°C (20). At this point of view, bacteria in seafood would not be detected with the conventional culture methods probably because they have been induced into the viable-but nonculturable (VBNC) state under stressful environments. It has been reported that *V. parahaemolyticus* was not detected only in oysters procured during spring and winter (21). The exact mechanism of bacteria entering into such a nonculturable state has not yet been fully elucidated; however, several studies revealed that bacteria in VBNC state expressed specific genetic patterns, which were not existed in the normal state, resulting in changes in the membrane composition. For instance, an increase in either the amount of saturated fatty acids with short chains or certain proteins-EFTU_ECOLI, FIU_ECOLI, and AG43_ECOLI that were involved in folding protection, iron uptake, and auto aggregation was observed in *E. coli* and *V. vulnificus* after these bacteria entered into the VBNC state (22,23). If pathogens were induced into VBNC state, it would be difficult to monitor the bacterial prevalence in seafood, implying that jacobever and plaice used in the present study may not be free from the microbial contamination.

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