RESEARCH LETTER

Development of a 16S–23S rRNA intergenic spacer-based quantitative PCR assay for improved detection and enumeration of *Lactococcus garvieae*

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Abstract

Lactococcus garvieae is an important foodborne pathogen causing lactococcosis associated with hemorrhagic septicemia in fish worldwide. A real-time quantitative polymerase chain reaction (qPCR) protocol targeting the 16S–23S rRNA intergenic spacer (ITS) region was developed for the detection and enumeration of *L. garvieae*. The specificity was evaluated using genomic DNAs extracted from 66 cocci strains. Fourteen *L. garvieae* strains tested were positive, whereas 52 other strains including *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *hordniae* and *Lactococcus lactis* ssp. *cremoris* did not show a specific signal. The minimal limit of detection was 2.63 fg of purified genomic DNA, equivalent to 1 genome of *L. garvieae*. The optimized protocol was applied for the survey of *L. garvieae* in naturally contaminated fish samples. Our results suggest that the qPCR protocol using ITS is a sensitive and efficient tool for the rapid detection and enumeration of *L. garvieae* in fish and fish-containing foods.

Introduction

Members of the genus *Lactococcus* are often isolated from food-related sources and are therefore generally regarded as safe. However, among seven *Lactococcus* species, *Lactococcus garvieae* is known as an important pathogen that causes disease in fish and mammals (Wang *et al.*, 2007; Li *et al.*, 2008). Lactococcosis outbreaks have been reported in ready-to-eat food products that are consumed without cooking (Novotny *et al.*, 2004). Recently, gastrointestinal disorders, endocarditis, bacteremia, peritonitis, liver abscess, and osteomyelitis have been associated with consumption of raw fish contaminated with *L. garvieae*; thus, the importance of controlling contamination of foods is increasing (James *et al.*, 2000; Wang *et al.*, 2007; Li *et al.*, 2008; Chan *et al.*, 2011). In South Korea, this microorganism has caused outbreaks in aquaculture, such as in black rockfish (Kang *et al.*, 2004), flounder (Baeck *et al.*, 2006; Jeong *et al.*, 2006), and has been isolated from fermented fish (Jung *et al.*, 2010).

Bacterial identification methods based on biochemical tests, such as the miniaturized API system, or conventional culture have traditionally been used for the identification of *L. garvieae* in fish (Zlotkin *et al.*, 1998). However, distinguishing this microorganism from other lactic acid bacteria commonly found in fermented foods, such as *Streptococcus thermophilus*, *Lactococcus lactis*, or *Enterococcus*-like strains, remains difficult and unreliable (Casalta & Montel, 2008; Ogier & Serror, 2010).

Real-time quantitative polymerase chain reaction (qPCR) is a popular method for identifying and monitoring contaminant bacterial populations in foods because of its sensitivity and time-efficiency (Casalta & Montel, 2008; Ogier & Serror, 2010). At present, qPCR with 16S rRNA gene-targeted primers is one of the most popular methods for identifying predominant bacterial populations (Matsuki *et al.*, 2004; Haarman & Knol, 2006). However, it has been demonstrated that the sensitivity of qPCR is not sufficient for the accurate quantification of subdominant populations in Gram-positive cocci

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(Matsuda *et al.*, 2009). Indeed, the ribosomal RNA gene primer set of *L. garvieae* also amplifies DNA fragments from other bacterial species. By contrast, the 16S–23S RNA intergenic spacer (ITS) is considered a good monitoring tool for bacterial identification and strain typing (Hoffmann *et al.*, 2010).

A real-time PCR for the detection of *L. garvieae* with primers targeting the 16S RNA gene has been applied directly to foods (Jung *et al.*, 2010). However, this qPCR method showed a false-positive amplification with *Lactococcus lactis* ssp. *lactis* strains (Dang *et al.*, 2012), and quantification of *L. garvieae* after an enrichment step has not been applied in foods. Despite the potential for foodborne transmission to humans, monitoring protocols that target *L. garvieae* in fish and fish-containing foods have not been developed. In this study, we reported an improved qPCR procedure targeting the ITS for detection and quantification of *L. garvieae* in fish and fermented fish products.

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. The strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA), the Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMG, Gent, Belgium), the Culture Collection of the University of Gothenburg (CCUG, Gothenburg, Sweden), the Deutsche Samm-lung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany), the Korean Collection for Oral Microbiology (KCOM, Gwangju, Korea), and the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). Lactococci, enterococci, and vagococci were grown aerobically at 30 or 37 °C for 18 h in trypticase soy yeast extract medium (Difco, Detroit, MI), and streptococci were grown under microaerophilic conditions on sheep blood agar (Asan Pharm Co., Seoul, Korea) at 37 °C for 20 h.

Genomic DNA preparation

Bacterial genomic DNA used for qPCR was extracted from cultivated bacteria, fresh and fermented fish-containing foods using the bead-beat method, as described previously (Jung *et al.*, 2010). Total bacterial DNA was quantified using the Infinite 200 NanoQuant (Tecan, Männedorf, Switzerland) at a wavelength of 260 nm.

Specificity of real-time PCR amplification

Real-time PCR assay was performed and monitored using SYBR Green chemistry with a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA). To amplify the 16S–23S rRNA ITS region of *L. garvieae*, we used two specific primers: ITSLg30F (5′-ACTTTATTCAGTTT TGAGGGGTCT-3′) positions 30–53 in *L. garvieae* KCTC 3772^T and ITSLg319R (5′- TTTAAAAGAATTCGCAGCTT TACA-3′) positions 296–319 in *L. garvieae* KCTC 3772^T (Dang *et al.*, 2012). The qPCR amplification was performed in a total volume of 20 μL containing 1 μL of each template DNA, 1 μL of 10 pmole primers, 7 μL nuclease-free water, and 10 μL SYBR Green I master mix (Roche Diagnostics, Indianapolis, IN). qPCR assays were carried out using a standard program: PCR conditions consisted of an initial denaturation step of 5 min at 95 °C, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. A melting curve analysis was then performed from 95 to 65 °C at a rate of 0.1 °C s^{-1} with the continuous acquisition of fluorescence data. All samples were analyzed in triplicate in MicroAmp 96-well reaction plates (Applied Biosystems). Real-time PCR assay specificity was optimized and tested in real-time PCRs using total DNA extracted from 14 *L. garvieae* strains and 17 non-*L. garvieae* bacterial strains belonging to ten different species.

Sensitivity of qPCR

The sensitivity of the qPCR assay was evaluated using *L. garvieae* KCTC 3772^T. qPCR detection was performed on a 10-fold dilution series of purified DNAs from *L. garvieae* cells (26.3×10^{-7} to 1 ng μL^{-1}). *Lactococcus garvieae* KCTC 3772^T concentrations were calculated using the viable cell plate count method. Serial 10-fold dilutions of the cultures were plated onto *Lactobacillus* MRS agar (Difco laboratories), which were subsequently incubated at 37 °C for 24 h, and CFUs were determined in triplicate.

Inhibition control

For confirmation of the qPCR-negative food samples from *L. garvieae* cell, 8 μL of the purified DNA preparation from the foods was spiked shrimp with 2 μL of the extracted DNA from *L. garvieae* KCTC 3772^T at a concentration corresponding to approximately 10^1–10^2 CFU per reaction. The qPCR was follow above same method, along with a positive and a negative control, to identify inhibition.

Standard curves

A calibration curve was constructed using fresh salmon and shrimp matrices that were negative for *L. garvieae*. For the construction of calibration curves in food samples, the strain of *L. garvieae* KCTC 3772^T was used. The

Table 1. Bacterial strains (n = 66) and PCR results

No.	Species	Designation	Isolation source	PCR
1	*Lactococcus garvieae*	KCTC 3772^T	Bovine mastitis	+
2	*Lactococcus garvieae*	LMG 8162	Bovine mastitis	+
3	*Lactococcus garvieae*	LMG 9472	Raw milk	+
4	*Lactococcus garvieae*	LMG 8501	Bovine mastitis	+
5	*Lactococcus garvieae*	KCTC 5620	Diseased yellowtail	+
6	*Lactococcus garvieae*	KCTC 5621	Turtle eye	+
7	*Lactococcus garvieae*	CAU 1101	Flounder	+
8	*Lactococcus garvieae*	CAU 1102	Pollack	+
9	*Lactococcus garvieae*	CAU 1103	Flounder	+
10	*Lactococcus garvieae*	CAU 1104	Pollack	+
11	*Lactococcus garvieae*	CAU 1105	Pollack	+
12	*Lactococcus garvieae*	CAU 1106	Flounder	+
13	*Lactococcus garvieae*	CAU 1107	Flounder	+
14	*Lactococcus garvieae*	CAU 1108	Flounder	+
15	*Lactococcus lactis* ssp. *lactis*	KCTC 2013	–	–
16	*Lactococcus lactis* ssp. *lactis*	KCTC 3115	–	–
17	*Lactococcus lactis* ssp. *lactis*	KCTC 3191	–	–
18	*Lactococcus lactis* ssp. *lactis*	KCTC 3899	Earthworm intestine	–
19	*Lactococcus lactis* ssp. *lactis*	KCTC 3769^T	–	–
20	*Lactococcus lactis* ssp. *lactis*	KCTC 3926	Dairy products	–
21	*Lactococcus lactis* ssp. *hordniae*	KCTC 3768^T	Leaf hopper	–
22	*Lactococcus lactis* ssp. *cremoris*	DSM 20069^T	–	–
23	*Lactococcus raffinolactis*	KCTC 3982^T	Raw milk	–
24	*Lactococcus plantarum*	DSM 20686^T	Frozen peas	–
25	*Lactococcus chungangensis*	KCTC 13185^T	Activated sludge foam	–
26	*Streptococcus anginosus*	ATCC 33397^T	Human oral cavity	–
27	*Streptococcus anginosus*	KCOM 1063	Human oral cavity	–
28	*Streptococcus australis*	KCOM 1439	Human oral cavity	–
29	*Streptococcus australis*	KCOM 1441	Human oral cavity	–
30	*Streptococcus gordonii*	KCTC 3286^T	Bacterial endocarditis	–
31	*Streptococcus infantis*	KCOM 1375	Human oral cavity	–
32	*Streptococcus intermedius*	KCTC 3268^T	Human oral cavity	–
33	*Streptococcus mitis*	KCTC 13047^T	Human oral cavity	–
34	*Streptococcus mitis*	KCTC 3556^T	Human oral cavity	–
35	*Streptococcus oralis*	KCTC 13048^T	Human plaque	–
36	*Streptococcus oralis*	DSM 20066	Human throat	–
37	*Streptococcus oralis*	DSM 20395	Human plaque	–
38	*Streptococcus oralis*	DSM 20379	Human plaque	–
39	*Streptococcus oralis*	ATCC 9811^T	Human mouth	–
40	*Streptococcus parasanguinis*	KCTC 13046^T	Human throat	–
41	*Streptococcus parasanguinis*	KCOM 1352	Human oral cavity	–
42	*Streptococcus pyogenes*	KCTC 3984^T	Scarlet fever	–
43	*Streptococcus pyogenes*	KCTC 3208	Pharynx of child	–
44	*Streptococcus pneumoniae*	KCTC 5080^T	Lower respiratory tract	–
45	*Streptococcus pseudopneumoniae*	CCUG 49455^T	Lower respiratory tract	–
46	*Streptococcus sanguinis*	KCTC 3284^T	Bacterial endocarditis	–
47	*Streptococcus sanguinis*	KCOM 1428	–	–
48	*Streptococcus sinensis*	KCOM 1017	Human sinus	–
49	*Streptococcus sinensis*	KCOM 1018	Human sinus	–
50	*Enterococcus hirae*	KCTC 3616^T	–	–
51	*Enterococcus mundtii*	KCTC 3630^T	Soil	–
52	*Enterococcus casseliflavus*	KCTC 3638^T	Plant material	–
53	*Enterococcus malodoratus*	KCTC 3641^T	Gouda cheese	–
54	*Enterococcus cecorum*	KCTC 3642^T	Chicken cecum	–
55	*Enterococcus saccharolyticus*	KCTC 3643^T	Straw bedding	–
56	*Enterococcus villorum*	KCTC 13904^T	Pig intestines	–

Table 1. Continued

No.	Species	Designation	Isolation source	PCR
57	*Enterococcus haemoperoxidus*	KCTC 13910^T	Service water	–
58	*Enterococcus moraviensis*	KCTC 13911^T	Service water	–
59	*Enterococcus phoeniculicola*	KCTC 3818^T	Uropygial gland	–
60	*Enterococcus solitarius*	KCTC 3923^T	Ear exudates	–
61	*Enterococcus raffinosus*	KCTC 5189^T	Blood culture	–
62	*Enterococcus avium*	KCTC 5190^T	Human feces	–
63	*Enterococcus faecalis*	KCTC 3206^T	–	–
64	*Vagococcus salmoninarum*	LMG 11491^T	Rainbow trout	–
65	*Vagococcus lutrae*	LMG 19537^T	Otter blood	–
66	*Vagococcus fluvialis*	LMG 9464^T	Chicken feces	–

+, PCR product was amplified with the ITSLg-F and ITSLg-R primer set; −, PCR product was not amplified with the ITSLg-F and ITSLg-R primer set.

strain was streaked on *Lactobacillus* MRS agar incubated at 37 °C for 24 h, and then the cells were taken from the agar surface and cultured in 3 mL of *Lactobacillus* MRS broth (Difco) for 24 h. The cell suspension was serially diluted in Ringer's solution and counted on *Lactobacillus* MRS agar incubated at 37 °C for 24 h. Each dilution was inoculated into a 10 g of fresh packed salmon and shrimp purchased from super markets in Seoul. Then, 40 mL of *Lactobacillus* MRS broth was added. The solid sample was homogenized using a Stomacher 80 laboratory blender (Seward Medical, London, UK) for 2 min at maximum speed. Subsequently, 1 mL was recovered and mixed with 9 mL of Ringer's solution, and 1 mL of the diluted sample was used for DNA extraction as described above (DNA at $t = 0$). The DNA extraction was also performed on the samples after 24 h ($t = 24$) of incubation at 37 °C in BHI broth. At $t = 24$, 1 mL of a 10-fold dilution in Ringer's solution was processed. One microliter of the $t = 0$ DNA (in triplicate) was used in qPCR amplifications, and calibration curves were constructed plotting the threshold cycle against the colony forming units (CFU g^{-1}). Similarly, the $t = 24$ DNA was used in amplification to determine the detection limit after overnight enrichment. The efficiency of the reactions was calculated according to the study by Rutledge & Cote (2003). Standard curves were constructed at least three times from three independent experiments. Contamination of background flora was checked using the *L. garvieae*-specific PCR analysis (Rutledge & Cote, 2003) and the API 20 Strep kit on *Lactobacillus* MRS medium. Suspected *L. garvieae* colonies from the *Lactobacillus* MRS agar plates were identified according to standard microbiological methods including the API 20 Strep method (BioMe′rieux sa, Marcy-l'Etoile, France) and *L. garvieae*-specific PCR analysis (Dang *et al.*, 2012), and then *L. garvieae* colonies were subjected to qPCR amplification as described above.

Lactococcus garvieae detection in potentially naturally contaminated samples

Forty-three samples of fresh fish and packaged foods containing fermented fish (Korean name: Jeotgal) were obtained from super markets in Seoul and Sokcho in South Korea. The samples used in this study were as follows: yellow corvenia, codfish, Alaska pollock, hairtail, anchovy, oyster, shrimp and fermented sample of yellow corvenia, codfish, pollack, anchovy, squid, octopus, prawn, nautilus, oyster, scallop, and clam. The packaging was removed under a laminar flow hood. From each package, 10 g of the fish or other seafood was aseptically cut and mixed with 40 mL of *Lactobacillus* MRS broth and homogenized as above. One milliliter of the homo genate was mixed with 9 mL of Ringer's solution, and 1 mL of this mix was used for DNA extraction ($t = 0$). At the same time, the 1–10 and 1–100 dilutions of the homogenate were plated on *Lactobacillus* MRS agar. Plates were incubated at 37 °C for 24 h in aerobic conditions. The homogenate was also incubated for 24 h at 37 °C, DNA extraction was carried out from a 10-fold dilution in Ringer's solution, and a loopful of the enriched homogenate was streaked on *Lactobacillus* MRS agar and incubated at 37 °C for 24 h ($t = 24$).

Results and discussion

The method for detection and quantification of *L. garvieae* presented was based on the amplification of the partial 16S–23S rRNA ITS gene. The specificity of the amplification was optimized and tested in qPCRs using total DNA extracted from 14 *L. garvieae* strains and 17 non-*L. garvieae* bacterial strains belonging to different species of *Lactococcus*, *Streptococcus*, *Enterococcus*, and *Vagococcus*. Amplification signals occurred only with the 14 *L. garvieae* DNA. In contrast, non-*L. garvieae* strains,

including closely related *Lactococcus* spp., did not show any signal results in the real-time PCR assay.

DNA was obtained from an *L. garvieae* culture at a concentration of 10^7 CFU mL^{-1}. Serial 10-fold dilutions were carried out to determine the sensitivity of our qPCR protocol. Each DNA dilution (26.3 ag to 26.3 ng) was used to construct a standard curve and a minimal limit of detection. The minimum limit of detection of *L. garvieae* genomic DNA using the new ITS gene-based qPCR assay was 2.63 fg (about 1 CFU), with a mean C_T value of 39.11 ± 1.54 (Table 2). The sensitivity of PCR amplification is dependent on the copy number, the age of the culture, and the method of cell lysis (Way *et al.*, 1993). One of the advantages of primers based on the ITS region is the multiple copies of rRNA operons. This minimal value is 12 times higher than that of the qPCR method targeting the 16S rRNA gene molecule (Jung *et al.*, 2010), indicating that our method is more sensitive for *L. garvieae* quantitation than the qPCR method targeting the corresponding 16S rRNA gene. The melting temperature for the amplicon from the *L. garvieae* type strain with the SYBR Green method was 87.5 °C, and the R^2 value was 0.99.

Real-time qPCR is regarded as the gold standard for accurate, sensitive, and rapid detection and enumeration of nucleic acid sequences. For this technique, SYBR Green I provides the simplest and most economical format for detecting and quantifying PCR products using the melting curve analysis (De Medici *et al.*, 2003; Audemard *et al.*, 2004). In our protocol, the primer sets with SYBR green fluorescence showed high amplification only to *L. garvieae* 10^1–10^2 CFU extracted DNA sample and have no other fluorescence signals from the spiked fish samples, such as shrimp. No amplification occurred in food samples indicating that this protocol is not interfering with SYBR green fluorescence and early contamination of *L. garvieae.*

A standard curve was created using the food matrices where there is a high incidence of *L. garvieae*. In particular, a standard curve was created starting from serially diluted *L. garvieae* cells in the salmon and shrimp samples. When inoculated salmon sample was used as a matrix, the mathematical expression of the log CFU mL^{-1} vs. the C_T values obtained was as follows: $y = -4.0942x + 38.074$ with an R^2 value of 0.99 (Fig. 1a). The number of bacteria in the inoculated shrimp sample ranged from $\log_{10}$ 1.32 ± 0.22 (at C_T values 34.83 ± 1.01) to $\log_{10}$ 2.98 ± 0.12 (at C_T values 23.47 ± 0.07) cells per gram according to the equation $y = -4.1285x + 39.163$ with an R^2 value of 0.99 (Fig. 1b). This genome equivalent was calculated assuming that one molecule of *L. garvieae* DNA corresponds to 2.6 fg of DNA, using a genome size of 2.5 Mb, and determined according to the following equation: DNA amount in fg = bp × 660 Da bp^{-1} × 1.6 × 10^{-27} kg Da^{-1} × 1 × 10^{-18} fg kg^{-1} (Park *et al.*, 2010). As the genome information of *L. garvieae* is not available from the current public databases, the genome size of 2.5 Mb for the *L. lactis* ssp. *lactis* KF147 (GenBank accession no. NC_013657) published on the NCBI GenBank database was utilized.

Table 2. C_T values for a dilution series of *Lactococcus garvieae* with 10^7 cells

DNA concentration (ng μL^{-1})	Cell number	C_T (mean ± SE)
26.3 × 10^{-0}	(5.96 ± 4.51) × 10^7	10.5 ± 0.01
26.3 × 10^{-1}	(5.96 ± 4.51) × 10^6	14.5 ± 0.02
26.3 × 10^{-2}	(5.96 ± 4.51) × 10^5	18.4 ± 0.10
26.3 × 10^{-3}	(5.96 ± 4.51) × 10^4	22.6 ± 0.02
26.3 × 10^{-4}	(5.96 ± 4.51) × 10^3	26.5 ± 0.04
26.3 × 10^{-5}	(5.96 ± 4.51) × 10^2	30.8 ± 0.07
26.3 × 10^{-6}	(5.96 ± 4.51) × 10^1	35.7 ± 0.48
26.3 × 10^{-7}	(5.96 ± 4.51) × 10^0	39.1 ± 1.54
0	0	ND

ND, not detected.

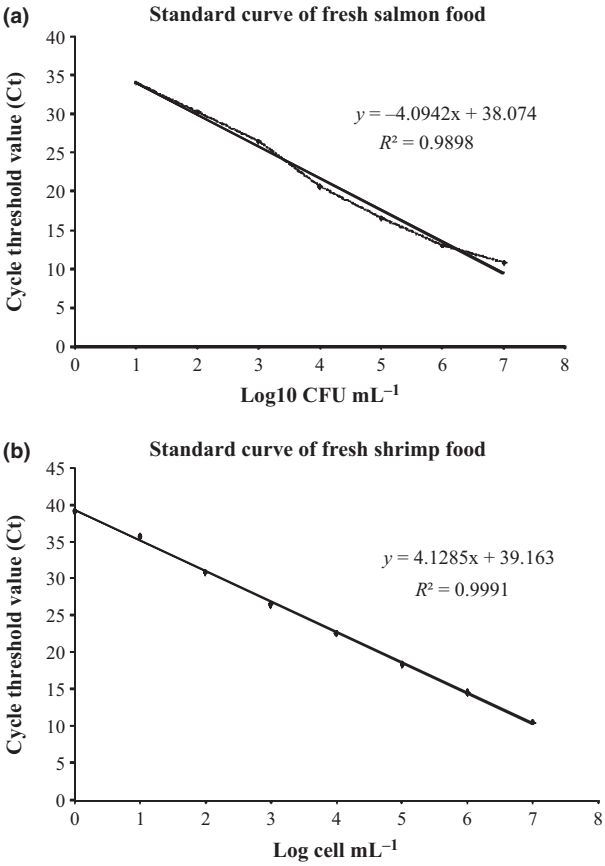

Fig. 1. Standard curves obtained via qPCR using ITSLg30F and ITSLg319R primers on 10-fold serial dilutions of *Lactococcus garvieae* in different food matrices (t = 0). (a) Inoculated *L. garvieae* in salmon, (b) inoculated *L. garvieae* in shrimp.

Table 3. Detection and enumeration of *Lactococcus garvieae* in fish by qPCR

Sample (no. of positive samples/no. of samples tested)	Results $t = 0$*		Results $t = 24$†	
	Traditional analysis (CFU g^{-1})	qPCR analysis (cell no. g^{-1})	Traditional analysis (CFU g^{-1})	qPCR analysis (cell no. g^{-1})
Yellow corvenia (0/3)	–	0	–	–
Codfish (0/3)	–	0	–	–
Alaska pollock (0/3)	–	0	–	–
Hairtail (0/3)	–	0	–	–
Anchovy (0/3)	–	0	–	–
Oyster (0/3)	–	0	–	–
Shrimp (0/3)	–	0	–	–
Fermented yellow corvenia (0/2)	–	0	–	–
Fermented codfish (0/2)	–	0	–	–
Fermented pollack (1/2)	48	1.72 ± 0.03	+	+
Fermented anchovy (1/2)	–	1.40 ± 0.16	–	+
Fermented squid (1/2)	–	1.52 ± 0.07	–	+
Fermented octopus (1/2)	592	2.98 ± 0.12	+	+
Fermented prawn (1/2)	–	1.32 ± 0.22	–	+
Fermented nautilus (1/2)	–	1.64 ± 0.08	–	+
Fermented oyster (1/2)	–	0	–	+
Fermented scallop (1/2)	–	0	–	+
Fermented clam (1/2)	–	0	–	+

*Results at $t = 0$ are reported as CFU g^{-1} as determined by the direct counts on *Lactobacillus* MRS and qPCR or based on the C(t) values obtained for the sample and the appropriate calibration curve.

†Results at $t = 24$ are reported as positive or negative. In the traditional analysis, positive refers to the presence of suspected colonies on *Lactobacillus* MRS plate and confirmed by qPCR, while in the qPCR analysis it refers to a fluorescence signal obtained during amplification.

The qPCR-based protocol was applied in parallel to traditional microbiological analysis to detect and quantify *L. garvieae* in food samples both at $t = 0$ and at $t = 24$. The results obtained by the two approaches are summarized in Table 3. At $t = 0$, *L. garvieae* was detected and enumerated using the ITS-based primer set, ITSLg30F and ITSLg319R, in six of 43 fresh and fermented fish samples (15%). At $t = 24$, *L. garvieae* was detected in nine of 43 tested samples (22.5%). The melting analyses of amplicons obtained from qPCR assays were 87.5 ° C ± 0.24. These results were confirmed via sequence analysis of the amplicons obtained through PCR and qPCR assays, which were 100% identical to the ITS fragment in the *L. garvieae* type strain KCTC 3772^T (GenBank accession no. HM241913). By traditional microbiological analysis, two of 43 samples (4.7%) at $t = 0$ and two of 43 samples (4.7%) at $t = 24$ were positive for *L. garvieae*. These results are from suspected *L. garvieae* colonies that were randomly selected from *Lactobacillus* MRS agar and subjected to qPCR analysis and identification using API 20 Strep kit. Melting curve analysis at the end of each run resulted in melting temperatures for the positive samples that were similar to those of the *L. garvieae* used as a control in the qPCR.

In our ITS-based procedures, *L. garvieae* was successfully detected in six samples ($t = 0$) ranging from $\log_{10}$ 1.32 ± 0.22 to $\log_{10}$ 2.98 ± 0.12 cells g^{-1} and nine fermented fish ($t = 24$) from a set of 43 tested samples. It is important to note that there is potential for transmission of *L. garvieae* to humans via the food chain. Rates of contamination of food products vary among samples. There are many documented cases of *L. garvieae* infection due to eating contaminated food (Wang *et al.*, 2007). The benefit of our new protocol is that it provides real-time results rather than next-day results, which may facilitate more appropriate responses.

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