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Development and Assessment of a Real-Time PCR Assay for Rapid and Sensitive Detection of a Novel Thermotolerant Bacterium, *Lactobacillus thermotolerans*, in Chicken Feces

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A new real-time PCR assay was successfully developed using a TaqMan fluorescence probe for specific detection and enumeration of a novel bacterium, *Lactobacillus thermotolerans*, in chicken feces. The specific primers and probe were designed based on the *L. thermotolerans* 16S rRNA gene sequences, and these sequences were compared to those of all available 16S rRNA genes in the GenBank database. The assay, targeting 16S rRNA gene, was evaluated using DNA from a pure culture of *L. thermotolerans*, DNA from the closely related bacteria *Lactobacillus mucosae* DSM 13345^T and *Lactobacillus fermentum* JCM 1173^T, and DNA from other lactic acid bacteria in quantitative experiments. Serial dilutions of *L. thermotolerans* DNA were used as external standards for calibration. The minimum detection limit of this technique was 1.84×10^3 cells/ml of an *L. thermotolerans* pure culture. The assay was then applied to chicken feces in two different trials. In the first trial, the cell population was 10^4 cells/g feces on day 4 and 10^5 cells/g feces on days 11 to 18. However, cell populations of 10^6 to 10^7 cells/g feces were detected in the second trial. The total bacterial count, measured by 4′,6-diamidino-2-phenylindole (DAPI) staining, was approximately 10^{11} cells/g feces. These results suggest that in general, *L. thermotolerans* is a normal member of the chicken gut microbiota, although it is present at relatively low levels in the feces.

Previously, we isolated *Lactobacillus thermotolerans*, a novel species, from chicken feces collected in Thailand (9). The preference of this bacterium for the chicken intestine may be due to the body temperature of chickens, 42°C (2), which corresponds to the optimum temperature for growth of this bacterium (42°C), as determined by measurement of the specific growth rate (9).

Our current interest in *L. thermotolerans* is to characterize this bacterium ecologically in the chicken intestine, since no studies have been conducted to date on the ecology of this new organism. Studies on the distribution and colonization of *L. thermotolerans* in different organs of the gastrointestinal tract should provide new insights into chicken intestinal microbiology. For this an effective method for enumeration of this bacterium is required. Development of a molecular ecological enumeration method appears to be particularly valuable in the case of *L. thermotolerans*, since conventional culture methods are insufficient due to the relatively high temperature required for culturing this bacterium. Real-time PCR offers significant advantages over other molecular enumeration techniques in terms of the speed at which assays are performed and the ability to quantify the target microbial population. Real-time PCR has already been established as a promising tool for studies of the composition of microbial communities in the gastrointestinal tract or feces of humans (1, 4, 5, 12), as well as chickens (13). However, most studies that have focused on the chicken microbiota have been conducted using conventional culture methods (6, 8, 10, 11). To the best of our knowledge, there has not yet been a report focusing on real-time monitoring of chicken lactobacilli, which are commonly used probiotic organisms in the avian industry. The use of probiotics to promote health and nutrition has attracted a great deal of attention, and claims have been made in this context with regard to daily weight gain, improvement in feed conversion, and resistance to disease (3).

In this paper, conditions for a real-time PCR assay of *L. thermotolerans* and successful application of this assay for monitoring the population dynamics of this bacterium in chicken feces are described.

MATERIALS AND METHODS

Bacteria and growth conditions. The strains used in the current study are listed in Table 1. *L. thermotolerans* JCM 11425^T was cultured using mixed gas (N_2-H_2-CO_2, 8: 1: 1) and Difco lactobacillus MRS broth (Becton Dickinson and Company) at 42°C overnight, and the reference strains were grown under the same conditions, except that the culture temperature was 37°C.

Chicken maintenance and sample collection. Two trials (trial 1 and trial 2) were conducted using five layer chicks (Boris Brown, 1 day old) in each trial at two different times. The chicks were obtained from the Hokuren Federation of Agricultural Cooperatives (Sapporo, Japan). They were kept in an individual section of a wooden box and were reared for 4 weeks for trial 1 and for 3 weeks for trial 2. The chickens were maintained and handled according to the recom-

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TABLE 1. Specificity of the primer-probe combination

Microorganism or prepn	Strain[a]	C_t[b]
Lactobacillus thermotolerans[c]	JCM 11425^T	18.57 (0.09)
Negative controls		
Lactobacillus salivarius subsp. *salicinius*	JCM 1044	34.87 (0.22)
Lactobacillus aviarius subsp. *araffinosus*	JCM 5667^T	34.56 (0.77)
Lactobacillus aviarius subsp. *aviarius*	JCM 5666^T	34.34 (1.46)
Lactobacillus crispatus	JCM 5810	34.45 (0.08)
Lactobacillus gallinarum	JCM 2011^T	34.61 (0.23)
Lactobacillus amylovorus	JCM 1126^T	35.28 (0.25)
Lactobacillus casei subsp. *casei*	JCM 1134^T	34.76 (0.81)
Lactobacillus helveticus	JCM 1120^T	32.27 (0.20)
Lactobacillus mucosae	DSM 13345^T	35.31 (0.54)
Lactobacillus fermentum	JCM 1173^T	33.77 (0.47)
Lactobacillus acidophilus	JCM 1132^T	34.57 (0.76)
Streptococcus equinus	JCM 7876	35.41 (0.80)
Lactococcus lactis subsp. *lactis*	JCM 1158	35.70 (0.41)
Enterococcus cecorum	JCM 8724^T	34.94 (0.35)
Pediococcus dextrinicus	JCM 5887^T	34.83 (0.32)
Leuconostoc mesenteroides subsp. *mesenteroides*	JCM 1564	34.46 (0.86)
Clostridium coccoides	JCM 1395^T	34.43 (0.26)
Clostridium butyricum	JCM 1391^T	35.91 (0.46)
Clostridium perfringens	JCM 3817	36.34 (0.43)
Bacillus coagulans	JCM 2257^T	36.11 (0.19)
SDW[d]		34.50 (0.48)

[a] Strains were obtained from the Japan Collection of Microorganisms (JCM) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM).
[b] The values in parentheses are standard deviations.
[c] *L. thermotolerans* was the target organism.
[d] Nontemplate control.

mendations of the ethics committee at our institute. The animals were given commercial feed (200 g/kg crude protein; energy, 2,950 kcal/kg) ad libitum once a day. Water was available during the entire experiment. Fresh feces were collected from individual chicks on days 4, 11, 18, and 27 in trial 1 and on days 1, 7, 14, and 21 in trial 2. A sterilized spatula was used for feces collection, and the samples were immediately transferred to the laboratory for analysis.

DNA extraction. The genomic DNAs were isolated from the culture broth of *L. thermotolerans* and of reference strains used as negative controls and from feces using an UltraClean soil DNA kit (MO BIO Laboratories, Inc., Solana Beach, CA) by following the manufacturer's instructions. Before DNA extraction, samples (0.1 g feces or 0.1 ml culture) were incubated with 50 μl of lysozyme (5 mg/ml) and 15 μl of *N*-acetylmuramidase (10.2 U/μl) at 37°C for 15 min. The quality of the extracted DNA was analyzed by electrophoresis on a 1.5% agarose gel. The DNA concentrations were determined by absorbance at 260 nm with a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). The DNA was then used for the real-time PCR assay.

Design of primers and probe. The PCR primers and probe were designed with Primer Express v2.0 (Applied Biosystems, Foster City, CA), and the specificity of the sequence was further checked against all the available data for 16S rRNA genes in the GenBank database. The probe was labeled with the fluorescent dye 6-carboxyfluorescein at the 5′ end and with 6-carboxytetramethylrhodamine at the 3′ end. The sequences were 5′-TGCACAGGATTGACGTTGGT-3′, 5′-GGCAGGTTGC CTACGTGTTACT-3′, and 5′-TCCCAACGAGTGGCGGACGG-3′ for forward primer 92F, reverse primer 157R, and TaqMan probe 113T, respectively.

5′ Nuclease PCR assay conditions. The amplification reactions were carried out 50-μl (total volume) mixtures. These mixtures contained 1× TaqMan Universal PCR master mixture (Applied Biosystems), which contained each of the *L. thermotolerans*-specific primers at a concentration of 900 nM, 250 nM fluorescence-labeled *L. thermotolerans*-specific probe, and 20 ng of DNA for each treatment. The amplification reactions were performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems), and the reactions were carried out by incubation for 2 min at 50°C (for activation of the uracil *N*-glycosylase) and for 10 min at 95°C (for activation of the AmpliTaq Gold DNA polymerase), followed by 15 s at 95°C (for denaturation) and 1 min at 63°C (for annealing and extension) for 40 cycles. Due to the positive signal of the closely related bacterium *Lactobacillus mucosae* (95% similarity), we increased the annealing temperature from 60 to 63°C. Data analysis was carried out using the ABI PRISM 7000 sequence detection system software (v1.0; Applied Biosystems). Each sample was analyzed in triplicate.

Specificity of the PCR assay. The DNAs extracted from the pure culture of *L. thermotolerans* and the reference strains were used to test the specificity of the primer-probe set. The most closely related strains, *L. mucosae* DSM 13345^T and *Lactobacillus fermentum* JCM 1173^T, had two mismatches in the probe that was designed, which enabled discrimination between strains. Other bacterial strains were discriminated from *L. thermotolerans* by evaluation of a threshold cycle (C_t) value. The C_t was defined as the cycle at which the fluorescence was significantly different from the background. Therefore, the C_t value provided an accurate measure of the number of target molecules originally present in the sample. Samples of the purified DNA (20 ng) were used as templates in the real-time PCR assay, which was carried out by using the thermal cycling conditions and data analysis procedure described above. Sterilized distilled water (SDW) was used as a nontemplate control.

Accuracy of the assay. In order to test the accuracy of the assay, 0.1-g samples of feces were mixed with various amounts (50, 100, or 150 μl) of a pure culture of *L. thermotolerans* (1.84×10^7 cells/ml); the mixtures were then subjected to DNA extraction, and then the numbers of cells were determined by real-time PCR. The assay was repeated twice.

Total count for the bacterial population as determined with DAPI. The total numbers of cells in the pure culture (*L. thermotolerans* cells/ml) and in the feces were determined after the samples were stained with a 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) *n*-hydrate solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 5 min at room temperature. The cells were examined with an Olympus BX50 microscope equipped with BX-FLA (Olympus Corporation, Tokyo, Japan). The DAPI signal was captured in 10 random microscopic fields.

RESULTS

Primer and probe design and specificity. The specificity of the assay with the primer-probe combination that we developed for the detection of *L. thermotolerans* was assessed using purified genomic DNA from a target strain and the negative controls listed in Table 1. Real-time PCR-generated C_t values of 18.57 and 34.85 (averages) were obtained for our target strain and the negative controls, respectively. However, a C_t value of 34.50 was obtained for SDW, which corresponded to the value obtained for the negative controls (Table 1). Because of the hypothesis (5) that the C_t values of the negative controls and the SDW might have been due to *Escherichia coli* DNA contamination of the *Taq* polymerase, we repeated the assay after treatment of the master mixture with DNase I (Promega Corporation, Madison, WI). However, even after this treatment, we were unable to remove the signal obtained with the negative control and the SDW samples.

Calibration curve and sensitivity of the assay. In order to construct a calibration curve and to determine the sensitivity of both primer-probe combinations, serial dilutions of DNA prepared from the pure culture of *L. thermotolerans* (1.84×10^7 cells/ml) were used for the PCR assay (Fig. 1). The minimum detection limit of the assay was 1.84×10^3 cells/ml of pure culture of *L. thermotolerans* (C_t, 32.22; SDW C_t, 34.50). The calibration curve was constructed by plotting the C_t values (Fig. 1) against known serial dilutions of *L. thermotolerans* DNA corresponding to cell concentrations between 1.84×10^3 and 1.84×10^7 cells/ml; the resulting curve is shown in Fig. 2. Each sample was analyzed in triplicate, and the variation in the C_t values of multiple replicate runs was found to be very low, as indicated by the standard deviation (Fig. 2). The efficiency of the curve was excellent (>94%), as the correlation coefficient was 0.9974, and a slope of −3.4652 was obtained. The effi-

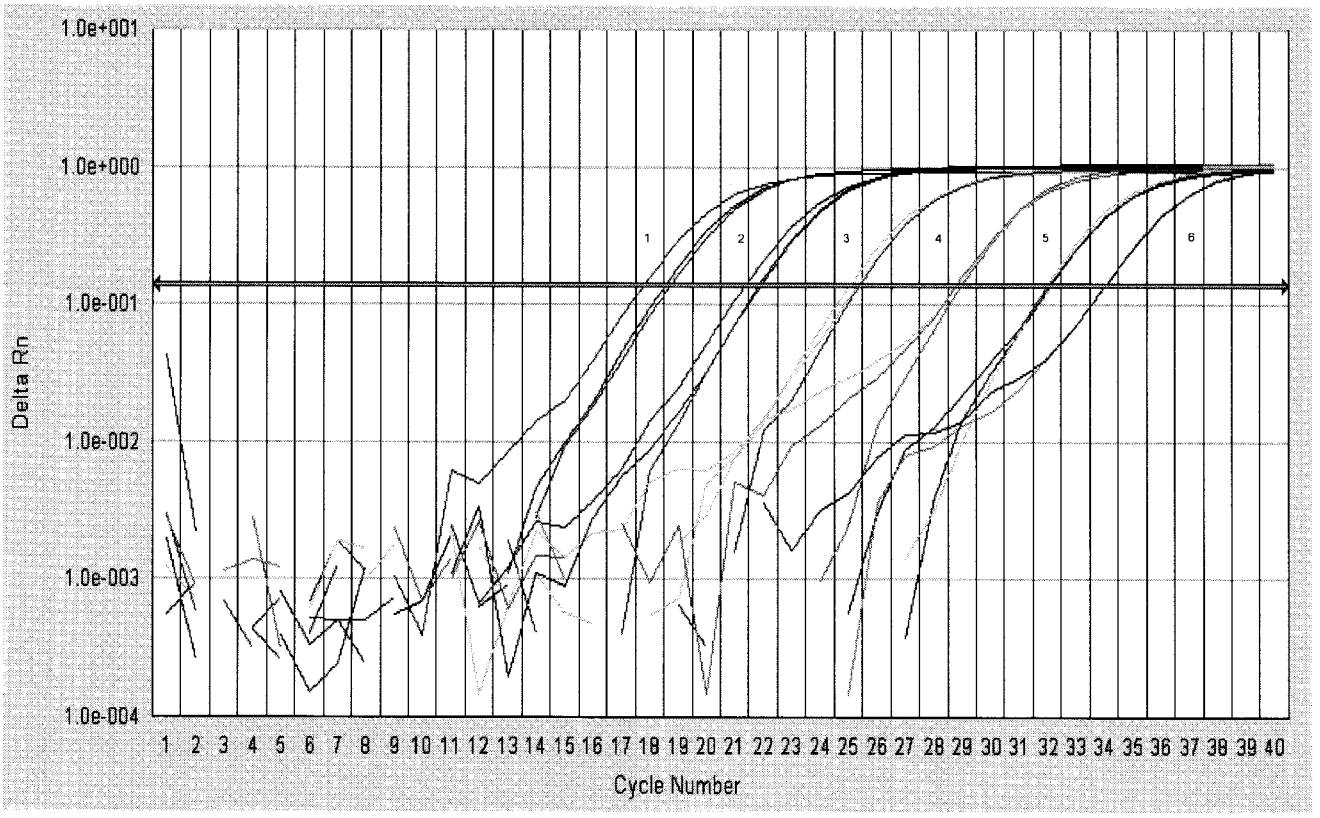

FIG. 1. Amplification sensitivity of the 5′ nuclease PCR assay for *L. thermotolerans*. DNA isolated from log-phase bacteria (1.84×10^7 cells/ml) was used in serial 10-fold dilutions. Delta Rn, fluorescence intensity after subtraction of the background signal. Line 1, signal of the original DNA sample corresponding to bacterial cells at a concentration of 1.84×10^7 cells/ml; line 2, 10-fold dilution of the original extracted DNA; line 3, 10^2-fold dilution of the original extracted DNA; line 4, 10^3-fold dilution of the original extracted DNA; line 5, 10^4-fold dilution of the original extracted DNA; line 6, nontemplate control.

ciency was calculated on the basis of 100% efficiency corresponding to a slope of −3.32192 (14).

Accuracy of the assay. In order to test the accuracy of the assay, 50-, 100-, and 150-μl pure cultures of *L. thermotolerans* containing 9.2×10^5, 1.8×10^6, and 2.8×10^6 cells (DAPI counts), respectively, were added to chicken feces (0.1 g, containing 2.1×10^5 *L. thermotolerans* cells; real-time monitoring), and this was followed by DNA extraction and quantification by real-time PCR. All of the samples yielded values that were somewhat high, but the cell numbers were almost the same as the expected cell numbers (Fig. 3), thus demonstrating correlation regression (R^2, 0.9976). The results obtained suggest that the recovery of the cells from the samples was quite accurate; therefore, this newly developed PCR method can be used to examine the bacterial content of chicken feces.

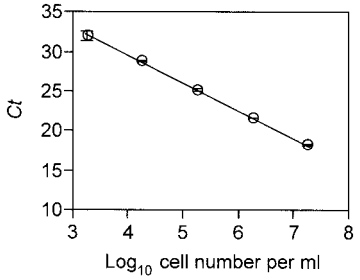

FIG. 2. Calibration curve. The C_t values obtained in Fig. 1 were plotted against known numbers of *L. thermotolerans* cells ranging from 1.84×10^3 to 1.84×10^7 cells/ml of pure culture. The R^2 value was 0.9974. The C_t values shown are the averages of three replicates. The error bars indicate standard deviations.

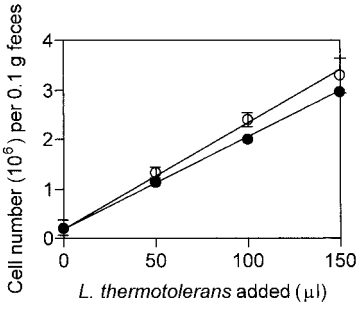

FIG. 3. Accuracy of real-time PCR assay. DNA from chicken feces samples (0.1 g) mixed with 50, 100, or 150 μl of a pure culture of *L. thermotolerans* (1.84×10^7 cells/ml) was extracted and detected by real-time PCR. The numbers of cells determined by real-time PCR (○) were compared to the expected values (●). Regression R^2 values of 0.9976 and 0.9996 were obtained. The error bars indicate standard deviations.

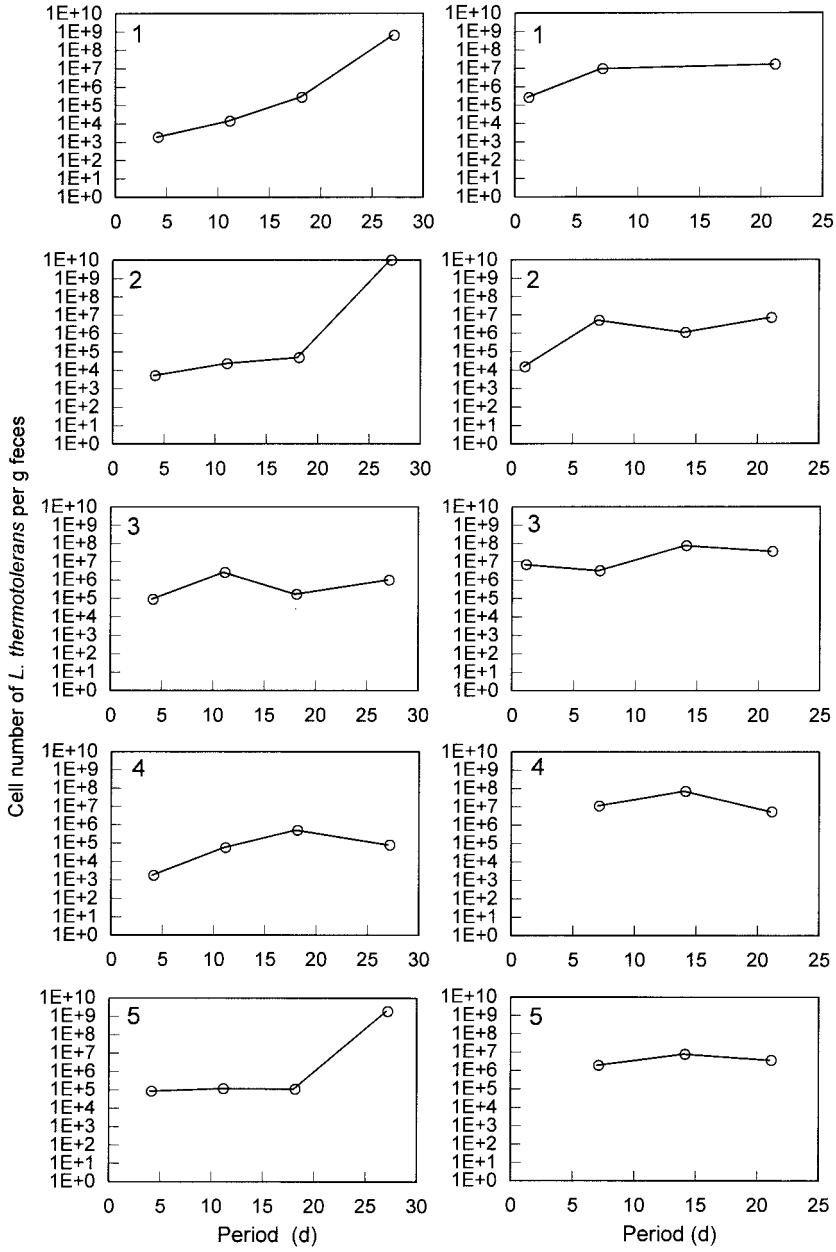

FIG. 4. Changes in the number of *L. thermotolerans* cells at different times for individual chickens, as detected by real-time PCR. The results of trials 1 (left) and 2 (right) are shown. Each trial was conducted using five chickens, and the designations of the chickens are indicated on the graphs.

Detection of *L. thermotolerans* in chicken feces. Figure 4 shows the populations of *L. thermotolerans* in the chicken feces of individual chickens in two trials monitored by real-time PCR. In trial 1, the population of *L. thermotolerans* ranged from 1.8×10^3 to 9.7×10^9 cells/g feces. In general, the chickens showed a tendency toward higher numbers of *L. thermotolerans* cells until day 27. In trial 2, the total *L. thermotolerans* results also showed a tendency toward slow increases in some chickens from day 1 to day 21.

Table 2 shows the average total *L. thermotolerans* count for each trial, as obtained by real-time PCR performed with chicken feces samples. In trial 1, a higher number of cells (2.4×10^9 cells/g feces) was observed on day 27 compared with the numbers observed from day 4 to day 18 (10^4 to 10^5 cells/g feces). In trial 2, the average cell populations increased 10-fold (10^6 to 10^7 cells/g feces) from day 1 to day 21. The total cell populations measured by DAPI staining were approximately 10^{11} cells/g feces in both trials. Thus, *L. thermotolerans* cells accounted for 9.2×10^{-6} to 8.5×10^{-1}% of the total cells in the feces in trial 1 and for 1.5×10^{-3} to 4.5×10^{-2}% of the total cells in trial 2.

DISCUSSION

Here, we developed a real-time PCR assay that is rapid, specific, and sensitive for monitoring *L. thermotolerans*. The

TABLE 2. Mean numbers of total cells and *L. thermotolerans* cells in chicken feces as determined by DAPI staining (total cells) and by real-time PCR (*L. thermotolerans*)

Trial	Day	*n*	Total cells/g of feces	*L. thermotolerans*	
				Cells/g of feces	% of total cells
1	4	5	4.0×10^{11} (2.0×10^{11})[a]	3.7×10^{4} (4.7×10^{4})	9.2×10^{-6}
	11	5	3.4×10^{11} (2.4×10^{11})	5.8×10^{5} (11.8×10^{5})	1.7×10^{-4}
	18	5	1.3×10^{11} (1.6×10^{11})	2.2×10^{5} (1.8×10^{5})	1.6×10^{-4}
	27	5	2.8×10^{11} (4.7×10^{11})	2.4×10^{9} (4.1×10^{9})	8.5×10^{-1}
2	1	3	1.6×10^{11} (1.5×10^{11})	2.4×10^{6} (3.9×10^{6})	1.5×10^{-3}
	7	5	2.0×10^{11} (1.2×10^{11})	6.2×10^{6} (4.8×10^{6})	3.1×10^{-3}
	14	5	8.5×10^{10} (6.2×10^{10})	3.9×10^{7} (4.0×10^{7})	4.5×10^{-2}
	21	3	1.3×10^{11} (1.1×10^{11})	2.0×10^{7} (6.2×10^{7})	1.5×10^{-2}

[a] The values in parentheses are standard deviations.

minimum sensitivity of our method was 1.84×10^{3} cells/ml of pure culture of *L. thermotolerans*, which is rather low compared to the sensitivities of other analyses, for which detection limits of 0 $\log_{10}$ have been reported (5, 13). This was because of the unusual fluorescence signal expressed in the negative controls and SDW (Table 1), probably due to contamination by nucleotides present in the PCR master mixture, as previously reported (5). Although we attempted to eliminate the signal by using DNase I, the C_t value remained unchanged. The specificity of the primer-probe set used in the newly developed real-time PCR assay for *L. thermotolerans* detection was established by discrimination between intestinal bacteria and food bacteria (Table 1). The accuracy of this real-time PCR assay was nonetheless demonstrated in another quantitative experiment, in which feces were mixed with a known amount of a pure culture of *L. thermotolerans* for evaluation of the recovery achieved with the assay described here (Fig. 3). All of the samples yielded slightly higher numbers of cells than the numbers expected, which may have been due to an error in the DAPI counting of the added cells in *L. thermotolerans* pure cultures. It has been reported previously that phenolic compounds from plants may bind to DNA and thus can interfere with PCRs (7). The results shown in Fig. 3 clearly indicate that our new method is free from PCR inhibitors associated with the fecal samples. In fact, the possibility of inhibition of the PCR when fecal samples from chickens reared on commercial feed are used is thought to be lower than the possibility associated with PCR studies of herbivores, whose feed contains phenolic compounds originating from plant materials. Based on our results, we concluded that the present method enabled accurate and sensitive detection of *L. thermotolerans* cells in chicken feces. This new method thus provides a powerful tool for monitoring the distribution and abundance of this bacterium in a complex microbial community.

In general, most of the chickens used in trial 1 showed an increase in the *L. thermotolerans* population in the feces samples collected from day 4 to day 27 (Fig. 4). These results suggest that *L. thermotolerans* colonizes slowly (i.e., until the second week of life) and then rapidly proliferates until day 27. The average cell population of *L. thermotolerans* on day 27 (Table 2) was found to be 10-fold greater than that of *Lactobacillus salivarius* (10^{8} cells/g feces on day 40) reported previously (11) in chicken feces. In trial 2, the initial number of target cells on day 1 in chicks 1 and 3 was higher than the number of cells in chickens in trial 1. Moreover, the increase in the level of the target strain with time tended to be slower than the increase in the level of the target strain observed in trial 1. In the present study, the two trials were conducted at different times using different batches of chicks. Moreover, the microbiota of the chicks was not controlled by the suppliers. Therefore, based on our results, we concluded that *L. thermotolerans* is indeed a normal member of the microbiota of the chicken intestine. Since the total bacterial population in chicken feces is approximately 10^{11} cells/g, our results demonstrated that *L. thermotolerans* accounts for a minor percentage of the bacterial diversity in chicken feces (Table 2).

In conclusion, we developed a highly sensitive and specific real-time PCR assay for detection of the *L. thermotolerans* present in chicken feces. This newly developed assay was successfully used to monitor the dynamics of this novel bacterium in chicken feces, thereby providing a powerful tool for studying the distribution and abundance of this bacterium in a complex microbial community. Our results also demonstrated that this novel bacterium is a normal member of the chicken intestinal microbiota.

We are currently developing a method involving fluorescent in situ hybridization coupled with confocal laser scanning microscopy to visualize epithelium surface colonization by *L. thermotolerans* in the chicken gastrointestinal tract. These studies, together with enumeration of this bacterium in the chicken intestine by real-time PCR, should provide much more comprehensive information about the ecology of *L. thermotolerans* in the chicken gastrointestinal tract.

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