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1 Title: **Development and assessment of a Real-Time PCR assay for the**
2 **rapid and sensitive detection of a novel thermotolerant bacterium,**
3 ***Lactobacillus thermotolerans*, in chicken feces**

4 Running title: **DETECTION OF *L. THERMOTOLERANS* IN CHICKEN FECES**

5 Byline: **SELIM *ET AL.***

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ABSTRACT

1
2 A new Real-Time PCR assay was successfully developed using a TaqMan[®]
3 fluorescence probe for specific detection and enumeration of a novel bacterium,
4 *Lactobacillus thermotolerans*, in chicken feces. The specific primers and probe
5 were designed based on the 16S rRNA gene of *L. thermotolerans* sequences and
6 their sequences were compared to those of all available 16S rRNA genes in
7 GenBank. The assay, targeting 16S rRNA gene, was evaluated using DNA from a
8 pure culture of *L. thermotolerans*, DNA from closely related bacteria,
9 *Lactobacillus mucosae* DSM 13345^T; *Lactobacillus fermentum* JCM 1173^T and
10 DNA from other lactic acid bacteria in quantitative experiments. Serial dilutions
11 of *L. thermotolerans* DNA were used as an external standard for calibration. The
12 minimum detection limit of this technique was 1.84×10^3 cells/ml of pure culture
13 of *L. thermotolerans*. The assay was then applied to chicken feces in two
14 different trials. In the first trial, the cell population was 10^4 cells/g feces on d4
15 and 10^5 cells/g feces on d11-18. However, cell population of 10^6 to 10^7 cells/g
16 feces were detected in the second trial. The total bacterial count, measured by 4',
17 6-diamidino-2-phenylindole (DAPI) staining, was approximately 10^{11} cells/g
18 feces. These results suggest that *L. thermotolerans* is in general distributed as a

1 normal member of the chicken gut microbiota, although it is present in relatively
2 low numbers in the feces.

3 Key words: *Lactobacillus thermotolerans*, Real-Time PCR, Chicken feces

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INTRODUCTION

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3 Previously we have isolated *Lactobacillus thermotolerans*, a novel species,
4 from chicken feces collected in Thailand (9). The preference of this bacterium
5 for the chicken intestine may be due to the body temperature of chickens,
6 42°C (2), which corresponds to the optimum temperature for growth (42°C) of
7 this bacterium, as determined by measurement of the specific growth rate (9).

8 Our current interest in *L. thermotolerans* is to characterize this bacterium
9 ecologically in chicken intestine, since no studies have been conducted to date
10 on the ecology of this new bacterium. Studies on the distribution and
11 colonization of *L. thermotolerans* in different organs of gastrointestinal tract
12 will provide a new insight in chicken intestinal microbiology. For this
13 purpose an effective method for enumeration of this bacterium is required.
14 The development of a molecular ecological enumeration method appears to be
15 of particular value in the case of *L. thermotolerans*, since conventional culture
16 methods are insufficient due to the relatively high temperature required for
17 culturing this bacterium. Real-Time PCR offers significant advantages over
18 other molecular enumeration techniques in terms of the speed by which assays
19 are performed and the ability to quantify the target microbial population.
20 Real-Time PCR has already been established as a promising tool for studies

1 on the composition of microbial communities in the gastrointestinal tract or
2 feces of humans (1, 4, 5, 12) as well as chickens (13). However, most studies
3 with a focus on chicken microbiota have been conducted using conventional
4 culture methods (6, 8, 10, 11). To the best of our knowledge, there has not yet
5 been a report focusing on the Real-Time monitoring of chicken lactobacilli, a
6 commonly used probiotic organism in the avian industry. The use of
7 probiotics to promote health and nutrition has attracted a great deal of
8 attention these days and claims have been made in this context with regard to
9 daily weight gain, improvement in feed conversion and resistance to disease
10 (3).

11 In this study, establishment of conditions for a Real-Time PCR assay of *L.*
12 *thermotolerans* and its successful application for monitoring population
13 dynamics of this bacterium in chicken feces will be described.

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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 (N₂: H₂: CO₂= 8: 1: 1) in Difco Lactobacilli MRS Broth (Becton, Dickinson and Company) at 42°C overnight, and the reference strains were grown under the same conditions, with the exception 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, one day old) in each trial in two different periods. The chicks were obtained from the Hokuren Federation of Agricultural Cooperatives (Sapporo, Japan) and were kept in an individual section of a wooden box and were reared for a period of 4 weeks for trial 1, and 3 weeks for trial 2. Chickens were maintained and handled according to the recommendations of the Ethics Committee at our institute. The animals were given commercial feed (crude protein, 200 g/kg; and energy, 2950 kcal/kg) *ad libitum* once a day. Water was available during the entire experiment. Fresh feces were collected from individual chicks on d4, 11, 18, and 27 in the case of trial 1, and on d1, 7, 14, and 21 in the case of trial 2. A

1 sterilized spatula was used for feces collection, and the samples were
2 immediately transferred to the laboratory for further use.

3 **DNA extraction.** The genomic DNAs were isolated from the culture
4 broths of *L. thermotolerans*, and reference strains used as negative controls,
5 and from feces using UltraClean™ Soil DNA Kit (MO BIO Laboratories, Inc.,
6 Solana Beach, CA), following the manufacturer's instructions. Before DNA
7 extraction, samples (0.1 g feces or 0.1 ml culture) were incubated with 50 µl
8 of lysozyme (5 mg/ml) and 15 µl of *N*-acetylmuramidase (10.2 u/µl) at 37°C
9 for 15 min. The quality of the extracted DNA was analyzed by electrophoresis
10 on 1.5% agarose gel. The DNA concentrations were measured by absorbance
11 at 260 nm in a Beckman DU 640 Spectrophotometer (Beckman Coulter, Inc.,
12 Fullerton, CA). The DNA was then used for the Real-Time PCR assay.

13 **Design of primers and probe.** The PCR primers and probe were designed
14 targeting 16S rRNA gene of *L. thermotolerans* with Primer Express™ v2.0
15 (Applied Biosystems, Foster City, CA), and the specificity of these sequences
16 was further checked against all the available data of 16S rRNA genes in
17 GenBank. The probe was labeled with fluorescent dye 6-carboxyfluorescein
18 (6-FAM) at the 5' end, and 6-carboxytetramethylrhodamine (6-TAMRA) at the

1 3' end. The sequences were 5'-TGCACAGGATTGACGTTGGT-3', 5'-
2 GGCAGGTTGCCTACGTGTTACT-3' and 5'-TCCCAACGAGTGGCGGACGG-3' for
3 the forward primer (92F), reverse primer (157R), and TaqMan[®] probe (113T
4 probe), respectively.

5 **5' nuclease PCR assay conditions.** The amplification reactions were
6 carried out in a total volume of 50 μ l, and the mixtures contained 1xTaqMan[®]
7 Universal PCR Master Mix (Applied Biosystems) including 900 nM each of
8 the *L. thermotolerans*-specific primers, a 250 nM fluorescence-labeled *L.*
9 *thermotolerans*-specific probe, and 20 ng DNA for each treatment.
10 Amplification reactions were performed on an ABI PRISM[®] 7000 Sequence
11 Detection System (Applied Biosystems), and the reactions were carried out
12 after 2 min at 50°C (activation of the uracil *N*-glycosylase) and 10 min at
13 95°C (activation of the AmpliTaq Gold DNA Polymerase), followed by 15 s at
14 95°C (denaturation) and 1 min at 63°C (anneal/extension) for 40 repeated
15 cycles. Due to the positive signal of the closely related bacterium, *L. mucosae*
16 (95% similarity), we increased the annealing temperature from 60 to 63°C.
17 Data analysis was carried out using ABI PRISM[□] 7000 Sequence Detection
18 System software v1.0 (Applied Biosystems). Each sample was analyzed in

1 triplicate.

2 **Specificity of the PCR assay.** The DNAs extracted from the pure culture
3 of *L. thermotolerans* and the reference strains were used to test the specificity
4 of the primer-probe set. The most closely related strains, *L. mucosae* DSM
5 13345^T and *L. fermentum* JCM 1173^T, had two mismatches in the designed
6 probe, which enabled discrimination between strains. The discrimination of
7 other bacterial strains from *L. thermotolerans* was carried out by evaluation of
8 a threshold cycle (*C_t*) or *C_t* value. The *C_t* was defined as the cycle at which
9 fluorescence is significantly different from the background. Therefore, the *C_t*
10 value provided an accurate measure of the number of target molecules
11 originally present in the sample. Samples of the purified DNA (20 ng) were
12 used as the template in the Real-Time PCR assay, which was carried out
13 according to the thermal cycling conditions and data analysis as described
14 above. Sterilized distilled water (SDW) was used as a non-template control.

15 **Accuracy of the assay.** In order to test the accuracy of the assay, a 0.1 g
16 feces sample was mixed with various amounts (50, 100, or 150 µl) of pure
17 culture of *L. thermotolerans* (1.84x10⁷ cells/ml); the mixture was then
18 subjected to DNA extraction, and then the number of cells was quantitated by

1 Real-Time PCR. The assay was repeated twice.

2 **Total count of bacterial population by DAPI.** The total cell number in
3 the pure culture (*L. thermotolerans* cells/ml) and that in the feces were
4 counted after staining the samples with 4', 6-diamidino-2-phenylindole
5 dihydrochloride (DAPI) n-hydrate solution (Wako Pure Chemical Industries,
6 Ltd., Osaka, Japan) for 5 min at room temperature. The cells were examined
7 under an Olympus BX50 microscope equipped with BX-FLA (Olympus
8 Corporation, Tokyo, Japan). The DAPI signal was captured in ten random
9 microscopic fields.

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RESULTS

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13 **Primer-probe design and specificity.** The specificity of the assay with
14 the primer and probe combination that we developed for the detection of *L.*
15 *thermotolerans* was assessed using purified genomic DNA from a target strain
16 and the negative controls listed in Table 1. Real-Time generated C_t values of
17 18.5 and 34.85 (average) were yielded for our target strain and the negative
18 controls, respectively. However, a C_t value of 34.50 was obtained for sterilized

1 distilled water (SDW), which corresponded to the value obtained for the
2 negative controls (Table 1). According to the hypothesis (5) that the C_t values
3 of the negative controls and the SDW might have been due to *Escherichia coli*
4 DNA contamination of the Taq polymerase, we repeated the assay after
5 treatment of the Master Mix with DNase I (Promega Corporation, Madison,
6 WI). However, even after the treatment, we were unable to remove the signal
7 obtained with the negative control and the SDW samples.

8 **Calibration curve and the sensitivity of the assay.** In order to construct
9 a calibration curve and to determine the sensitivity of both primer-probe
10 combinations, serial dilutions of DNA prepared from the pure culture of *L.*
11 *thermotolerans* (1.84×10^7 cells/ml) were used for the PCR assay (Fig. 1). The
12 minimum detection limit of the assay was 1.84×10^3 cells/ml of pure culture of
13 *L. thermotolerans* (C_t 32.22, while SDW was 34.50). The calibration curve
14 was constructed by plotting the C_t values (Fig. 1) against known serial
15 dilutions of *L. thermotolerans* DNA corresponding to cell concentrations
16 between 1.84×10^3 and 1.84×10^7 cells/ml; the resulting curve is shown in Fig. 2.
17 Each sample was analyzed in triplicate, and the variation in the C_t values of
18 multiple replicate runs were found to be very low as indicated by standard

1 deviation (Fig. 2). The efficiency of the curve was excellent (above 94%), as
2 the correlation coefficient was 0.9974, and a slope of $\square 3.4652$ was obtained.
3 The efficiency was calculated on the basis of 100% efficiency correspond to
4 slope of $\square 3.32192$ (14).

5 **Accuracy of the assay.** In order to test the accuracy of the assay, pure
6 cultures of *L. thermotolerans* at volumes of 50, 100, and 150 μl containing
7 9.2×10^5 , 1.8×10^6 , and 2.8×10^6 cells (DAPI count), respectively, were added to
8 chicken feces (0.1 g, containing 2.1×10^5 *L. thermotolerans* cells, Real-Time
9 monitoring), followed by DNA extraction and quantification by Real-Time
10 PCR. All of the samples yielded values that were slightly high, but almost the
11 same cell numbers as those expected were observed (Fig. 3), thus
12 demonstrating correlation regression, an R^2 value of 0.9976. The results
13 obtained here suggest that the recovery of the cells from these samples was
14 quite accurate; therefore, this newly developed PCR method is applicable for
15 examination of the bacterial content in chicken feces.

16 **Detection of *L. thermotolerans* in chicken feces.** In Fig. 4, the cell
17 population of *L. thermotolerans* in the chicken feces of individual chickens in
18 two trials monitored by Real-Time PCR is shown. In trial 1, the cell

1 population of *L. thermotolerans* ranged between 1.8×10^3 to 9.7×10^9 cells/g
2 feces. In general, the chickens showed a tendency toward higher numbers of *L.*
3 *thermotolerans* cells until d27. In trial 2, the total *L. thermotolerans* results
4 also showed a tendency toward slow increases in some chickens from d1 to
5 d21.

6 Table 2 shows the average total *L. thermotolerans* count for each trial, as
7 obtained by Real-Time PCR carried out on chicken feces samples. In trial 1, a
8 higher number of cells (2.44×10^9 cells/g feces) were observed on d27, as
9 compared with those observed from d4 to d18 (10^4 to 10^5 cells/g feces). In trial
10 2, the average cell populations increased tenfold (10^6 to 10^7 cells/g feces) from
11 d1 to d21. The total cell populations measured by DAPI staining were
12 approximately 10^{11} cells/g feces in both trials. Thus, in percentage *L.*
13 *thermotolerans* ranged between 9.2×10^{-6} to 8.5×10^{-1} in trial 1, and 1.5×10^{-3} to
14 4.5×10^{-2} for trial 2 in feces against total cells in this study.

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DISCUSSION

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18 Here, we developed a Real-Time PCR assay that is rapid, specific and

1 sensitive for the monitoring *L. thermotolerans*. The sensitivity of our method
2 was a minimum of 1.84×10^3 cells/ml of pure culture of *L. thermotolerans*,
3 which is rather low sensitivity as compared to the other cases where the
4 detection limit at the level of $0 \log_{10}$ have been reported (5, 13). This was
5 because of the unusual fluorescence signal expressed in the negative controls
6 and SDW (Table 1) probably due to the contamination of nucleotides present
7 in the PCR Master Mix as has previously been reported (5). Although we
8 attempted to eliminate the signal by using DNase I, the *Ct* value remained
9 unchanged. The specificity of primer-probe sets used in the newly developed
10 Real-Time PCR assay for *L. thermotolerans* detection was established by
11 discrimination between intestinal bacteria and food bacteria (Table 1). The
12 accuracy of this Real-Time PCR assay was nonetheless demonstrated in
13 another quantitative experiment, in which feces were mixed with a known
14 amount of pure culture of *L. thermotolerans* for evaluation of the recovery
15 achieved by the present assay (Fig. 3). All of the samples yielded slightly
16 higher cell numbers than those expected, a result which may have been due to
17 an error in the DAPI count of the added cell number in *L. thermotolerans* pure
18 culture. It has previously been reported that phenolic compounds from plant

1 may bind to DNA, and thus can interfere with PCR reactions (7). The results
2 shown in Fig. 3 clearly indicate that our new method is free from PCR
3 inhibitors associated with the fecal samples. In fact, the possibility of an
4 inhibition of PCR when using fecal samples from chickens reared on
5 commercial feed is thought to be lower than that associated with PCR studies
6 of herbivores, whose feed contains phenolic compounds originating from
7 plant materials. Based on our results, it was concluded that the present method
8 enabled the accurate and sensitive detection of *L. thermotolerans* cells in
9 chicken feces. This new method thus provides a powerful tool to monitor the
10 distribution and abundance of this bacterium in a complex microbial
11 community.

12 In general, most of the chickens used in trial 1 showed an increase in
13 the *L. thermotolerans* population in the feces samples collected from d4 to
14 d27 (Fig. 4). These results suggest that *L. thermotolerans* colonize slowly, i.e.,
15 until the second week of life, and then rapid proliferation on d27. The average
16 cell population of *L. thermotolerans* on d27 (Table 2) was found to be tenfold
17 that of *L. salivarius* (10^8 cells/g feces on d40) reported previously (11) in
18 chicken feces. In trial 2, the initial number of target cells on d1 in chicks 1 and

1 3 was higher than that of chickens in trial 1. Moreover, the increase in the
2 target strain with time tended to be slower than that of the target strain
3 observed in trial 1. In the present study, the two trials were conducted during
4 different periods of time using different batches of chicks. Moreover, the
5 microbiota of these chicks was not controlled by the suppliers. Therefore,
6 based on the present results, we were also able to conclude that *L.*
7 *thermotolerans* is indeed a normal member of the microbiota of the chicken
8 intestine. Since the total bacterial population in chicken feces is approximately
9 10^{11} cells/g, our results demonstrated that *L. thermotolerans* accounts for a
10 minor percentage of the bacterial diversity in the chicken feces (Table 2).

11 In conclusion, we developed a highly sensitive and specific Real-Time
12 PCR assay for the detection of *L. thermotolerans* present in chicken feces.
13 This newly developed assay was successfully applied to monitor the dynamics
14 of this novel bacterium in chicken feces thereby providing a powerful tool to
15 study the distribution and abundance of this bacterium in a complex microbial
16 community. The present results also demonstrated that this novel bacterium is
17 a normal member of the chicken intestinal microbiota.

18 We are currently developing fluorescent *in situ* hybridization coupled with

1 confocal laser scanning microscopy method to visualize epithelium surface
2 colonization of *L. thermotolerans* in chicken gastrointestinal tract. These
3 studies together with the enumeration of this bacterium in chicken intestine
4 by Real-time PCR method will provide much more comprehensive
5 knowledge about ecology of *L. thermotolerans* in chicken gastrointestinal
6 tract.

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9

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- 3

FIGURE LEGENDS

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3 FIG. 1. Amplification sensitivity of 5' nuclease PCR assay for *L.*
4 *thermotolerans*. DNA isolated from log-phase bacteria (1.84×10^7 cells/ml) was
5 used in serial 10-fold dilutions. Delta Rn, fluorescence intensity after the
6 subtraction of the background signal. 1, Signal of the original DNA sample
7 corresponding to bacterial cells at 1.84×10^7 cells/ml; 2, 10-fold; 3, 10^2 -fold; 4,
8 10^3 -fold; 5, 10^4 -fold dilution of the original extracted DNA. 6, non-template
9 control.

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11 FIG. 2. Calibration curve. The C_t (threshold cycle) values obtained in Fig.
12 1 were plotted against the known cell number of *L. thermotolerans*, ranging
13 from 1.84×10^3 to 1.84×10^7 cells/ml of the pure culture. The R^2 value was
14 0.9974. The C_t values shown are the average of three replicates. The error bar
15 shows standard deviation.

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17 FIG. 3. Accuracy of Real-Time PCR assay. DNA from chicken feces
18 samples (0.1 g) mixed with 50, 100, or 150 μ l of a pure culture of *L.*

1 *thermotolerans* (1.84×10^7 cells/ml) was extracted and detected by Real-Time
2 PCR. The cell numbers determined by Real-Time PCR (\square) were compared to
3 the expected values (\bullet). Regression R^2 values of 0.9976 and 0.9996 were
4 obtained. The error bar shows standard deviation.

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6 FIG. 4. Changes in the cell number of *L. thermotolerans* at different time
7 points for individual chickens, as detected by Real-Time PCR. The results of
8 trials 1 and 2 are shown. Each trial was conducted using 5 chickens, the
9 number of which is indicated in the graph.

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TABLE 1. Specificity of the primers-probe combination

Microorganism	Strain no. ^a	<i>C_t</i> ^b
Target strain		
<i>Lactobacillus thermotolerans</i>	JCM 11425 ^T	18.57 (0.09)
Negative control		
<i>L. salivarius</i> subsp. <i>salicinius</i>	JCM 1044	34.87 (0.22)
<i>L. aviarius</i> subsp. <i>araffinosus</i>	JCM 5667	34.56 (0.77)
<i>L. aviarius</i> subsp. <i>aviarius</i>	JCM 5666 ^T	34.34 (1.46)
<i>L. crispatus</i>	JCM 5810	34.45 (0.08)
<i>L. gallinarum</i>	JCM 2011 ^T	34.61 (0.23)
<i>L. amylovorus</i>	JCM 1126 ^T	35.28 (0.25)
<i>L. casei</i> subsp. <i>casei</i>	JCM 1134 ^T	34.76 (0.81)
<i>L. helveticus</i>	JCM 1120 ^T	32.27 (0.20)
<i>L. mucosae</i>	DSM 13345 ^T	35.31 (0.54)
<i>L. fermentum</i>	JCM 1173 ^T	33.77 (0.47)
<i>L. acidophilus</i>	JCM 1132 ^T	34.57 (0.76)
<i>Streptococcus equines</i>	JCM 7876	35.41 (0.80)
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	JCM 1158	35.70 (0.41)
<i>Enterococcus cecorum</i>	JCM 8724 ^T	34.94 (0.35)
<i>Pediococcus dextrinicus</i>	JCM 5887 ^T	34.83 (0.32)
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>	JCM 1564	34.46 (0.86)
<i>Clostridium coccooides</i>	JCM 1395 ^T	34.43 (0.26)
<i>C. butyricum</i>	JCM 1391 ^T	35.91 (0.46)
<i>C. perfringens</i>	JCM 3817	36.34 (0.43)
<i>Bacillus coagulans</i>	JCM 2257 ^T	36.11 (0.19)
SDW (Sterilized distilled water) ^c	–	34.50 (0.48)

^a Strains are from the Japan Collection of Microorganisms (JCM), and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

^b Threshold cycle. Values in the parentheses indicate standard deviation.

^c Non-template control.

TABLE 2. Mean counts of total cells and number of *L. thermotolerans* in chicken feces as determined by DAPI staining (total cells) and by Real-Time PCR (*L. thermotolerans*)

Trial 1	(cells/g feces)			
	d4	d11	d18	d27
	(n=5)	(n=5)	(n=5)	(n=5)
Total cells	4.0 (2.0) ^a x 10 ¹¹	3.4 (2.4) x 10 ¹¹	1.3 (1.6) x 10 ¹¹	2.8 (4.7) x 10 ¹¹
<i>L. thermotolerans</i>	3.7 (4.7) x 10 ⁴	5.8 (11.8) x 10 ⁵	2.2 (1.8) x 10 ⁵	2.4 (4.1) x 10 ⁹
Percentage (%) ^b	9.2 x 10 ⁻⁶	1.7 x 10 ⁻⁴	1.6 x 10 ⁻⁴	8.5 x 10 ⁻¹
Trial 2	(cells/g feces)			
	d1	d7	d14	d21
	(n=3)	(n=5)	(n=5)	(n=3)
Total cells	1.6 (1.5) x 10 ¹¹	2.0 (1.2) x 10 ¹¹	8.5 (6.2) x 10 ¹⁰	1.3 (1.1) x 10 ¹¹
<i>L. thermotolerans</i>	2.4 (3.9) x 10 ⁶	6.2 (4.8) x 10 ⁶	3.9 (4.0) x 10 ⁷	2.0 (6.2) x 10 ⁷
Percentage (%) ^b	1.5 x 10 ⁻³	3.1 x 10 ⁻³	4.5 x 10 ⁻²	1.5 x 10 ⁻²

^aValues in the parentheses indicate the standard deviation

^bPercentage of *L. thermotolerans* relative to the total number of cells

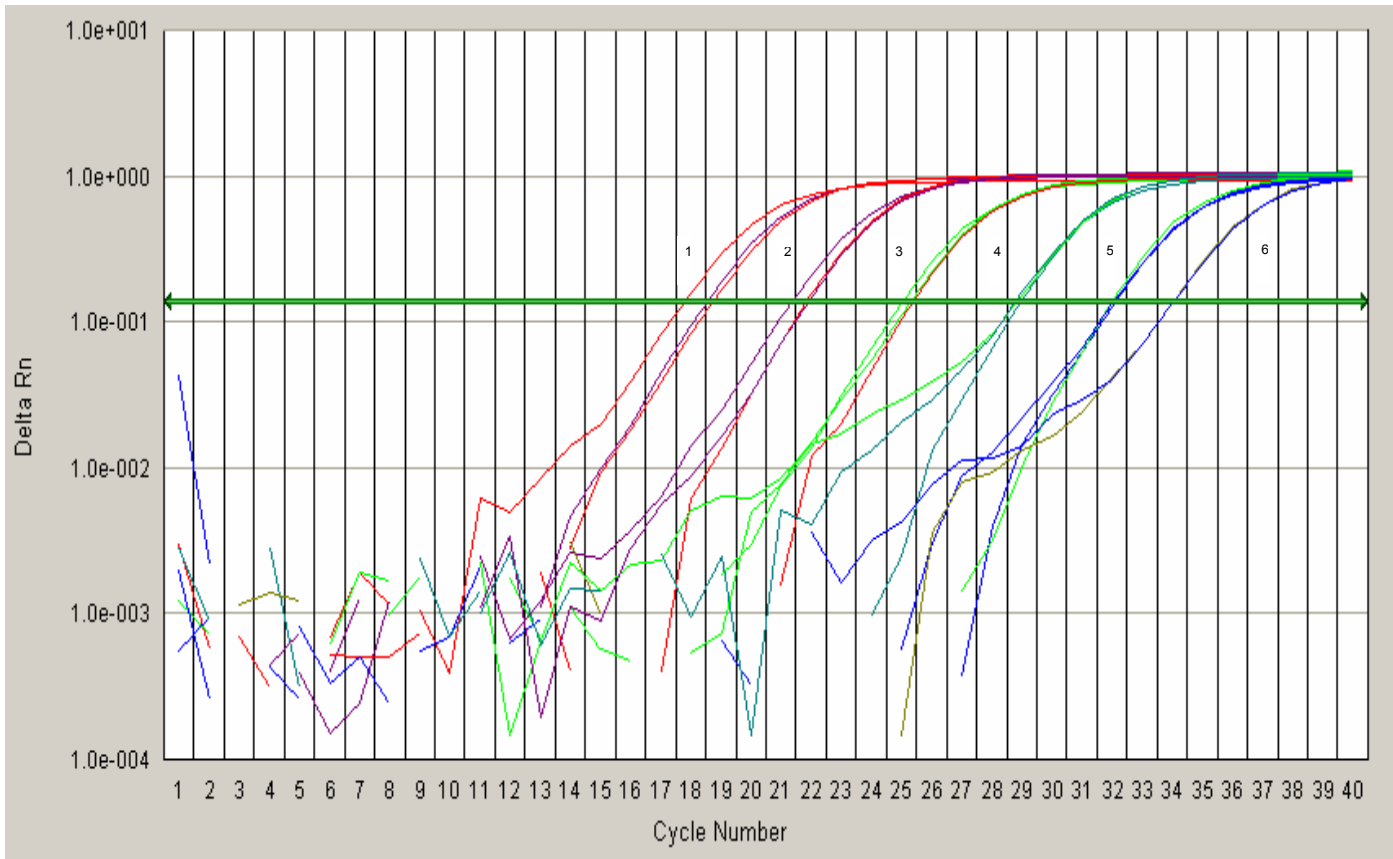


Fig. 1 Selim *et al*

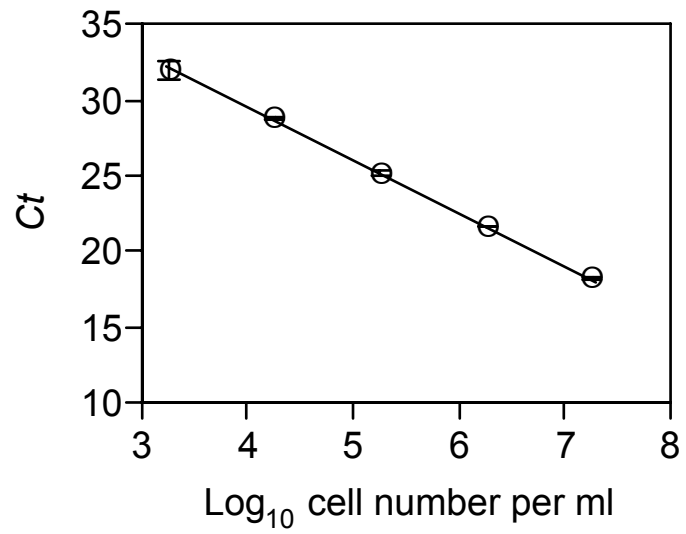


Fig. 2. Selim et al.

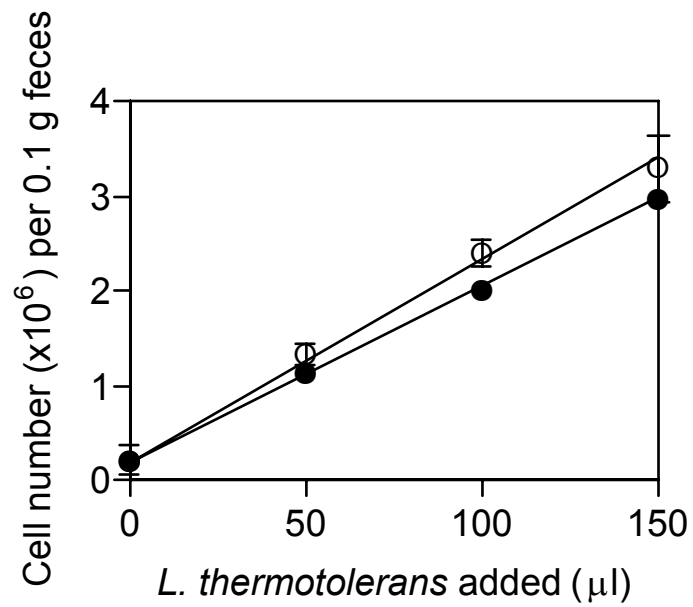


Fig. 3. Selim et al.

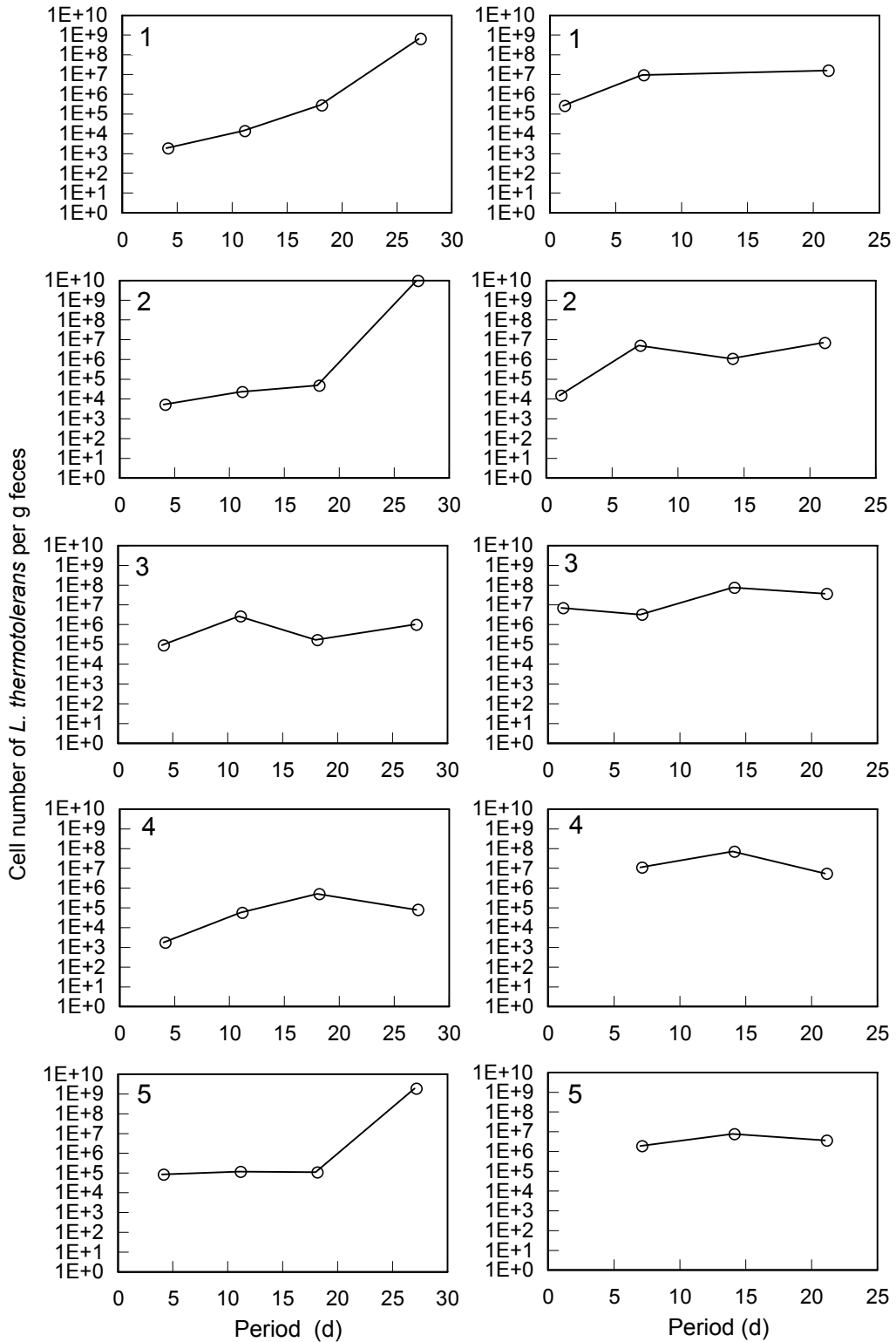


Fig. 4 Selim *et al*