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ORIGINAL PAPER

Validation of reference genes for real-time quantitative PCR studies in gene expression levels of *Lactobacillus casei* Zhang

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Abstract *Lactobacillus casei* Zhang, a potential probiotic strain isolated from homemade koumiss in Inner Mongolia of China, has been sequenced and deposited in GenBank. Real-time quantitative PCR is one of the most widely used methods to study related gene expression levels of *Lactobacillus casei* Zhang. For accurate and reliable gene expression analysis, normalization of gene expression data using one or more appropriate reference genes is essential. We used three statistical methods (geNorm, NormFinder, and BestKeeper) to evaluate the expression levels of five candidate reference genes (*GAPD*, *gyrB*, *LDH*, *16s rRNA*, and *recA*) under different culture conditions and different growth phases to find a suitable housekeeping gene which can be used as internal standard. The results showed that the best reference gene was *GAPD*, and a set of two genes, *GAPD* and *gyrB* (which were the most stable reference genes), is recommended for normalization of real-time quantitative PCR experiments under all the different experimental conditions tested. The systematic validation of candidate reference genes is important for obtaining reliable analysis results of real-time quantitative PCR studies in gene expression levels of *Lactobacillus casei* Zhang.

Keywords *Lactobacillus casei* Zhang · Reference genes · Real-time quantitative PCR

Introduction

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Food and Agriculture Organization of United Nations; World Health Organization FAO/WHO, 2001). Probiotic products usually incorporate lactic acid bacteria (LAB) because many strains of LAB are capable of exerting their beneficial effect by balancing the intestinal flora and eventually competing with pathogens for gut colonization [23].

Understanding the expression pattern of genes which are involved in LAB metabolism and response to environmental stresses should provide useful information about gene function and regulatory networks. The development of biological technology provided new tools for the research of LAB; for example, real-time quantitative PCR has been used as a powerful tool for gene expression analysis in LAB [17, 22, 31]. For accurate real-time quantitative PCR analysis, a good internal standard is very important to eliminate the sample-to-sample and run-to-run variation of RNA isolation and reverse transcription [4, 6, 28]. However, it is well documented that the expression levels of rRNA and reference genes in bacterial cells are highly variable under most experimental conditions [4, 27, 30]. Therefore, it is crucial to determine a suitable

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reference gene which is sufficient for interpretation of expression data in different conditions.

Lactobacillus casei Zhang is a potential probiotic strain isolated from homemade kumiss in Inner Mongolia of China. This strain has been sequenced and deposited in GenBank (CP001084) [32, 33]. Understanding the gene expression pattern of this organism will be useful in investigating its adaptation to different culture conditions, such as lactic acid stress, and is likely to provide target genes for improving the probiotic characteristics of *L. casei* Zhang through genetic engineering. However, little is known concerning the appropriate reference gene which could be used for normalization in the study of gene expression of *L. casei*. To date, a number of statistical methods have been proposed to evaluate the stability of gene expression and to select the best reference genes in a given experimental condition.

In the present work, we evaluated the capability of five previously used reference genes (*GAPD*, *gyrB*, *LDH*, *16s rRNA*, and *recA*) for real-time quantitative PCR experiments under different culture conditions and in different growth phases. Three different statistical tools, geNorm [29], NormFinder [1], and BestKeeper [19], were used to analyze the candidate reference genes.

Table 1 Experimental groups and different experimental culture conditions of *L. casei* Zhang

Experimental groups	Culture conditions
Different pH (Dif pH)[a]	Culture pH was kept at 5.3, 5.9, or 6.5
Different aeration (Dif Aera)[a]	Aerated with air (0.02 and 0.1 vvm) or nitrogen[b]
Different lactate concentration (Dif LacC)[a]	With 0, 30, or 60 g/l lactate in the medium before inoculation
Different growth phase with pH free (Dif GP pH free)	Cultured without pH control and sampled at initial and late exponential stage and stationary stage ($OD_{600\ nm}$ = 0.2, 1.4, and 1.6, detected after 5 times dilution)
Different growth phase with pH 5.9 (Dif GP pH5.9)	Cultured with pH keeping at 5.9 and sampled at initial and late exponential stage and stationary stage ($OD_{600\ nm}$ = 0.2, 1.8, and 2.1, detected after 10 times dilution)

[a] In these experimental groups, samples were sampled at exponential stage with OD_{600} = 1.2 ± 0.1 (detected after 10 times dilution)

[b] Aerated with nitrogen means injecting nitrogen gas periodically

Materials and methods

Bacterial strain and medium

L. casei Zhang was obtained from the Key Laboratory of Dairy Biotechnology and Bioengineering, Ministry of Education of China. A semisynthetic medium was used in all the experiments which contains 80 g/l glucose, 10.4 g/l yeast extract, 10.4 g/l soy peptone, 3.6 g/l K_2HPO_4, 15 g/l sodium acetate, 2.4 g/l sodium citrate, 1.0 g/l $MgSO_4{\cdot}7H_2O$, 0.054 g/l $MnSO_4{\cdot}5H_2O$, and 1.0 g/l Tween 80.

Fermentor and culture conditions

Bacteria were precultivated (9 h) in a sterile culture medium, and then 300 ml of this culture was inoculated into a fermentor (3 l working volume, BIOF-2000, GaoJi Biotechnology, China) filled with 2,700 ml of sterilized culture medium. Temperature was controlled at 37°C, with stirring speed of 300 rpm, and the pH value was controlled at 5.9 by the automatic addition of 17.5% aqueous ammonia. Anaerobic fermentation was maintained by supplying nitrogen gas periodically.

On the basis on the above culture conditions, the pH values and aeration conditions were changed to investigate the gene expression in different growth conditions. The influence of lactic acid production during fermentation on the gene expression level was tested by adding lactate to the medium in different concentration before the inoculation. In addition, gene expression levels at different growth phases with and without pH control were tested. All experimental groups were designed as described in Table 1. Each test was repeated at least three times.

RNA isolation and quality evaluation

The samples were first treated with rifampicin to eliminate the effects of centrifugation on bacterial transcriptome. Samples were then centrifuged at 5,000*g* for 5 min at 4°C to harvest *L. casei* Zhang cells. The cell pellets were quickly frozen by using liquid nitrogen, and ground to powder in a mortar filled with liquid nitrogen. The bacterial powder was suspended in 1 ml Trizol reagent (Invitrogen, USA) and then manipulated according to the manufacturer's instructions. To avoid residual genomic DNA contamination, RNA samples were treated with DNase I (Takara Biotechnology, Japan) and the PCR amplification was conducted to confirm the elimination of DNA. The integrity of the total RNA was determined by electrophoresis on 2% (w/v) agarose gels and the RNA concentrations were quantified by using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA).

Table 2 Primers of the candidate reference genes of *L. casei* Zhang

Gene symbol	Gene name	Accession number	Primers 5′–3′ (forward, reverse)
16s rRNA	Ribosomal gene	LSEI_r0258	ATCCAACCAGAAAGCCACGTCTAATCCTGTTCGCTACCC
recA	Recombinase A	LSEI_0923	TGCGACTGTTCGGTTAGACAGCGTGGTTGTCAAGATA
GAPD	Glyceraldehyde-3-phosphate dehydrogenase	LSEI_0967	CTTTCCCTGGTGAAGTTAGGTTCAGGAAGTAAGCCATT
gyrB	DNA gyrase B	LSEI_0005	GACAAGACAAAGGCATCACGTGCTTCACGGAAACAACC
LDH	Lactate dehydrogenase	LSEI_2549	TATCGCTCAGGAAATCGGTGGGACCATACAGGGAAT

Reverse transcription

The reverse transcriptions (RT) were performed by using the PrimerScript™ RT reagent kit (Takara Biotechnology Japan) according to the manufacturer's instructions. One microgram RNA template from each sample was converted into cDNA in 50 μl volume. The reverse transcription products were quantified by using Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and subsequently used to assess the various reference gene transcription levels in gene-specific real-time quantitative PCR assays.

Primers design and amplification efficiency evaluation

Five candidate reference genes (*GAPD*, *gyrB*, *recA*, *16s rRNA*, and *LDH*) were selected from the genes previously used in real-time quantitative PCR assay of *Lactobacillus* or other bacteria (Table 2) [3, 13, 14, 22, 25, 26]. The primers were designed by using Primer 5 software based on the available DNA sequences of *Lactobacillus casei* ATCC334 (GenBank accession number CP000423). A standard curve was constructed to calculate the gene-specific PCR efficiency from a 5-fold series dilution of cDNA template for each primer pair. The correlation coefficients (R^2) and slope values can be obtained from the standard curve, and the efficiency (E) of PCR was calculated according to the equation $E = (10^{-1/\text{slope}} - 1) \times 100$ [12].

Real-time quantitative PCR

The real-time quantitative PCR reactions were performed in the LineGene K instrument (Bioer Technology Hangzhou China) in a total reaction volume of 20 μl. The reaction mixture contained 10 μl 2 × SYBR Premix Ex Taq (Takara SYBR PrimeScript™ RT–PCR reagent kit), 1 μl of a cDNA template (previously diluted), 1 μl of each primer (final concentration 0.5 μM), and 7 μl DNase-free water. The PCR program consisted of an initial denaturation step at 95°C for 2 min to activate the Taq DNA polymerase, followed by 40 cycles of denaturation at 95°C for 30 s, at the specific annealing temperature (59°C) for 30 s and at 72°C for 1 min during which fluorescence was measured. A melt curve was produced to confirm a single gene-specific peak and to detect primer dimer formation by heating the samples from 70 to 95°C in 0.5°C increments with a dwell time at each temperature of 1 s while continuously monitoring the fluorescence. In addition, all the reaction products were further subject to electrophoresis on 2.0% agarose gels stained with ethidium bromide to confirm the accuracy of the amplified products.

Analyses of candidate reference genes

The reactions for each gene were performed three times per sample. Data on the expression level of the candidate reference genes were obtained in the form of Ct values (cycle threshold) based on the 'second derivative maximum' method by the LineGene K software (Bioer Technology Hangzhou China). The Ct values could be transformed into the starting quantities by using the formula obtained from the standard curve.

The distribution of the expression levels (Ct values) for each reference gene under the five experimental conditions was displayed as box and whiskers plots. The stability and suitability of reference genes were validated by geNorm, NormFinder, and BestKeeper programs.

Results and discussion

RNA quantity and quality

It is well known that RNA quality and quantity are critical for successful gene expression analysis. The input RNA for the reverse transcription step should be identical if various groups of samples are compared [8]. This requires quantification of the isolated RNA as well as assessment of the RNA integrity. In this study, RNA from different experimental conditions were quantified by Nanodrop ND-1000 and intact rRNA subunits of 23S and 16S were observed on

the gel electrophoresis, indicating that the degradation of the RNA was minimal.

Another critical factor for real-time qPCR is the prevention of amplification of contaminating genomic DNA which would result in an overestimation of the amount of RNA present. After the DNase treatment and before cDNA synthesis, each isolated RNA sample was subjected to real-time qPCR to test whether the contaminated genomic DNA is removed completely, and the results showed that all RNA samples were sufficient to use in real-time qPCR reactions.

Standard curve, PCR efficiency, and product specificity analyses

Using 5-fold serial dilutions of pooled cDNA, we determined the PCR reaction efficiency of each gene assay, as listed in Table 3, from the respective cDNA versus Ct efficiency plots. The efficiency plots for each of the candidate genes were found to have linear correlation coefficient (R^2) $\geq$0.996. All gene assays were found to have efficiencies $\geq$96%, with several being 100.5–106.8%, which met the experimental requirements of the real-time quantitative PCR. Meanwhile, the efficiencies higher than 100% may result from the calculation method, which is an overestimate of the "real efficiency" [10]. Electrophoresis analysis of all the amplified products from real-time qPCR showed a specific band with the expected sizes, and no primer dimer was observed. The melt curve analysis also indicated that all primer pairs produced only a single PCR product.

Table 3 Different parameters derived from real-time quantitative PCR analysis of the candidate reference genes of *L. casei* Zhang

Gene symbol	Amplicon length (bp)	Slope	Correlation (R^2)	PCR efficiency (%)
16s rRNA	306	−3.17	−0.996	106.8
recA	287	−3.31	−0.999	97.6
GAPD	335	−3.38	−0.998	101.8
gyrB	247	−3.28	−0.999	96.5
LDH	477	−3.41	−0.998	100.5

Transcription profiling of the candidate reference genes

The Ct values of all the five genes in all the samples were covered by the range of the standard curves within 7.2–23.6 cycles. The transcriptional levels of the five candidate reference genes are presented as box and whiskers plots in Fig. 1. Ct values for the different candidate reference genes were in highly variable quantity ranges, depending on the gene or the culture condition groups examined (Table 1). *16s rRNA* showed the highest expression with Ct values around 7.67, followed by *GAPD* and *LDH* with Ct values about 16.24, and *recA* and *gyrB* showed lower expression levels with Ct values at 21.47. For each gene, the fluctuations of Ct values varied among different experimental groups. In different growth phases, the genes with the smallest Ct variance were *16s rRNA* for different growth phases (Dif GP) at pH 5.9 and *GAPD* for Dif GP pH free. For different aeration conditions (Dif Aer), different pH (Dif pH), and different lactate concentration (Dif LacC), the genes with the smallest Ct variance were *LDH*, *GAPD*, and *16 s rRNA*, respectively.

Among all condition factors, the Ct values of *16S rRNA* were the lowest because of the many copy numbers in the genome. Sometimes it is unsuitable to use rRNA as an internal control for quantification of genes with relatively low expression levels [21]. Meanwhile, we found that the expression levels of the five reference genes were more variable in the Dif LacC group than in the other experiment groups. With increasing lactate concentration, both the growth rate and the expression level (Ct) of candidate reference genes decreased (data not show). Pieterse et al. reported that the combined lactic acid and a lower growth rate resulted in suppressing the expression of genes involved in metabolic processes [20]. Similarly, with a growth-inhibiting concentration, acetate inhibited exponential growth of *Escherichia coli* cells and caused expression decline of many genes involved in transcription and translation [2]. Thus, we thought that lactate may play a greater role in regulating gene expression.

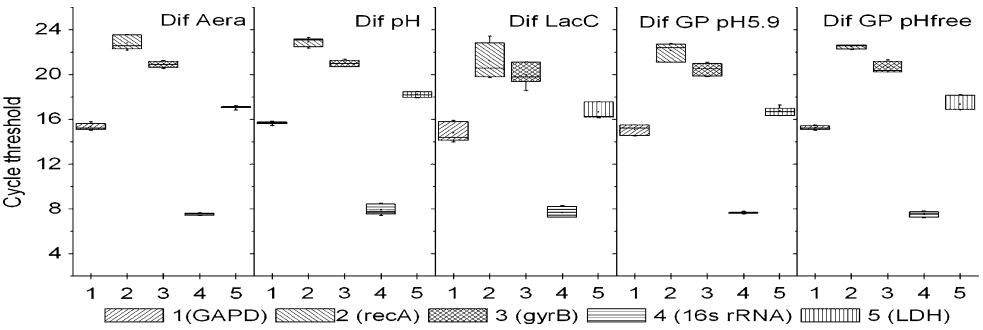

Fig. 1 Transcriptional levels of candidate reference genes (Ct values) among different conditions. *Boxes* indicate the 25th/75th percentiles, the *line marks* the median, and *whiskers* indicate the ranges. Samples were normalized to the total amount of RNA

Expression stability of the candidate reference genes

Because the approach of analyzing the distribution of Ct values does not consider real-time qPCR efficiencies and is not dependent on the experimental conditions [8], it does not provide satisfactory resolution for statistical evaluation of small differences between experimental groups.

Several statistical tools have been developed to identify stably expressed genes [5, 9, 11, 24]. These tools include qBasePlus [7], REST (Relative Expression Software Tool) [18], and Q-Gene [16]. In this work, we evaluated the expression stability of the analyzed genes according to three different statistical approaches (geNorm, NormFinder, and Bestkeeper).

The geNorm program relies on the principle that the expression ratio of two perfect reference genes should be identical in all samples, regardless of the experimental conditions or cell type. Increasing variation in this ratio corresponds to decreasing expression stability. The program calculates the gene stability measure *M* by determining the average pairwise variation between a particular reference gene and all the other control genes. Genes with lower values of *M* correspond to the most stable genes and hence are the most appropriate ones for normalization. For each gene, Ct values of samples were transformed into the starting quantities with the formula obtained from the standard curve, thereby taking into account the efficiency of the real-time qPCR reaction. The starting quantities were analyzed with geNorm to determine gene expression stability over the different culture conditions and growth phases by measuring the *M* value for each gene. In this case, the *M* values of the five candidate reference genes were less than 1 over all the different experimental conditions (the stability cutoff value defined in the program is 1.5). Thereby, these genes are stable enough in expression [29], which means they were capable of acting as reference genes in the mentioned conditions.

Interestingly, the most stable reference gene (with the lowest *M*) varied for each experiment group. By stepwise exclusion of unstable genes and subsequent recalculation of the average *M* values, the stability of genes was ranked based on their *M* values as described in Fig. 2. Starting from the least stable gene at the left, the genes are ranked according to increases in expression stability, with the two most stable genes at the right. In different aeration conditions, *16s rRNA* and *LDH* were the most stable genes, but they were least stable in different growth phases with pH 5.9. These results indicated that a different reference gene corresponds to different conditions and the most suitable reference gene is required to test for new experimental conditions.

Additionally, these results demonstrated that measuring expression levels by using multiple reference genes was more accurate than just using one [29]. Consequently, a normalization factor based on the expression levels of the best-performing reference genes must be calculated. An accurate normalization should not include the unstable genes. If all candidate reference genes are relatively stable, the calculation of geNorm algorithm was recommended to start with two most stable reference genes (normalization factor, NF_n, $n = 2$). The stepwise inclusion of an extra, less stable reference gene until the $(n + 1)$th gene has no significant contribution to the newly calculated normalization factor (NF_{n+1}) (pairwise variation V_n/V_{n+1} less than 0.15). The normalization factor should be achieved with as few genes as possible. Figure 3 shows that the pairwise variation V_n/V_{n+1} between two sequential normalization factors NF_n and NF_{n+1}. Since the inclusion of a third gene has no significant effect (low $V_{2/3}$ value) on the NF, the two most stable genes were required for calculating the NF (NF_2) for each experimental group and combination. For the Dif LacC group, it is remarkable that the pairwise variation of $V_4/V_5 > 0.15$, which means that the fifth gene (*recA*) has a significant effect on the calculation of NF.

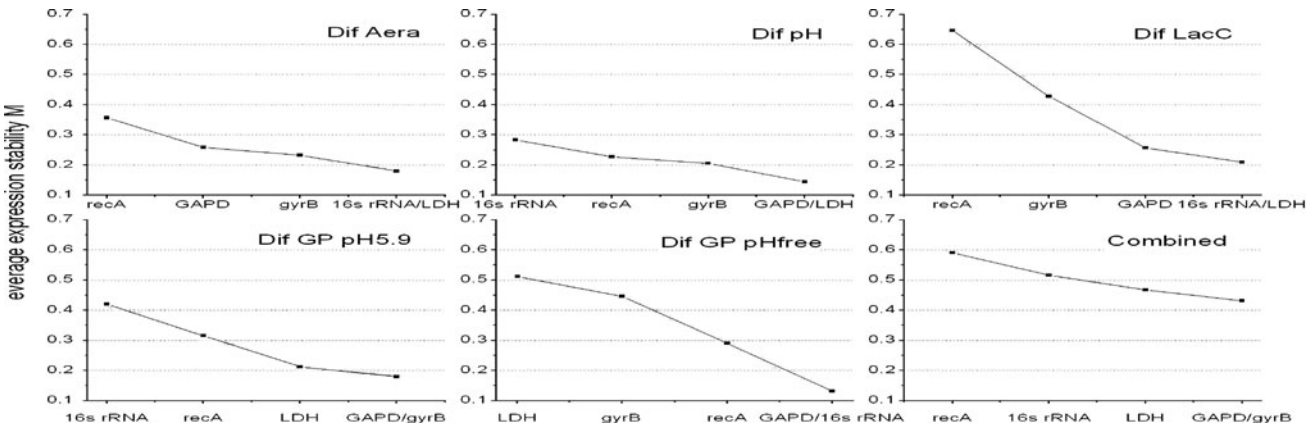

Fig. 2 Candidate reference genes ranked according to geNorm among different experimental conditions and combined

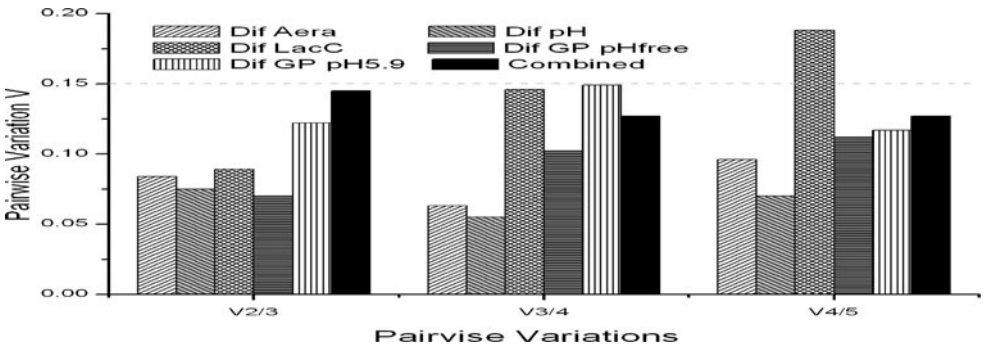

Fig. 3 Optimal number of control genes for normalization as determined by geNorm for different experimental conditions and combined analysis. The recommended upper limit of the pairwise variation value *V* is set at 0.15

Table 4 Stability values of candidate reference genes for *L. casei* Zhang according to NormFinder

	GAPD	*recA*	*gyrB*	*16S rRNA*	*LDH*	Best gene (best combination)
Combined all experimental conditions	0.111	0.260	0.180	0.288	0.232	*GAPD* (*GAPD* and *gyrB*)

Table 5 Descriptive statistics of the five candidate reference genes based on their cycle threshold values

Factor	*GAPD*	*recA*	*gyrB*	*16S rRNA*	*LDH*
N	15	15	15	15	15
GM (CP)	15.25	22.30	20.61	7.67	17.21
AM (CP)	15.25	22.33	20.62	7.67	17.22
Min (CP)	14.08	19.79	19.00	7.25	16.23
Max (CP)	15.86	23.58	21.32	8.48	18.48
SD (± CP)	0.39	0.73	0.50	0.23	0.58
CV (% CP)	2.58	3.26	2.43	2.95	3.37
Min (*x*-fold)	−2.22	−5.80	−3.09	−1.35	−1.93
Max (*x*-fold)	1.52	2.44	1.65	1.80	2.35
SD (± *x*-fold)	1.31	1.65	1.41	1.17	1.49

We thought that this result may be attributed to the obvious expression fluctuation in response to the different lactate concentration in the medium during fermentation (Fig. 1).

The NormFinder program is a model-based variance estimation approach applied to evaluate the expression stabilities of candidate reference genes for all samples, and it automatically calculates the stability value for all candidate reference genes tested on a sample set containing any number of samples organized in any given number of groups [1]. It allows comparison of intra- and intergroup variation and calculation of expression stability. For each gene, the average Ct value of each duplicate reaction was converted to quantities by using the standard curve, and the stability value was calculated with NormFinder [15]. The lower average expression stability values indicated the more stably expressed genes.

The results of analysis are shown in Table 4. The results showed that the ranking of the stability of reference gene was slightly different from that obtained by using geNorm. The best gene was *GAPD*, followed by *gyrB*, *LDH*, *recA*, and *16S rRNA*. Meanwhile, we found that when we combined all the experimental conditions, the best gene was *GAPD* whose stability value was 0.111, and the best combination of two genes was *GAPD* and *gyrB* whose stability value was 0.112; this conclusion was consistent with the result obtained by using geNorm.

The BestKeeper program is another Excel-based software tool. The average Ct value of each duplicate reaction is used (without conversion to quantity) to analyze the stability of candidate reference gene. BestKeeper evaluates the stabilities of candidate reference genes based on the coefficient of correlation to the BestKeeper index. This index is the geometric mean of the Ct values of all candidate reference genes. BestKeeper also calculates the standard deviation (SD) of the Ct values between the whole data set. All the Ct values were analyzed as total data.

Table 6 Repeated pairwise correlation analysis HKG vs. BestKeeper index

BestKeeper vs.	HKG1-*GAPD*	HKG2-*recA*	HKG3-*gyrB*	HKG4-*16SrRNA*	HKG5-*LDH*
Coeff. of corr. (*r*)	0.946	0.930	0.870	0.682	0.842
P value	0.001	0.001	0.001	0.005	0.001

Descriptive statistics of the average cycle threshold were computed for each candidate reference gene, and the results are listed in Table 5. The parameters computed included the geometric mean (GM), arithmetic mean (AM), minimal (Min) and maximal (Max) value, standard deviation (SD), coefficient of variance (CV), and the multiple factor of their minimal and maximal values expressed as the *x*-fold ratio and its standard deviation are presented. Bestkeeper analysis revealed that *16S rRNA* was the gene with the lowest overall variation, and *recA* was the highest from the list of selected genes with an SD of 0.23 and 0.73, respectively.

The gene with the highest coefficient of correlation with the BestKeeper index indicates the highest stability. The five candidate reference genes evaluated in our analysis correlated well between genes and gene with the Bestkeeper index (Table 6). The best correlation between one housekeeping gene and the Bestkeeper index was obtained for *GAPD* ($r = 0.946$) with a *P* value of 0.001, followed by *recA*, *gyrB*, *LDH*, and *16S rRNA*. The statistically significant correlation shown by *GAPD* with the Bestkeeper index was consistent with the analysis results of this gene obtained by geNorm and NormFinder.

GAPD has been commonly selected as a reference gene when evaluating mRNA expression. Nevertheless, some studies showed that the gene expression of *GAPD* was variable in certain experimental conditions [5, 9, 24]. Therefore, it is necessary to validate the stability of the reference gene in each experimental condition. In our study, *GAPD* was proved to be the best reference gene. Furthermore, we showed that a set of two genes, *GAPD* and *gyrB* (which were the most stable reference genes in our study), is recommended for the normalization of real-time quantitative PCR experiments under all the different experimental conditions tested.

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