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Identification of reference genes for expression studies in the liver and spleen of laying hens housed in cage and cage-free systems

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Abstract

Background: The liver and spleen play a pivotal role in metabolism and immune response. During stress, neuroendocrine response induces changes in gene expression, and its assessment demands the validation of the stability of the reference genes to perform relative gene expression experiments.

Aim: The objective of this study was to determine the expression stability of four reference genes (*GAPDH, ACTB, RNA18S*, and *HMBS*) in the liver and spleen tissues from laying hens housed in a conventional cage (CC) and cage-free (CF) egg production systems.

Methods: Liver and spleen from Hy-Line Brown hens housed in CC and CF egg production systems were used. mRNA transcript levels were determined by quantitative polymerase chain reaction (qPCR), and the gene expression stability was evaluated using geNorm, BestKeeper, and NormFinder algorithms.

Results: The most stable gene from liver tissue was *ACTB* in CC, CF, and CC-CF groups (overall data). In the spleen, the most stable genes were *GAPDH* (CC), *HMBS* (CF), and *ACTB* (CC-CF).

Conclusion: The *ACTB* gene was the most stable gene in the liver, and *GAPDH* and *HMBS* genes were stable in spleen tissues that could be used for the normalization in qPCR experiments performed in liver and spleen tissues of laying hens housed CC and CF production systems.

Keywords: Laying hens, Liver, mRNA, Reference genes, Spleen.

Introduction

Chicken meat and eggs are the most consumed foods worldwide and continue to be a primary source of quality animal protein in developed and developing countries, contributing to global food security (Mench *et al.*, 2011; Mottet and Tempio, 2017). Currently, the concern about animal welfare, particularly in food production animals, has been growing among consumers (Schuck-Paim *et al.*, 2021). As a result, intensive commercial egg production systems are being evaluated, particularly in conventional cages (CCs), due to the restriction of moving and the inability to perform natural behaviors such as nesting, perching, and dust bathing, generating stress in birds (Løtvedt *et al.*, 2017). Stress factors produce physiological behaviors and effective performance changes and affect gene transcription (Guo *et al.*, 2020; Rostagno, 2020).

In the stress responses, activation of the hypothalamic-pituitary-adrenal axis allows the release of glucocorticoids producing a physiologic response that affects the immune function and induces apoptosis of immature spleen and thymus cells, leading to the degeneration of primary lymphoid tissues, including the spleen, thymus, and bursa (Guo *et al.*, 2020). The spleen is the largest immune organ of birds and is primarily responsible for regulating cellular and humoral immunity. However, the neuroendocrine response induced by stress alters the regulation of immune-related genes (El-Lethey *et al.*, 2003; Zhang *et al.*, 2018). Furthermore, in the case of the liver, multiple metabolic functions, including plasma protein synthesis, vitamin, glycogen storage, and fatty acid synthesis, have been reported in birds, which can be affected by glucocorticoids allowing the development of diseases in the long term (Hu *et al.*, 2019).

In studies of gene expression, variables such as cDNA concentration and differences in tissues and cells' gene expression must be controlled (Vandesompele *et al.*, 2002). Therefore, the relative gene expression method needs a reference gene as an internal control whose

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expression under experimental conditions is unaffected (Vandesompele *et al.*, 2002). Therefore, validating the reference genes' stability is necessary to consider them adequate for practical needs (Rocha-Martins *et al.*, 2012). This study aimed to determine the expression stability of four reference genes (*GAPDH, ACTB, RNA18S*, and *HMBS*) in the liver and spleen tissues of laying hens housed in CC and CF egg production systems.

Materials and Methods

Study population

The study was carried out using tissue samples from a previous project in the Laboratory of Immunology and Molecular Biology at the University of Tolima. Briefly, under commercial conditions, approximately 60,000 Hy-Line Brown pullets were placed in cages with a density of 16 pullets/cage (314.645 cm^2/bird). Pullets were reared with the same sanitary conditions, management, and feed program until 15 weeks of age. Later, they were transferred into two different production systems, conventional cage (CC) and cage-free (CF), on the same farm, up to 82 weeks of age. A total of 45,000 hens were housed in CCs with 4 hens per cage (450 cm^2/hen) and 15 replicates of 12 cages each (48 birds/replicate). For the CF system (deep litter), approximately 14,500 hens (1,111 cm^2/bird) were distributed in 2 poultry houses, 15 rooms with 990 hens/room. Both systems offered water and the same diet, as well as health and nutritional management following company policies (Rodríguez-Hernández *et al.*, 2021).

Samples, RNA extraction, cDNA synthesis, and endpoint polymerase chain reaction (PCR)

At 80 weeks of production, six hens (n = 6) from CC and six hens (n = 6) from CF were randomly selected, and 0.5 g of liver and spleen were extracted. Total RNA was extracted from tissue samples using an RNA-solv reagent kit (OMEGA, Norcross, GA) according to the manufacturer's instructions. RNA concentration and quality were measured using the NanoDrop One (Thermo Scientific, Wilmington, DE), and cDNA was synthesized with GoScript™ Reverse Transcription System kit (Promega, Madison, WI) following the manufacturer's instructions. Endpoint PCR and agarose gel electrophoresis of all genes were carried out to determine the cDNA quality and the amplicon size.

The reaction had a total volume of 25 µl, composed of 14.8 µl of distilled-deionized water, 5 µl of 5× green GoTaq® Flexi Buffer (Promega, Madison, WI), 1 µl of dNTPs (1.5 mM) (Invitrogen, Carlsbad, CA), 1 µl of each primer (forward and reverse) (10 pmol/µl) (Table 1), 1 µl $MgCl_2$ (25 mM), 0.125 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, WI) and 1 µl of the cDNA as template. The amplifications were carried out in a ProFlex PCR System (Applied Biosystems, Carlsbad, CA) with an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and a last step of final extension at 72°C for 5 minutes. Amplicons were revealed on 1% agarose gel by electrophoresis (PowerPac™ HC, Bio-Rad, Bio-Rad, Hercules, CA) stained with HydraGreen™ (ACTGene, Piscataway, NJ) and visualized under UV light, using the ENDURO™ GDS gel documentation system (Labnet International, Inc, Woodbridge, NJ).

Quantitative PCR (qPCR)

Relative gene expression of *GAPDH, ACTB, RNA18S,* and *HMBS* genes (Table 1) was measured by qPCR using Luna® Universal qPCR Master Mix (New England BioLabs Inc., Beverly, MA) in a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA), by fast ramp program. Thermal cycling conditions were initial denaturation of 1 minute at 95°C, then 40 cycles of denaturation for 15 seconds at 95°C, and annealing of 30 seconds at 60°C. Subsequently, a melting step was performed at 95°C for 1 second, 60°C for 20 seconds, and a continuous rise in temperature to 95°C at a rate of 0.15°C per second. Each sample was run in triplicate.

Analysis of reference gene expression stability

Expression levels of the tested reference genes were quantified by the cycle of quantification (Cq) values obtained through qPCR. The three technical replicates were averaged and transformed by the $2^{-\Delta Ct}$ method (Zhang *et al.*, 2021). Those values were used as input data on geNorm, and NormFinder, to evaluate the gene expression stability (Vandesompele *et al.*, 2002; Andersen *et al.*, 2004). In the case of the BestKeeper, original data was used (Pfaffl *et al.*, 2004). In addition, analysis of comprehensive data was performed regardless of its origin (CC-CF group).

Ethical approval

All procedures were approved by the Ethics Committee of the University of Tolima, Act 007-2020, based on the Colombia Laws.

Results

Primer specificity

All the primers used were specific, and a single peak in melt curve analysis indicated no contamination with genomic DNA, primer dimers, or nonspecific PCR products (Fig. 1).

Expression profiles of reference genes

The Cq values of the four reference genes from the liver and spleen from laying hens from the CC ranged between 9.30 and 32 and in CF between 9.10 and 27.49. In the CC group, the *RNA18S* gene is the gene with the highest expression, with Cq values being from 9.30 to 14.10, followed by *ACTB* (16.13–25.52), *GAPDH* (16.82–24.88) and *HMBS* (24.21–32.42) (Fig. 2). On the other hand, in the CF group, as well, the gene most highly expressed was *RNA18S* (9.10–11.01), followed by *ACTB* (15.10–19.58), *GAPDH* (18.34–20.06), and *HMBS* (24.04–27.49).

Table 1. Primers used in the selection of candidate reference genes.

Gene	Primer sequence	Tm (°C)	Amplicon size (bp)	References
GAPDH	F- GAGGGTAGTGAAGGCTGCTG	60	113	Rodríguez-Hernández *et al.* (2021)
	R- CATCAAAGGTGGAGGAATGG	56		
ACTB	F- GCCCCCAAAGTTCTACAAT	55	110	
	R- AGGCGAGTAACTTCCTGTA	55		
RNA18S	F- CGAAAGCATTTGCCAAGAAT	55	98	
	R- GGCATCGTTTATGGTCGG	55		
HMBS	F- GGCTGGGAGAATCGCATAGG	60	131	
	R- TCCTGCAGGGCAGATACCAT	60		

(Tm): melting temperature.

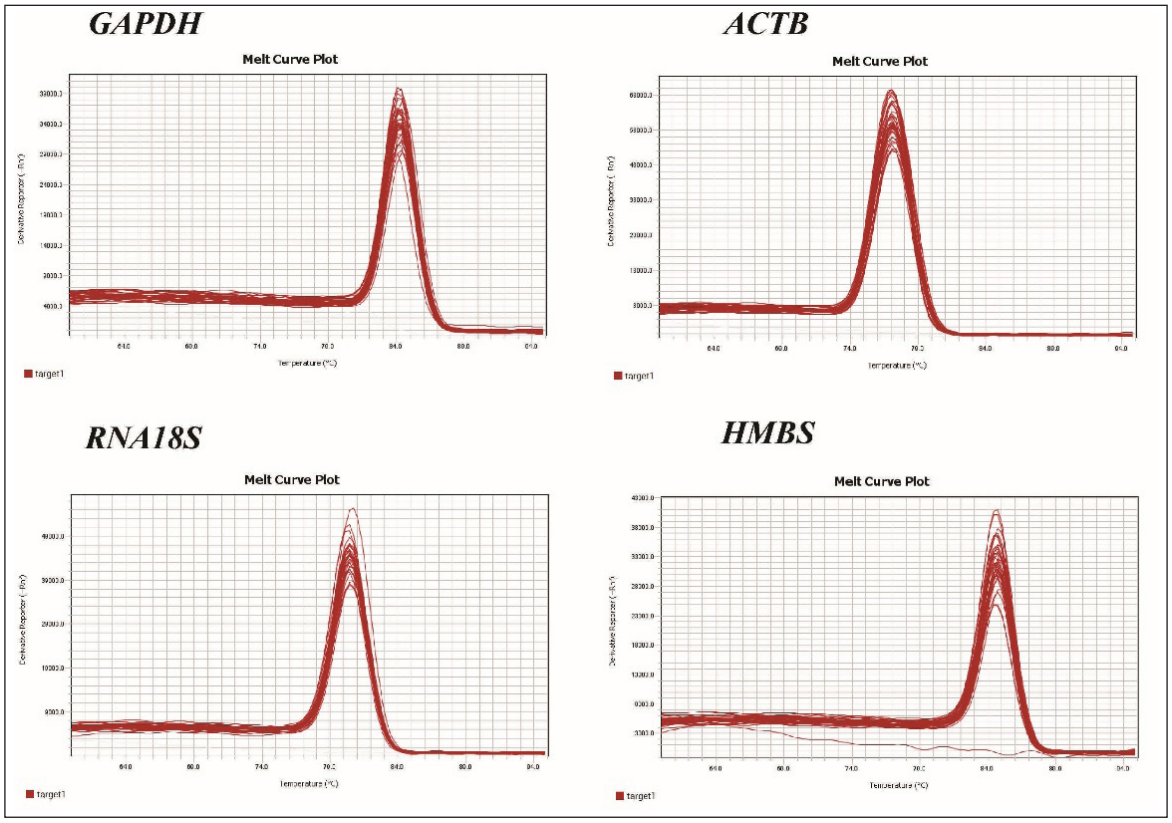

Fig. 1. Melting curve of *GAPDH*, *ACTB*, *RNA18S*, and *HMBS* genes in the liver and spleen of laying hens.

Reference gene stability

According to the geNorm algorithm, a stable reference gene has a *M* value below 1.5, and in our study, except for the *RNA18S* gene in the liver (CC) (Fig. 3), all the reference genes were under this value. In the liver, the most stable gene was *ACTB,* regardless of the production system; and in the spleen, the most stable genes were *GAPDH* (CC), *HMBS* (CF), and *ACTB* (CC-CF) (Table 2). On the other hand, BestKeeper software indicates that the most stable reference gene has a standard deviation (SD) <1, and in the liver, the *GAPDH* is the most stable gene with an SD of 0.19 (CC), 0.11 (CF), and 0.17 (CC-CF). Furthermore, the genes *HMBS* (CC and CC-CF) and *RNA18S* (CF) were stable in the spleen (Table 2). Additionally, NormFinder results showed that *ACTB* was the stable gene for the liver and spleen in CC-CF. Furthermore, the best combination of genes was *ACTB* and *HMBS* for the liver (0.086) and *GAPDH* and *ACTB* (0.11) for the spleen (Table 3).

Discussion

Transcriptome analysis using technologies such as DNA microarrays, RNA-Seq, and other methods is in demand to evaluate gene expression (Lovén *et al.*, 2012; Hasanpur *et al.*, 2022). However, the qPCR remains a valid and preferred tool to validate the

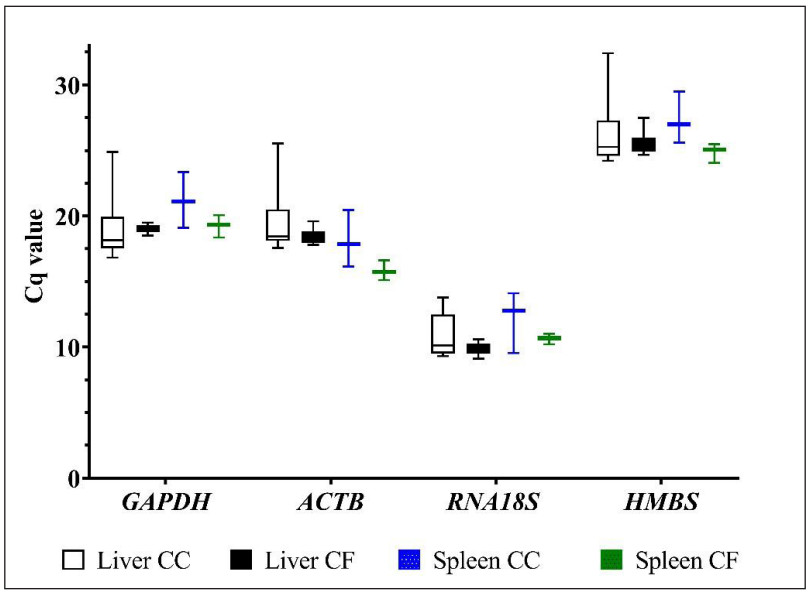

Fig. 2. Cq values for four reference genes, *GAPDH, ACTB, RNA18S,* and *HMBS* genes in the liver and spleen of laying hens. The Cq values of the *GAPDH, ACTB, RNA18S,* and *HMBS* reference genes from liver CC (white boxes), liver CF (black boxes), spleen CC (blue boxes), and spleen CF (green boxes). The box indicates the 25th and 75th percentiles, the lines represent the median, the squares represent the means, and the whiskers represent the maximum and minimum values.

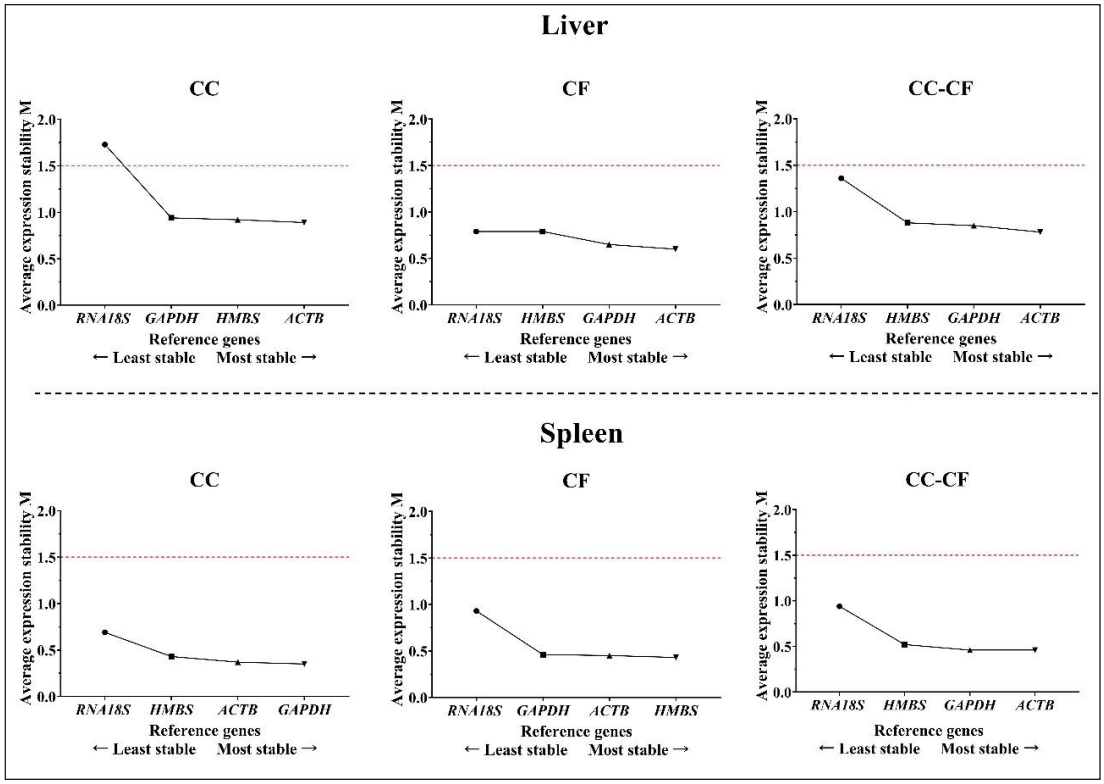

Fig. 3. *M* value of four candidate reference genes in liver and spleen tissues from laying hens housed in a cage and cage-free systems used the geNorm algorithm. CC: conventional cage; CF: cage-free; CC-CF: overall data.

Table 2. Reference gene stability value ranked by geNorm and BestKeeper algorithm in liver and spleen of laying hens of the 80 weeks of production.

Tissue	geNorm								BestKeeper							
	CC			CF			CC-CF			CC			CF			CC-CF		
	Ranking		M value	Ranking		M value	Ranking		M value	Ranking		Standard Deviation	Ranking		Standard Deviation	Ranking		Standard Deviation
Liver	1	*ACTB*	0.89	1	*ACTB*	0.60	1	*ACTB*	0.78	1	*GAPDH*	0.19	1	*GAPDH*	0.11	1	*GAPDH*	0.17
	2	*HMBS*	0.92	2	*GAPDH*	0.65	2	*GAPDH*	0.85	2	*ACTB*	0.20	2	*RNA18S*	0.16	2	*ACTB*	0.20
	3	*GAPDH*	0.94	3	*HMBS*	0.79	3	*HMBS*	0.88	3	*HMBS*	0.24	3	*ACTB*	0.17	3	*RNA18S*	0.21
	4	*RNA18S*	1.73	4	*RNA18S*	0.79	4	*RNA18S*	1.36	4	*RNA18S*	0.27	4	*HMBS*	0.20	4	*HMBS*	0.23
Spleen	1	*GAPDH*	0.35	1	*HMBS*	0.43	1	*ACTB*	0.46	1	*HMBS*	0.33	1	*RNA18S*	0.14	1	*HMBS*	0.28
	2	*ACTB*	0.37	2	*ACTB*	0.45	2	*GAPDH*	0.46	2	*GAPDH*	0.36	2	*ACTB*	0.22	2	*RNA18S*	0.31
	3	*HMBS*	0.43	3	*GAPDH*	0.46	3	*HMBS*	0.52	3	*ACTB*	0.36	3	*HMBS*	0.24	3	*GAPDH*	0.31
	4	*RNA18S*	0.69	4	*RNA18S*	0.93	4	*RNA18S*	0.94	4	*RNA18S*	0.38	4	*GAPDH*	0.25	4	*ACTB*	0.32

CC: conventional cage; CF: cage-free; CC-CF: overall data.

Table 3. Reference gene stability value ranked by NormFinder algorithm in liver and spleen of laying hens of the 80 weeks of production.

Tissue	CC			CF			CC-CF			The best combination of two genes	
	Ranking		Stability value	Ranking		Stability value	Ranking		Stability value	Genes	Stability value
Liver	1	*ACTB*	0.31	1	*ACTB*	0.22	1	*ACTB*	0.08	*ACTB* and *HMBS*	0.086
	2	*GAPDH*	0.36	2	*HMBS*	0.29	2	*HMBS*	0.12		
	3	*HMBS*	0.39	3	*GAPDH*	0.30	3	*GAPDH*	0.16		
	4	*RNA18S*	0.75	4	*RNA18S*	0.35	4	*RNA18S*	0.25		
Spleen	1	*GAPDH*	0.09	1	*HMBS*	0.17	1	*ACTB*	0.08	*ACTB* and *GAPDH*	0.111
	2	*ACTB*	0.15	2	*ACTB*	0.18	2	*GAPDH*	0.13		
	3	*HMBS*	0.20	3	*GAPDH*	0.20	3	*HMBS*	0.21		
	4	*RNA18S*	0.34	4	*RNA18S*	0.49	4	*RNA18S*	0.39		

CC: conventional cage; CF: cage-free; CC-CF: overall data.

results obtained by transcriptomic analyses (Garrido *et al.*, 2020; Hasanpur *et al.*, 2022). Normalization strategies are needed to achieve accurate results, and reference genes as markers of stability is a common and effective method used for qPCR normalization in the relative quantification method (Vandesompele *et al.*, 2002; Samiullah *et al.*, 2017; Garrido *et al.*, 2020). Nevertheless, the selection and validation of the stability of reference genes for each condition are suggested due to the variation among different experimental conditions and tissues (Wong and Medrano, 2005; Greer *et al.*, 2010; Peng *et al.*, 2018).

Several studies have been conducted on screening reference genes in various tissues of laying hens and geese (Nascimento *et al.*, 2015; Zhang *et al.*, 2021). For example, using next-generation sequencing data, Hasanpur *et al.* (2022) report the *Ap2m1* gene as a suitable reference gene for the liver and the *Rpl6* gene as a relatively appropriate reference gene for the spleen in chicken. In contrast, Mogilicherla *et al.* (2022) reported the *ALB* gene in the liver and the *GAPDH* gene in the spleen as the most stable reference genes in *Gallus gallus*. Alternatively, Zhang *et al.* (2021) identified the *ACTB* gene as a good reference gene in different tissues of 120-day-old Hy-line brown layer hens. Nevertheless, the reference genes cited previously were defined for other experimental conditions.

The geNorm, BestKeeper, and NormFinder algorithms were used to evaluate the stability of the reference genes. The geNorm algorithm calculates the gene expression stability (*M*) as the mean, the standard deviation of the log-transformed expression ratios for every candidate reference gene under different experimental conditions, where genes with the lowest *M* represent the most stable gene expression (Vandesompele *et al.*, 2002; Garrido *et al.*, 2020). Regarding NormFinder, this algorithm calculates the most stable gene with the lowest stability value calculated by combining the intragroup and intergroup variations of each gene (Andersen *et al.*, 2004). Finally, the BestKeeper algorithm determines gene expression stability based on the standard deviation and the coefficient of variation (Pfaffl *et al.*, 2004). In this study, the rankings of reference genes were different when using different algorithms. The discrepancy in results delivered by different algorithms has been reported in previous studies (Velada *et al.*, 2015; Gao *et al.*, 2017; Peng *et al.*, 2018). Therefore, the most stable reference genes were selected based on the results from the three algorithms.

The liver plays a determining role in the physiological adaptation of animals to changes in the environment, and its gene expression could be affected by different stressors (Kumar *et al.*, 2019; Xu *et al.*, 2019). In the liver, the *ACTB* gene was classified as the most stable in two of the three algorithms applied. This gene encodes an actin protein involved in cell motility, structure, and integrity (Gonzaga *et al.*, 2020). This result agrees with the findings of Gonzaga *et al.* (2020), in which the *ACTB* gene was considered the most stable gene in the liver of two chicken genotypes under heat stress. Furthermore, numerous studies commonly use the *ACTB* gene as a reference gene (Chapman and Waldenström, 2015). This result differs from the findings of Mogilicherla *et al.* (2022), where the *ALB* gene was the most stable in the liver. However, this study's results suggest that the *ACTB* gene may not be affected by the egg production system (CC or CF). In addition, using more than one reference gene is recommended to get a more robust, accurate, and reliable normalization of gene expression data (Vandesompele *et al.*, 2002). In this study, the use of *GAPDH* with the *ACTB* gene is recommended due to the stable results.

In the case of the spleen, it mediates the immune response, and distinct stress factors could alter the regulation of immune response genes (Zhang *et al.*, 2018). The best combination of genes in this tissue was *ACTB*, *GAPDH,* and *HMBS* in all the algorithms applied. Comparable results were demonstrated in chickens under the infectious bronchitis virus infection, where the *ACTB* gene was suggested as a stable reference gene (Khan *et al.*, 2017). Also, Mogilicherla *et al.* (2022) described using the *GAPDH* gene in the spleen for normalization in gene expression studies in chickens. Similarly, Boo *et al.* (2020) reported that chickens could use *GAPDH* and *HMBS* as reference genes. Moreover, in yellow-feathered broilers, the use of the genes *ACTB* and *HMBS* were reported as reference genes in different tissues (Zhang *et al.*, 2018). Previously, in magnum tissue of hens housed in CF systems, the most stable gene was *HMBS,* similar to our results in the spleen of hens of the CF (geNorm and NormFinder) and comprehensive data (CC-CF; BestKeeper) group (Rodríguez-Hernández *et al.*, 2021). On the other hand, the *HMBS* gene was reported as the most stable gene in the spleen of quail (Macario *et al.*, 2022) and codifying for HMBS protein, a vital enzyme in the heme biosynthetic pathway (Wang *et al.*, 2020).

In this study, in the overall data (CC-CF), the *ACTB* gene was the most stable gene according to two of three software used, which indicate that under the experimental conditions, CC and CF systems do not affect the stability of the gene. Therefore, using the *ACTB* gene may allow more excellent reliability of qPCR data analysis of hens under the two egg production systems. Furthermore, the best combination of genes was ACTB/HMBS (Liver) and *ACTB/GAPDH* (Spleen) references genes.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

María Herrera-Sánchez and Roy Rodríguez-Hernández were responsible for the design of the study; María Herrera-Sánchez and Kelly Lozano-Villegas performed the experiments; María Herrera-Sánchez analyzed the data; María Herrera-Sánchez and Kelly Lozano-Villegas wrote the manuscript; María Herrera-Sánchez, Kelly Lozano-Villegas, Roy Rodríguez-Hernández, and Iang Rondón-Barragán reviewed and editing the paper. Iang Rondón-Barragán revised the manuscript critically. All authors read and approved the final manuscript.

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