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# Screening of reliable reference genes for the normalization of RT-qPCR in chicken gastrointestinal tract

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**ABSTRACT** The application of reverse transcription quantitative real-time PCR technology for the production of gene tissue expression profiles is a widely employed approach in molecular biology research. It is imperative to ascertain internal reference genes that exhibit stable expression across diverse tissues to ensure the precision of tissue gene expression profiles. While there have been studies documenting the most suitable reference genes for various tissues in chickens, there is a dearth of research on the identification of reference genes in the gastrointestinal (**GI**) tract of chickens. This study utilized 4 different algorithms (Delta CT, BestKeeper, NormFinder, and Genorm) to assess the stability of 19 internal reference genes in various GI tract tissues, including individual GI tract tissues, the anterior and posterior GI tract, and the entire GI tissue. The RefFinder software was employed to comprehensively rank these genes. The research findings successfully identified the most appropriate internal reference genes for each type of GI tissue. Furthermore, *TBP, DNAJC24, Polr2b, RPL13,* and *Ap2m* exhibited stable expression in the entire and posterior GI tract, whereas *HMBS, TBP, Ap2m, GUSB, DNAJC24*, and *RPL13* demonstrated stable expression in the anterior GI tract. However, the internal reference genes commonly utilized, namely *β-Actin, 18s RNA*, and *ALB*, exhibit poor stability and are not advised for future investigations concerning gene expression in the GI region. Consequently, *MUC2* and *CDX1*, 2 genes that specifically express in the gut, were chosen for examination to ascertain the stability of the aforementioned internal reference genes in this particular study. In summary, this study presents a relatively stable set of internal reference genes that can be employed to enhance the precision of quantifying mRNA expression levels in functional genes within the chicken GI tract.

**Key words:** RT-qPCR, reference gene, gene expression profile, gastrointestinal tract

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## INTRODUCTION

Gene expression analysis has become a crucial component in numerous molecular biology investigations. In comparison to recent advancements in high-throughput transcriptome sequencing technology, reverse transcription quantitative real-time polymerase chain reaction (**RT-qPCR**) offers several advantages, including cost-effectiveness, ease of use, rapid detection, heightened sensitivity, and reliable reproducibility (Kubista et al., 2006). Consequently, RT-qPCR assumes a pivotal role in contemporary cellular and molecular biology research (Dheda et al., 2005). Nevertheless, the precision of RT-qPCR quantification hinges upon the quality, concentration, and reverse transcription efficiency of RNA (Sanders et al., 2014). The utilization of reference genes, also known as housekeeping genes, for the purpose of gene expression homogenization is a frequently employed approach to overcome this constraint (Huggett et al., 2005). The optimal reference genes are characterized by their consistent expression across various tissue types and cells, unaffected by external factors such as the environment, experimental conditions, or other variables. Nevertheless, the absence of a universally ideal reference gene applicable to all tissues necessitates caution (Hasanpur et al., 2022; Mogilicherla et al., 2022), as certain commonly utilized reference genes, including glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***), beta-actin (***β-Actin***), exhibit unsuitability in specific tissues due to their inherent variability (Glare et al., 2002; Barber et al., 2005; Lin and Redies, 2012).

In recent years, there has been a significant increase in scholarly investigations concerning reference genes in various tissues of domesticated animals (Bonnet et al., 2013; Vorachek et al., 2013; Cieslak et al., 2015; Park et al., 2015; Faheem and Khaliq, 2019; Wang et al., 2022).



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**Table 1.** Information of the 19 selected reference genes in this study.

| Gene name | Full name | Applicable tissues |
|---|---|---|
| *HMBS* | Hydroxymethylbilane synthase | Chicken skeletal muscle (Nascimento et al., 2015), proliferation of primary preadipocytes (Na et al., 2021), magnum of laying hens (Rodríguez Hernández et al., 2021) |
| *PPIA* | Peptidylprolyl isomerase A | Proliferation of primary preadipocytes (Na et al., 2021) |
| *TBP* | TATA box binding protein | Chicken liver (Bagés et al., 2015), chicken abdominal adipose tissue and primary preadipocytes (Na et al., 2021) |
| *RPL4* | Ribosomal protein L4 | Chicken spleen (Mogilicherla et al., 2022) |
| *RPL5* | Ribosomal protein L5 | Chicken muscle tissue (Cedraz De Oliveira et al., 2017), Gizzard (Mogilicherla et al., 2022), quail intestine and abdominal fat (de Sousa et al., 2021) |
| *RPL13* | Ribosomal protein L13 | Chicken and turkey spleen, liver, caecum and cecal tonsil (Mitra et al., 2016), Thigh muscle (Mogilicherla et al., 2022) |
| *HSP10* | Heat shock protein 10 | Chicken breast muscle (Mogilicherla et al., 2022) |
| *GUSB* | Glucuronidase beta | Chicken spleen (Mogilicherla et al., 2022) |
| *DNAJC24* | DnaJ heat shock protein family member C24 | A suitable reference gene for chicken breast muscle, thigh muscle, heart, liver, spleen, gizzard, and bursa (Mogilicherla et al., 2022) |
| *MRPS30* | Mitochondrial ribosomal protein S30 | Chicken muscle tissues (Cedraz De Oliveira et al., 2017), quail breast muscle (de Sousa et al., 2021) |
| *HPRT* | Hypoxanthine guanine phosphoribosyl transferase | Chicken skeletal muscle (Nascimento et al., 2015), quail breast muscle, abdominal fat, liver, and intestine (de Sousa et al., 2021), chicken ovarian and uterine (Hassanpour et al., 2019) |
| *Ap2m* | Adaptor-related protein complex 2 mu 1 subunit | Predict a suitable gene for chicken liver and has a good preference in small intestine (Hasanpur et al., 2022) |
| *Polr2b* | RNA polymerase II subunit B | Predict a suitable gene for kidney and had a high score in small intestine (Hasanpur et al., 2022) |
| *Nelfcd* | Negative elongation factor complex member C/D | Predict had a high score in small intestine (Hasanpur et al., 2022) |
| *ATP5b* | ATP synthase subunit beta, mitochondrial | Predict had a high score in small intestine (Hasanpur et al., 2022) |
| *ALB* | Albumin | Chicken Liver and spleen (Mogilicherla et al., 2022) |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | Widely used reference gene |
| *β-Actin* | Beta actin | Widely used reference gene |
| *18s RNA* | 18s ribosomal RNA | Widely used reference gene |

Notably, in the case of chickens (*Gallus gallus*), several studies have successfully identified stable reference genes under different conditions (Cedraz De Oliveira et al., 2017; Hassanpour et al., 2019; Rodríguez Hernández et al., 2021), and the evaluation of genes employed for gene expression analysis in major chicken organs has also been established (Hasanpur et al., 2022). However, the absence of a dependable reference gene applicable to multiple tissues remains a challenge. A previous study showed that the selected reference genes exhibit significant variation between different tissues, indicating that no single reference gene exhibits consistent expression across all the tissues examined (Mogilicherla et al., 2022). To mitigate the influence of unstable reference genes on gene expression profiles, numerous studies have employed a dual or triple reference genes strategy, albeit at the cost of increased workload (Gharbi et al., 2015; Zheng et al., 2022). Nevertheless, when examining gene expression profiles across multiple tissues, the inclusion of stable reference genes becomes imperative. Given the organ-specific expression of certain genes, particularly within the gastrointestinal (**GI**) tract, investigations pertaining to the chicken GI tract have experienced notable growth in recent years.

Historically, the study of GI health has played a significant role in the field of animal nutrition science research (Chee et al., 2010; Moran, 2017). In recent years, the focus on the intestinal microbiome has further propelled this research (Clavijo and Florez, 2018; Wen et al., 2021; Zhang et al., 2022). The GI tract, being a lengthy and complex system, undergoes similar developmental stages (Gao et al., 2012; Kitazawa et al., 2017), making it crucial to compare the variations in gene expression profiles across different sections. Presently, there is a dearth of reference primers tailored specifically for targeting the GI tract. Although Hasanpur et al. have proposed candidate reference primers based on resequencing data, their suitability for partial intestinal breaks remains unverified through experimental validation (Hasanpur et al., 2022).

The assessment of genes utilized for normalizing gene expression across various tissues is currently insufficient and imperative. Given the resemblance of chicken GI tissues, we were motivated to authenticate reference genes in various GI tract tissues, including individual GI tract tissues, the anterior and posterior GI tract, and the entire GI tissue respectively. This investigation involved evaluating the stability of 19 potential reference genes, comprising both conventional and newly discovered reference genes suitable for intestinal tissues as identified through next-generation sequencing (Table 1). Four statistical algorithms, namely Delta CT (Silver et al., 2006), BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), and Genorm (Vandesompele et al., 2002), were utilized in this study as they are widely recognized methods for assessing reference gene stability (Piazza et al., 2017; Schwarz et al., 2020). The software RefFinder (Xie et al., 2012; Xie et al., 2023) was employed to rank the aforementioned algorithms, which is also commonly used in reference gene stability analysis (Volland et al., 2017; Kumar et al., 2022). Lastly, mucin 2 (***MUC2***) and caudal type homeobox 1 (***CDX1***), 2 genes that specifically express in the gut (Grainger et al., 2013; Liu et al., 2020), were chosen for examination to ascertain the stability of the stable reference genes selected in this study. The outcome of this investigation

will establish reliable internal reference genes for gene expression profiling in various chicken GI tissues, including individual GI tissues, the entire anterior GI tract, the entire posterior GI tract, and the entire GI tract.

## MATERIALS AND METHODS

### Animals and Sample Collection

The animal protocols were approved by the Institutional Animal Care and Use Committee (**IACUC**) of Jiangsu University of Science and Technology (G2022SJ13, Zhenjiang, China). Animal care and handling practices were followed by the IACUC guidelines.

The sample utilized in this study was obtained from a prior investigation conducted within our laboratory (Zheng et al., 2022). Specifically, in this experiment, 6 AA broilers with similar body weights were used for subsequent analysis. All chicks were housed under identical environmental conditions and provided unrestricted access to water. Tissue samples, including the crop, proventriculus, gizzard, duodenum, jejunum, ileum, and colon, were promptly frozen in liquid nitrogen and stored at −80°C to facilitate subsequent RNA extraction.

### Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from each individual tissue using the Vezol Reagent (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. The quality and concentration of the samples were assessed using the Nanophotometer N60 Touch (IMPLEN, Munich, Germany). The extracted total RNA samples were then frozen and stored at −80°C until cDNA synthesis. The cDNA synthesis was performed using the HiScript II 1st Strand cDNA Synthesis Kit with gDNA wiper (Vazyme), using 1 $\mu$g of the extracted total RNA. After synthesis, the cDNA was diluted to a 1:3 ratio with nuclease-free water and stored at −20°C. To minimize quantitative errors resulting from reverse transcription efficiency, all 48 individual RNA samples were reverse transcribed in the same batch for this study. A negative control (no cDNA) and a reverse transcription control (no reverse transcription) were used for comparison.

### Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction

The reverse transcription quantitative real-time polymerase chain reaction (**RT-qPCR**) was performed using a Bio-Rad Light Cycler 96 Real-Time PCR system with 20 $\mu$L reaction volumes containing 1 $\mu$L cDNA, 10 $\mu$L MagicSYBR Mixture (Jiangsu Cowin Biotech Co., Ltd., Taizhou, China), 1 $\mu$L of each of the forward and reverse primers (10 $\mu$M), and 7 $\mu$L deionized water. The qPCR amplification procedure was as follows: 95°C for 15 min, 40 cycles of 95°C for 10 s, 58°C for 20 s, 72°C for 30 s, and an extension for 10 min at 72°C. All reactions were conducted in triplicate. A total of 19 excellent reference genes validated in previous experiments and predicted based on transcriptome data were selected for this study (Table 1). The RT-qPCR gene-specific primers for the 19 reference genes, *MUC2* and *CDX1* were designed using Primer Premier 5.0 software (Table S1), and were synthesized by Shangya Biotechnology Co., Ltd. (Hangzhou, Zhejiang, China). The melting curves were analyzed to determine if the primer was single banded, then Sanger sequencing (Shangya) was performed to confirm its specificity. The efficiency of primer amplification for each reference gene was assessed through slope analysis using a linear regression model. Relative standard curves were generated by performing 4 serial dilutions of cDNA (1:1, 1:5, $1:5^2$, $1:5^3$, $1:5^4$). The efficiency of each primer was calculated using the equation: $E = (10\ (-1/\text{slope})-1) \times 100\%$.

### Bioinformatics and Statistical Analysis

Data were collected and analyzed using Bio-Rad CFX96 Manage software. The CT values of genes were exported to Microsoft Excel for further analysis. The relative gene expression levels were then normalized using the $2^{-\triangle CT}$ method, as previously described (Schmittgen and Livak, 2008). The $\triangle$CT value was calculated by subtracting the smallest CT value within each group. Both the CT raw value and the relative gene expression levels were utilized for subsequent gene stability analysis using various software programs. To assess the statistical significance of differences between 2 groups, a 2-sided Student $t$ test was conducted, with significance considered at $P \leq 0.05$. To assess the statistical significance among different groups, a 1-way ANOVA was used, with significance considered at $P \leq 0.05$.

To determine stability rankings across samples obtained from various chicken GI tissues, we employed 4 publicly accessible algorithms, namely Delta CT, Bestkeeper, NormFinder, and Genorm, which have been validated for in silico prediction of reference gene stability. The Delta CT method facilitated the assessment of the level of deviation, either increased or decreased, by comparing the CT values. A lower degree of deviation indicates reduced variability in gene expression within the samples (Silver et al., 2006). BestKeeper uses the Pearson correlation coefficient matrix to calculate the standard deviation. Genes exhibiting high correlation were consolidated into a composite measure known as the BestKeeper index. BestKeeper determines the gene with the lowest coefficient of variance and standard deviation (Pfaffl et al., 2004). The NormFinder software employs a model-based methodology to enable the estimation of expression variation among various organ groups. It further ranks genes based on the similarity of their expression profiles and generates a stability measure, which signifies enhanced stability in gene expression when it exhibits a low value (Andersen et al., 2004). The Genorm algorithm was employed to ascertain the average

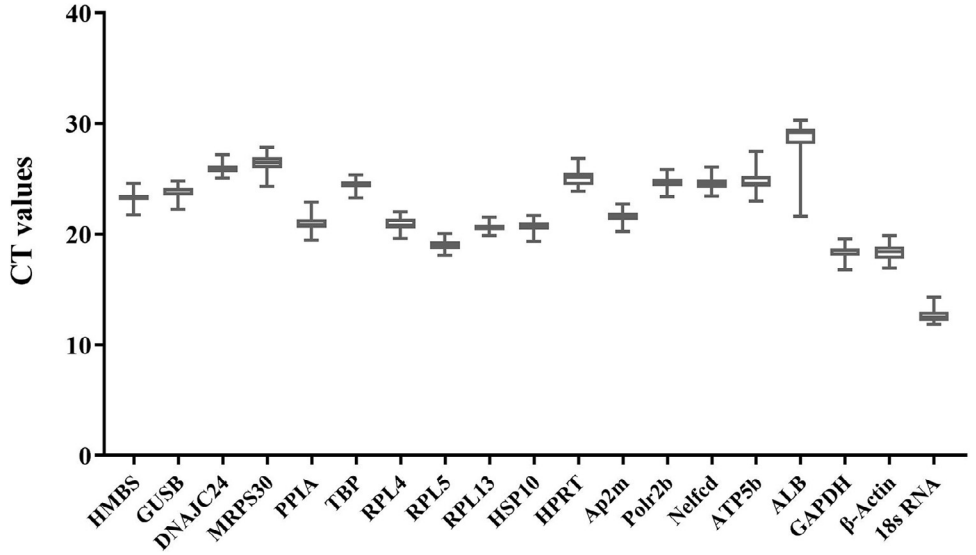


**Figure 1.** The average CT values of the 19 reference genes in the entire gastrointestinal tract of broilers. CT values presented as mean ± SD.

expression stability of the genes under investigation. This was achieved by computing the average pairwise variation between each gene and all other potential reference genes. The gene exhibiting the lowest M value was deemed to be the most stable. Additionally, Genorm determined the ratio Vn/Vn+1 for consecutive numbers of reference genes, a value of Vn/Vn+1 > 0.15 indicated the need for an additional reference gene (Vandesompele et al., 2002). RefFinder (http://blooge.cn/RefFinder/), a freely accessible online in silico prediction tool, to assess and prioritize the stability of potential reference genes. This tool integrates 4 algorithms mentioned earlier, assigning a value to each gene based on their rankings from each algorithm (Xie et al., 2012, 2023).

## RESULTS

### Expression Levels Analysis of 19 Reference Genes in Chicken Gastrointestinal Tract

In order to enhance the precision of detecting reference gene expression levels, an initial examination was conducted to assess the specificity of 19 primer pairs. The findings revealed that the melting curves of all 19 pairs of reference gene primers exhibited clarity and singularity (Figure S1). Moreover, the standard curve correlation coefficients ($R^2$) for all 19 reference genes surpassed 0.98, while the amplification efficiency of the primer pairs ranged from 91.568% to 97.925% (Table S1). These outcomes confirmed that the 19 primer pairs adhere to the quality control criteria of RT-qPCR and are suitable for subsequent analyses.

In this study, the expression levels of 19 reference genes were assessed in chicken whole GI tract tissues using RT-qPCR. The average expression levels of these 19 reference genes were categorized into 3 levels of abundance based on CT values: high, medium, and low. The 18s RNA reference gene exhibited high abundance expression, with an average CT value of 12.63. On the other hand, *β-Actin, GAPDH, RPL5, RPL13, HSP10, RPL4, PPIA, Ap2m, HMBS, GUSB, TBP, Nelfcd, Polr2b*, and *ATP5b*, with average CT values ranging from 17.04 to 24.81, were reference genes expressed at a medium level of abundance. *HPRT, DNAJC24, MRPS30*, and *ALB* were identified as reference genes with low abundance expression, as indicated by their average CT values ranging from 25.05 to 28.28 (Figure 1). The *18s RNA* and *ALB* genes exhibited the highest (CT = 12.63) and lowest (CT = 28.28) transcript abundance, respectively. Among these 19 reference genes, *ALB* displayed the greatest variation in CT values across different tissues and was particularly unstable in the duodenum. This instability may introduce uncertainty in the analysis of gene expression profiles.

### Expression Stability Analysis of 19 Reference Genes in Each GI Tract Tissues Respectively

In order to determine the most suitable reference genes for various alimentary tract tissues, we conducted an analysis of the expression stability of 19 reference genes using 4 different algorithms. The results indicated that the optimal reference genes for the Delta CT method in crop tissue were *TBP* (0.35), *HMBS* (0.38), and *RPL13* (0.38). According to the BestKeeper algorithm, the most stable genes were *RPL5* (0.10), *RPL13* (0.15), *HMBS* (0.16). NormFinder suggested that *TBP* (0.054), *HPRT* (0.095), and *Polr2b* (0.113) were the most suitable reference genes. Genorm analysis indicated *RPL13*|*TBP* (0.108) and *HMBS* (0.131) is optimal. The comprehensive ranking provided by RefFinder recommended *TBP* (1.5), *RPL13* (2.21), and *HMBS* (3.08) as the top choices.

The Delta CT method in proventriculus tissue suggested that *GUSB* (0.39), *TBP* (0.39), and *GAPDH* (0.40) were the most appropriate reference genes. The BestKeeper algorithm identified *HMBS* (0.08),

*DNAJC24* (0.11), and *GUSB* (0.11) as the most stable genes. NormFinder analysis indicated that *Polr2b* (0.046), *TBP* (0.059), and *GUSB* (0.067) were the most suitable reference genes. Genorm analysis suggested *HMBS*|*GUSB* (0.051) and *GAPDH* (0.074) was optimal. The comprehensive ranking provided by RefFinder recommended *GUSB* (1.73), *HMBS* (2.38), and *TBP* (3.31) as the top choices.

The Delta CT method in gizzard tissue identified *GAPDH* (0.64), *TBP* (0.65), and *HMBS* (0.67) as potential reference genes. The BestKeeper algorithm determined *RPL5* (0.30), *RPL13* (0.34), and *RPL4* (0.49) as the most stable genes. NormFinder suggested that *GAPDH* (0.111), *HMBS* (0.125), and *TBP* (0.153) were suitable options. Genorm analysis indicates *RPL4*|*GUSB* (0.153) and *GAPDH* (0.218) was optimal. RefFinder's comprehensive ranking recommended *GAPDH* (2.06), *RPL4* (3.22), and *GUSB* (4.00) as the top choices.

The Delta CT method in duodenum tissue identified *RPL13* (0.47), *Polr2b* (0.48), and *RPL4* (0.50) as potential reference genes. The BestKeeper algorithm determined that *GAPDH* (0.10), *MRPS30* (0.15), and *RPL13* (0.16) exhibited the highest stability. NormFinder suggested that *HMBS* (0.049), *RPL13* (0.071), and *HSP10* (0.078) were the most suitable genes. Genorm analysis indicated *TBP*|*Polr2b* (0.092) and *RPL5* (0.096) was optimal. RefFinder's comprehensive ranking recommended *RPL13* (2.34), *Polr2b* (2.99), and *GAPDH* (3.98) as the top choices.

The Delta CT method in jejunum tissue identified *HSP10* (0.35), *Nelfcd* (0.35), and *Ap2m* (0.36) as potential reference genes. According to the BestKeeper algorithm, the most stable genes were *DNAJC24* (0.21), *HPRT* (0.25), and *RPL13* (0.29). NormFinder suggested that *Ap2m* (0.073), *HMBS* (0.08), and *DNAJC24* (0.115) were the most suitable genes. Genorm analysis indicated *Ap2m*|*Nelfcd* (0.107), and *HMBS* (0.08) was optimal results. Finally, RefFinder's comprehensive ranking recommended *Ap2m* (2.28), *Nelfcd* (3.16), and *HSP10* (3.36) as the top choices.

The Delta CT method in ileum tissue identified *HMBS* (0.35), *DNAJC24* (0.35), and *HPRT* (0.36) as the optimal reference genes. The BestKeeper algorithm determined *TBP* (0.11), *Ap2m* (0.12), and *HMBS* (0.13) as the most stable genes. NormFinder suggested *HMBS* (0.048), *DNAJC24* (0.058), and *Ap2m* (0.065) as suitable options. Genorm analysis indicated *HMBS*|*Ap2m* (0.096) and *DNAJC24* (0.113) is the optimal choice. RefFinder provides a comprehensive ranking, recommending *HMBS* (1.32), *Ap2m* (2.21), and *TBP* (3.31) as the top choices.

The Delta CT method in cecum tissue identified *Polr2b* (0.28), *HSP10* (0.28), and *Ap2m* (0.29) as the optimal reference genes. The BestKeeper algorithm determined that the most stable genes were *18s RNA* (0.28), *HPRT* (0.39), and *TBP* (0.40). NormFinder suggested that *Polr2b* (0.104), *Ap2m* (0.11), and *HSP10* (0.124) were the most suitable genes. Genorm analysis indicates *GAPDH*|*β-Actin* (0.082) and *MRPS30* (0.103) was the optimal choice. The comprehensive ranking provided by RefFinder recommended *Polr2b* (2.71), *GAPDH* (3.94), and *HSP10* (4.53) as the top choices.

The Delta CT method in colon tissue suggested that the optimal reference genes were *GAPDH* (0.33), *TBP* (0.33), and *DNAJC24* (0.34). The BestKeeper algorithm identified *18s RNA* (0.18), *HSP10* (0.23), *TBP* (0.28) as the most stable genes. NormFinder suggested that *TBP* (0.036), *GAPDH* (0.048), *DNAJC24* (0.084) were suitable choices. Genorm analysis indicated that the combination of *DNAJC24* | *GAPDH* (0.088), *HMBS* (0.104) was optimal. RefFinder provides a comprehensive ranking, recommending *GAPDH* (2.06), *TBP* (2.21), *DNAJC24* (2.59) as the top choices. Detailed scores for other internal reference genes can be found in Tables S2 to S9.

## Expression Stability Analysis of 19 Reference Genes in the Whole GI Tract

The expression stability of the 19 reference genes in the entire GI tract of broilers was assessed using 4 different algorithms. According to the Delta CT method, the ranking order from highest to lowest stability was as follows: *TBP, DNAJC24, Polr2b, Ap2m, RPL13, HMBS, HSP10, Nelfcd, RPL4, RPL5, GAPDH, PPIA, MRPS30, HPRT, 18s RNA, β-Actin, ATP5b, GUSB*, and *ALB* (Figure 2A). BestKeeper analysis identified the following genes as stable across all organs: *RPL13, RPL5, DNAJC24, 18s RNA, TBP, HSP10, HMBS, Polr2b, Ap2m, MRPS30, HPRT, RPL4, Nelfcd, GAPDH, PPIA, ATP5b, β-Actin, GUSB*, and *ALB* (Figure 2B). The analysis conducted using NormFinder revealed the hierarchical arrangement of genes in various organs, specifically *TBP, DNAJC24, Ap2m, HSP10, Polr2b, HMBS, RPL13, Nelfcd, GAPDH, RPL4, RPL5, PPIA, MRPS30, 18s RNA, HPRT, ATP5b, β-Actin, GUSB*, and *ALB*, as depicted in Figure 2C. According to the Genorm stability criteria, the expression stability of the 19 reference genes was assessed and ranked from high to low. The ranking, in descending order, was as follows: *Polr2b*|*Nelfcd, TBP, DNAJC24, RPL4, RPL13, RPL5, Ap2m, HMBS, HSP10, GAPDH, MRPS30, PPIA, HPRT, β-Actin, 18s RNA, ATP5b, GUSB*, and *ALB*. It is worth noting that the *ALB* gene did not meet the stability criteria. However, all the other reference genes fell within the threshold range of M value <1, indicating a relatively reliable stability (Figure 2D). RefFinder combined the calculations from all above-mentioned algorithms and suggested stable genes in all organs: *TBP, DNAJC24, Polr2b, RPL13, Nelfcd, Ap2m, RPL5, HSP10, HMBS, RPL4, 18s RNA, GAPDH, MRPS30, PPIA, HPRT, β-Actin, ATP5b, GUSB*, and *ALB*, respectively (Figure 2E).

We conducted expression stability analysis of 19 reference genes in the anterior and posterior segments of the GI tract of broilers using 4 different algorithms. In the anterior segments of the GI tract, the Delta CT method yielded the following ranking order from highest to

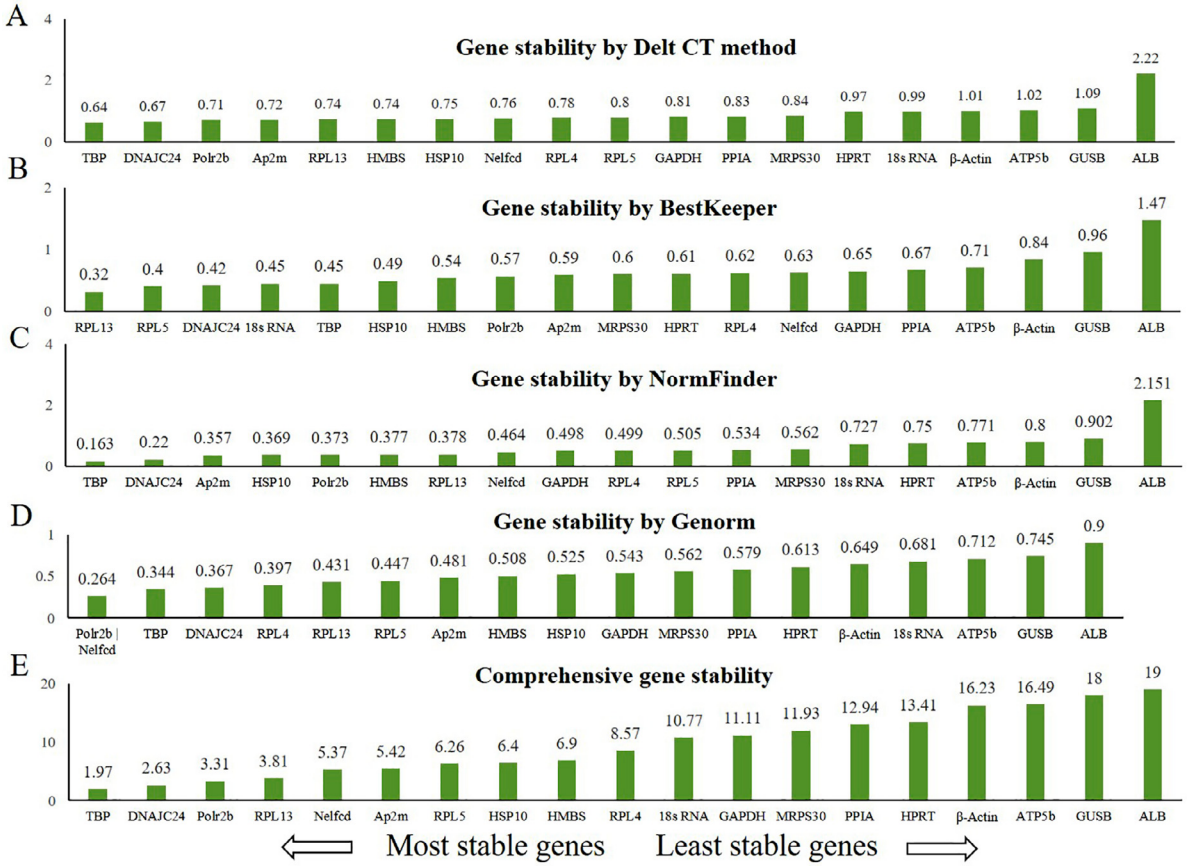


**Figure 2.** Five software analyses of 19 reference genes for stability in the entire gastrointestinal tract of broilers. (A) Delta CT method analysis; (B) BestKeeper analysis; (C) NormFinder analysis; (D) Genorm analysis; (E) RefFinder analysis.

lowest: *HMBS, TBP, DNAJC24, Ap2m, GUSB, GAPDH, RPL13, HPRT, HSP10, Polr2b, Nelfcd, PPIA, RPL5, MRPS30, RPL4, 18s RNA, ATP5b, β-Actin*, and *ALB* (Figure 3A). Similarly, the BestKeeper method produced the following ranking order from highest to lowest: *GUSB, RPL13, HSP10, RPL5, HPRT, 18s RNA, HMBS, DNAJC24, TBP, MRPS30, Ap2m, ATP5b, PPIA, GAPDH, RPL4*, and *Polr2b, Nelfcd, β-Actin*, and *ALB* (Figure 3B). The Normfinder method yielded the following ranking order, from highest to lowest: *HMBS, TBP, DNAJC24, Ap2m, GUSB, RPL13, HPRT, HSP10, GAPDH, RPL5, Polr2b, Nelfcd, PPIA, MRPS30, 18s RNA, RPL4, ATP5b, β-Actin*, and *ALB* (Figure 3C). Similarly, the Genorm method produced the ranking order, from highest to lowest: *Ap2m|TBP, HMBS, Polr2b, Nelfcd, GAPDH, DNAJC24, GUSB, RPL13, HSP10, HPRT, PPIA, RPL5, RPL4, MRPS30, 18s RNA, ATP5b, β-Actin*, and *ALB* (Figure 3D). Lastly, the RefFinder method recommended the comprehensive ranking order, from highest to lowest, as follows: *HMBS, TBP, Ap2m, GUSB, DNAJC24, RPL13, HSP10, HPRT, GAPDH, RPL5, Polr2b, Nelfcd, 18s RNA, PPIA, MRPS30, RPL4, ATP5b, β-Actin*, and *ALB* (Figure 3E). In contrast to the stability analysis results of reference genes throughout the GI tract, the stability of *HMBS, Ap2m*, and *GUSB* has been improved in the analysis of the anterior segments of the GI tract. Conversely, the performance of the gut-specific internal reference genes *Polr2b* and *Nelfcd* was poor, indicating a significant disparity between the anterior and posterior regions of the GI tract. Overall, internal reference genes such as *TBP, DNAJC24*, and *RPL13* exhibited a relatively stable nature in both the anterior segments of the GI tract and the entire intestinal tissue.

Following the excision of the tissues comprising the entire anterior segments of the GI tract, encompassing the crop, proventriculus, and gizzard, the posterior segments of the GI tract, commonly referred to as the intestinal tract, were obtained. Upon comparing the outcomes of the entire GI tract, it was observed that the relative rankings of the 5 software programs exhibited no significant alterations. Notably, the *GUSB* gene displayed a lower score in the entirety of the GI tract, but a higher score in the intestinal tissue, specifically ranking eighth, particularly in the CT method. This finding suggested that *GUSB* holds potential as a prospective candidate for subsequent selection as an intestinal reference gene (Figure S2).

## *Comparative Analysis of the Quantitative Effects of Reference Genes Based on the Gene Expression Profile of* MUC2 *and* CDX1 *in GI Tract*

In order to illustrate the efficacy of the validated candidate reference genes, we opted to conduct a

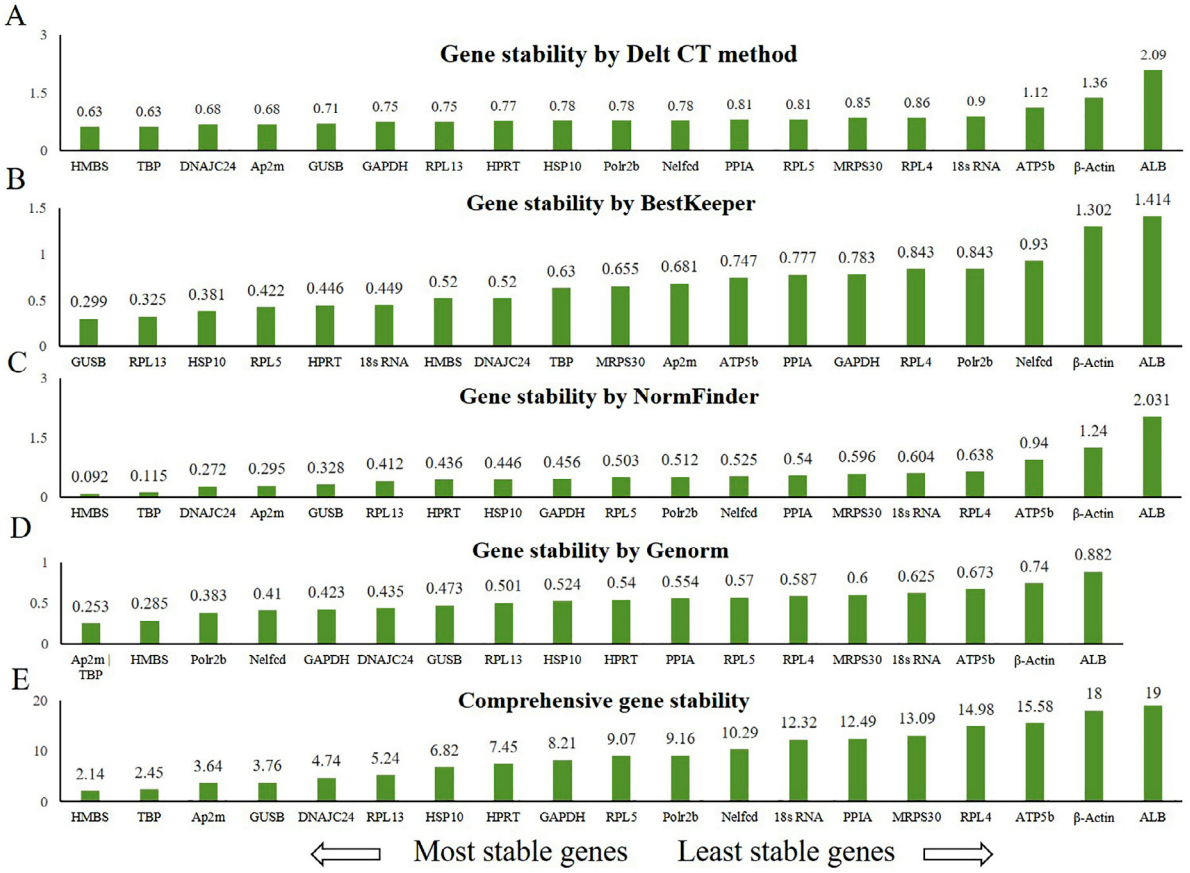


**Figure 3.** Five software analyses of 19 reference genes for stability in the entire anterior gastrointestinal tract of broilers (A). Delta CT method analysis; (B) BestKeeper analysis; (C) NormFinder analysis; (D) Genorm analysis; (E) RefFinder analysis.

comparative analysis on 2 intestine-specific genes, namely *MUC2* and *CDX1*. This analysis involved the utilization of 2 stable reference genes (*DNAJC24* and *RPL13*) and 2 commonly used reference genes (*β-Actin* and *ALB*), despite their instability. These reference genes were experimentally validated using all 5 aforementioned software. The utilization of *DNAJC24* and *RPL13* as reference genes revealed predominantly high expression levels of *MUC2* in the jejunum. Conversely, when *β-Actin* and *ALB* were used as reference genes, the relative expression varied among different intestinal tissues, resulting in insignificant changes. Particularly in the *ALB* group, *MUC2* and *CDX1* were not expressed in the duodenum. These findings differed from the analysis conducted using the 2 stable reference genes (Figures 4 and 5).

Moreover, the utilization of *DNAJC24* and *RPL13* as reference genes revealed a distinct upregulation of *MUC2* in the duodenum compared to the colon ($P < 0.05$), alongside consistent expression of *CDX1* in both tissues. Conversely, when *β-Actin* and *ALB* are employed as reference genes, the outcomes differ significantly and exhibit instability (Figures 4 and 5). Overall, the housekeeping reference genes chosen in this investigation adequately facilitate gene expression profiling. However, the conventional reference genes *β-Actin* and *ALB* demonstrate an unstable nature, consequently yielding inconsistent results.

## DISCUSSION

RT-qPCR is a widely employed method in contemporary molecular biology research (Gadkar and Filion, 2014), and the precise quantification of RT-qPCR relies on the identification of stable internal reference genes (Huggett et al., 2005; Sanders et al., 2014). The GI tract of chickens holds significant importance in terms of food digestion and nutrient absorption, exhibiting distinct morphological variations from mammals, particularly in the anterior GI tract (Gao et al., 2012; Kitazawa et al., 2017). Each segment of the chicken GI tract serves a distinct functional purpose, which is attributable to the selective expression of genes. The identification of internal reference genes in the GI tract of chickens is essential for conducting a comprehensive investigation into the expression patterns of specific genes within the GI tract. In this study, we employed various software tools, namely Genorm, NormFinder, BestKeeper, Delta CT, and RefFinder, to assess the stability of internal reference genes concurrently. Given the variability in algorithms across these softwares, it is imperative to employ multiple algorithms to accurately determine the stability of internal reference genes. In this study, a total of 19 candidate reference genes were evaluated for their expression stability in various sections of the chicken GI tract, including the anterior, posterior, and entire GI tract.

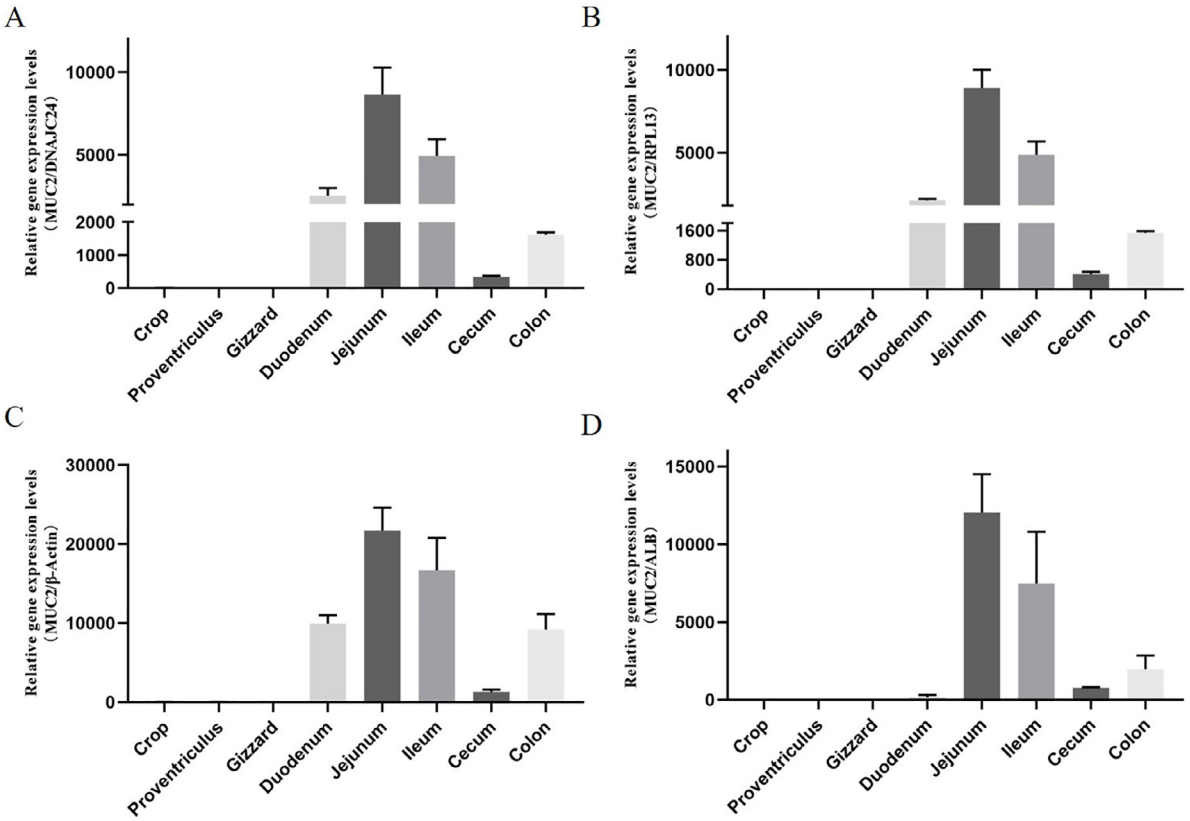


**Figure 4.** The *MUC2* gene expression profile analysis in the entire gastrointestinal tract. (A) Relative *MUC2* expression by normalization to *DNAJC24*; (B) Relative *MUC2* expression by normalization to *RPL13*; (C) Relative *MUC2* expression by normalization to *β-Actin*; (D) Relative *MUC2* expression by normalization to *ALB*.

Ideally, it is desirable for an internal reference gene used in quantitative detection to exhibit stability and moderate expression levels. The 19 internal reference genes selected for this investigation demonstrated comparable expression levels, with CT values below 30, thereby satisfying the screening criteria for internal reference genes. However, it is noteworthy that the *ALB* gene, which serves as a housekeeping gene (Thiel et al., 2015; Mogilicherla et al., 2022), exhibits the highest variability in its CT value and possesses the lowest stability score among all software analyses. Consequently, it is evident that in future investigations, the utilization of *ALB* as an internal reference for gene expression in the chicken GI tract is not recommended.

We have demonstrated the suboptimal performance of housekeeping genes (*β-Actin* and *GAPDH*) in this study, particularly due to their unstable expression levels throughout the entire GI tract, which aligns with previous studies (Glare et al., 2002; Barber et al., 2005; Lin and Redies, 2012). Nevertheless, it is important to acknowledge that certain segments of the GI tract have exhibited satisfactory performance of specific housekeeping genes in particular tissues, such as *GAPDH* in the proventriculus, gizzard, duodenum, cecum, and colon tissues; *18s RNA* in the cecum, and colon tissues; *β-Actin* in the cecum. Conversely, *HPRT* and *HMBS*, which exhibit commendable performance across various tissues (Nascimento et al., 2015; Hassanpour et al., 2019; Na et al., 2021; Rodríguez Hernández et al., 2021), do not exhibit elevated scores. The diminished scores of reference genes, such as *HPRT* and *HMBS*, which demonstrate proficiency in multiple tissues, within this intestinal tissue further underscore the distinct characteristics of different tissues and the varying requirements for internal reference genes.

In the combined analysis for all organs, 3 of 5 algorithms showed that *TBP* and *DNAJC24* are the most stable genes. *RPL13* and intestinal reference genes *Polr2b, Ap2m*, and *Nelfcd* screened based on sequencing data have shown good performance (Hasanpur et al., 2022) (ranking in the top 9) in the 5 software programs. Particularly, *Ap2m* in jejunum, ileum, and cecum, *Polr2b* in the crop, proventriculus, duodenum, and cecum, and *Nelfcd* in the jejunum all had a well performance. This study employed experimental methods to demonstrate that reference primers selected from transcriptomes can be utilized in RT-qPCR. However, it is important to consider the relative expression of genes. For example, we observed that the highly ranked gene *Eif2b5*, identified through resequencing (Hasanpur et al., 2022), exhibits high CT values in actual testing and some tissues show minimal or no expression of *Eif2b5*, rendering it unsuitable as a reference gene for tissue expression profile analysis.

Given the distinct characteristics of the chicken's anterior and posterior GI tracts, our analysis focused on examining the stability of reference genes separately. We found that the stability of reference genes in the posterior GI tract, as well as the overall GI tract, exhibited similarities. However, in the anterior GI tract, aside

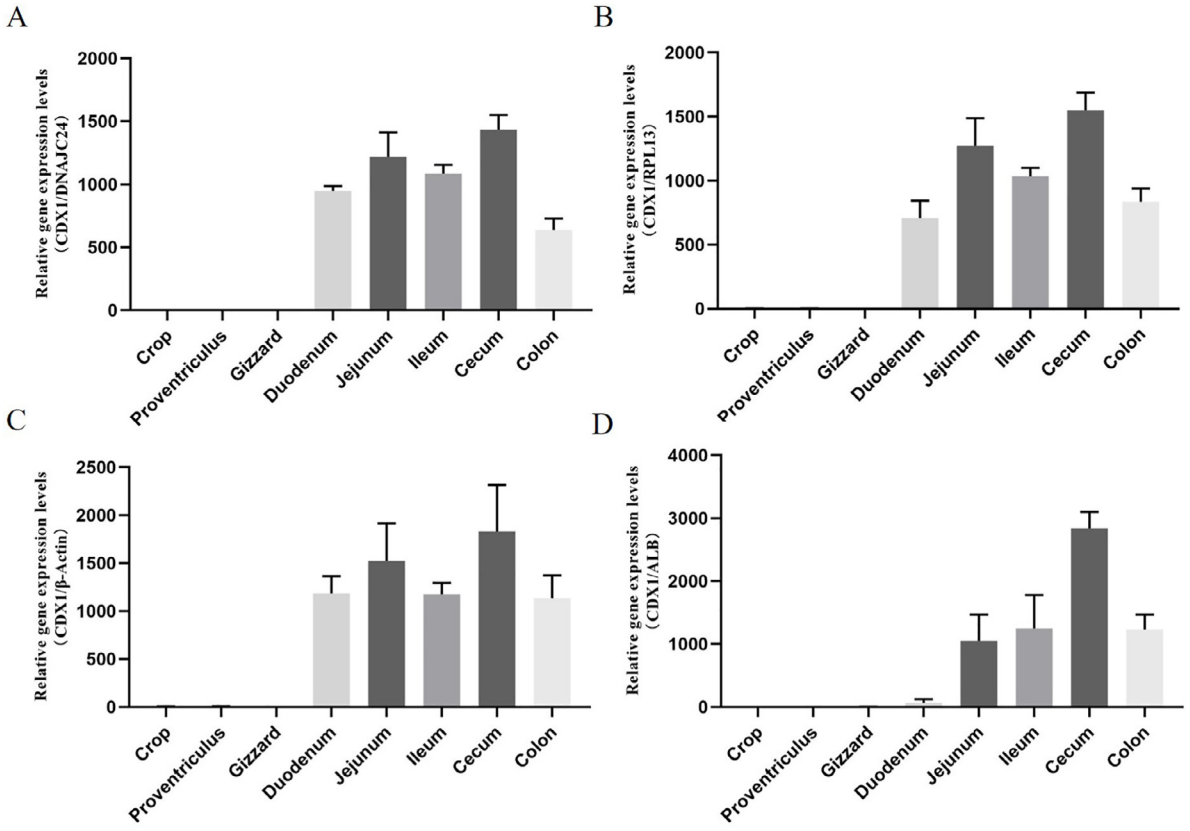


**Figure 5.** The *CDX1* gene expression profile analysis in the entire gastrointestinal tract. (A) Relative *CDX1* expression by normalization to *DNAJC24*; (B) Relative *CDX1* expression by normalization to *RPL13*; (C) Relative *CDX1* expression by normalization to *β-Actin*; (D) Relative *CDX1* expression by normalization to *ALB*.

from genes such as *TBP* and *DNAJC24*, reference genes like *HMBS* and *GUSB* also demonstrated relatively high stability (ranking within the top 5). This observation may be attributed to morphological disparities between the anterior and posterior segments of the GI tract (Kitazawa et al., 2017). Furthermore, *GUSB* exhibited favorable performance in the analysis of both the anterior and posterior GI tracts, as evidenced by its ranking of 8 and 4 respectively in the RefFinder. However, its performance was subpar when considering the entire GI tract, which could potentially be attributed to variations in *GUSB* expression levels between the anterior and posterior GI tracts. Genorm analysis revealed that the paired variation analyses of reference genes in the anterior GI tract, posterior GI tract, and the entire GI tract yielded V2 = 3 values that were all below the recommended threshold of 0.15. This suggests that 2 reference genes should be utilized as standardized correction factors in the RT-qPCR experiments. In order to ensure precise normalization, the utilization of 2 reference genes from the top 5 selected by RefFinder is deemed satisfactory.

Subsequently, to validate the efficacy of the recently identified reference genes throughout the entire GI tract, *CDX1* and *MUC2* were employed for gene expression profile analysis. *MUC2*, a significant secretory protein present in the intestinal tract, plays a crucial role in maintaining intestinal homeostasis (Liu et al., 2020). Previous studies have reported high expression levels of chicken *MUC2* in the GI tract using RT-qPCR (Jiang et al., 2013), which lacks the ability to accurately quantify the relative expression levels of the gene. *CDX1* encode homeodomain transcription factors related to the *Drosophila caudal* gene, and exhibit transcriptional specificity in the intestine tissue (Lohnes, 2003; Grainger et al., 2013). Currently, there is a lack of comprehensive analysis on the expression of chicken *CDX1* throughout the entire GI tact. The gene expression profiles obtained using *DNAJC24* and RPL13 as reference genes appear to be more stable than those obtained using *β-Actin* and *ALB*. These findings provide a foundation for future investigations on the expression of *MUC2* and *CDX1* in the chicken GI tissues.

In conclusion, this study assessed the suitability of 19 reference genes for various GI tissues, including individual GI tissues, the entire anterior GI tract, the entire posterior GI tract, and the entire GI tract as a whole. Our findings indicate that different GI tissues require different reference genes, allowing researchers to select appropriate reference genes based on specific tissues. According to the ranking order generated by the RefFinder software, *TBP* and *DNAJC24* were identified as the most reliable reference genes for gene expression profile analysis in the whole GI tract and posterior GI tract, while *HMBS* and *TBP* were deemed the most reliable for the anterior segment of the GI tract. The findings from these studies contribute to the selection of optimal reference genes for normalizing RT-qPCR data for studies involving the gene expression of chicken GI tract.

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## DISCLOSURES

The authors declare no conflicts of interest.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2023.103169.

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