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Reference gene stability in the *Longissimus thoracis et lumborum muscle* of Colombian Creole sheep

Estabilidade de genes de referência no músculo *Longissimus thoracis et lumborum* de ovelhas crioulas colombianas

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Highlights

Algorithms were used to analyze the stability of reference genes.
TUBB2A gene showed a low correlation coefficient in female Colombian Creole sheep.
GAPDH gene showed the highest stability in *Longissimus thoracis et lumborum muscle.*

Abstract

The Colombian Creole sheep breed has a high economic and social importance for Colombia. Both males and females of this breed are multipurpose animals, and evaluating the production and meat quality of both sexes is important for small farmers in Colombia. This requires the use of tools that help to evaluate critical production points, such as Real-time quantitative polymerase chain reaction (RT-qPCR), which is a widely used molecular tool for the relative quantification of candidate genes in various tissues. For its correct use, the use of housekeeping genes with stable expression, so-called "reference genes", is required. However, recent studies have shown that the expression of these reference genes can vary among tissues and can be modulated by breed, sex, or external stimuli. Likewise, there is little information regarding the expression of these genes in the *Longissimus thoracis et lumborum* muscle of male and female Colombian Creole sheep. In this study, the stability in the expression of seven reference genes (*ACTB, YWHAZ, SDHA, GAPDH, TUBB2A, B2M,* and *PGK1*) in the *Longissimus thoracis et lumborum* muscle of male and female Colombian Creole sheep was compared since they are used in RT-qPCR studies to determine the most stable ones for this breed. Twelve animals, six males and six females, with a body weight of 26 ± 4 kg and 12 ± 3 months of age, were used under grazing conditions. Biopsies of the *Longissimus thoracis et lumborum* muscle were taken, from which RNA was extracted and cDNA was synthesized. Expression was determined using RT-qPCR, and its stability was analyzed by computational algorithms using the geNorm, Normfinder, and BestKeeper software packages, which were integrated using the RefFinder software package. The results indicate that *GAPDH, ACTB,* and *SDHA* have the highest stability, whereas the most variable expression

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was found for *B2M.* These data provide the basis for more precise results in RT-qPCR studies of gene expression in the muscle of Colombian Creole sheep.
Key words: cDNA. Normalization. Pair-wise correlation. Relative quantifications. RT-qPCR.

Resumo

A raça de ovinos Crioula Colombiana tem grande importância econômica e social para a Colômbia. Avaliar a produção e a qualidade da carne de machos e fêmeas é importante para pequenos produtores do país e, assim, faz necessário o uso ferramentas que ajudam a avaliar os pontos críticos de produção, como a reação em cadeia da polimerase em tempo real (Real-time quantitative polymerase chain reaction [RT-qPCR]). Esta é uma ferramenta molecular amplamente usada para a quantificação relativa de genes candidatos em vários tecidos. Para o seu uso correto, é necessário o uso de genes com expressão estável denominados genes de referência. No entanto, estudos recentes têm mostrado que a expressão desses genes de referência pode variar entre os tecidos e pode ser modulada por raça, sexo ou estímulos externos. Da mesma forma, existem poucas informações sobre a expressão desses genes no músculo *Longissimus thoracis et lumborum* de ovinos machos e fêmeas da raça Crioula Colombiana. Neste estudo foi comparada a estabilidade na expressão de sete genes de referência (*ACTB, YWHAZ, SDHA, GAPDH, TUBB2A, B2M* e *PGK1*) no músculo *Longissimus thoracis et lumborum* de ovinos Crioulo Colombiano machos e fêmeas, por serem genes utilizados em estudos de RT-qPCR visando determinar os mais estáveis para esta raça. Doze animais com peso corporal de 26 ± 4 kg e 12 ± 3 meses de idade foram utilizados em condições de pastejo. Foram realizadas biópsias do músculo *Longissimus thoracis et lumborum,* de onde o RNA foi extraído e o cDNA foi sintetizado. A expressão foi determinada usando RT-qPCR e sua estabilidade foi analisada por algoritmos computacionais usando geNorm, Normfinder e BestKeeper pacote de software, os quais foram integrados usando RefFinder pacote de software. Os resultados indicam que *GAPDH, ACTB* e *SDHA* apresentam maior estabilidade, enquanto a expressão mais variável foi para *B2M.* Esses dados fornecem a base para resultados mais precisos em estudos de RT-qPCR de expressão gênica em músculos defeminino ovinos da raça Crioula Colombiana.
Palavras-chave: cDNA. Correlação de pares. Normalização. Quantificação relativa. RT-qPCR.

Introduction

Colombian Creole sheep are widely used by sheep farmers in Colombia because they can tolerate high temperatures, are more resistant to parasites compared to breeds of European origin, can digest forages with a high amount of fiber and have a high prolificity and a short production cycle (Ángel & Ramírez, 2014; Hernandez et al., 2019). However, due to the limited information about the management of the breed, several issues may appear in the production, including an inadequate diet and high parasitic loads, resulting in a limited use of its genetic potential and a low yield in terms of quality and meat production (Vergara, Valencia, & Herrera, 2019). Various factors, such as the age of the animals at slaughter, supplementation, diet and gender, can affect the muscular characteristics and the meat quality (Lind, Berg, Morten, Hersleth, & Olav, 2011; Guerrero, Valero, Campo, & Sañudo, 2013; Gagaoua et al., 2016; Cafferky et al., 2019). It is therefore necessary to incorporate

technologies that facilitate the study of the meat quality in the Colombian Creole sheep, such as molecular tools that allow the analysis of the levels of expression of candidate genes.

Real-time quantitative polymerase chain reaction (RT-qPCR) is a tool that allows the quantification of transcripts, facilitating the detection of changes in their modulation with high specificity, sensitivity and precision (Hildyard, Finch, & Wells, 2019). When RT-qPCR performs a relative quantification of transcripts, it is necessary to normalize the data by the quantification of constitutive genes with constant expression (Huggett, Dheda, Bustin, & Zumla, 2005; Rapacz, 2013). However, the expression of some reference genes, such as β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-2-microglobulin (B2M), can vary depending on conditions such as tissue, breed, sex or environmental stimuli (Sikand, Singh, Ebron, & Shukla, 2012; Sahu et al., 2018), thus generating a source of fluctuation in normalization. Therefore, both the reference genes and the genes of interest must be chosen specifically to the conditions to be evaluated (Rapacz, 2013; Wang, Zhang, Liu, Liu, & Ding, 2017; Hildyard et al., 2019).

The present study investigated the occurrence of stable reference genes in the *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep for analysis using RT-qPCR.

Materials and Methods

Experimental animals and sampling

The procedures were endorsed by the bioethics committee of the "Universidad del Tolima" (Approval number 02, 2017). In total, 12 clinically healthy Colombian Creole sheep with a body weight of 26 ± 4 kg and 12 ± 3 months of age were used; they were separated by gender into two groups of six animals each. The animals were kept under grazing conditions on a pasture of *Cynodon plectostachyus* and legumes, such as *Guazuma ulmifolia* and *Gliricidia sepium,* on the farm "El Recreo" Guamo, Tolima-Colombia, at an elevation of 321 meters above sea level, with an average temperature of 28°C, a relative humidity of 74.9% and a total rainfall of 1,000 to 1,400 mm, classified according to Holdridge (1987) as tropical dry forest. *Longissimus thoracis et lumborum* muscle samples were taken by excisional biopsy following the procedures described by Bradley (1978), under dissociative anesthesia using xylazine (0.2 mg kg^{-1} intramuscular), ketamine (2 mg kg^{-1} intravenous) and lidocaine (5-10 ml) (Jassim, 2014; Moolchand, Kachiwal, Soomro, & Bhutto, 2018). The muscle samples were preserved in vials with RNAlater™ (Thermo Fisher Scientific, USA) and stored at -20°C until use.

RNA extraction and cDNA synthesis

Total RNA was extracted from muscle tissue samples using the TRizol Reagent® protocol (Thermo Fisher Scientific, USA) and adding chloroform. The RNA concentration and purity were determined spectrophotometrically using NanoDrop™ One (Thermo Fisher Scientific, USA), selecting values of 1.8-2.1 in the A260/A280 ratio and 2.0-2-2 in the A260/A230 ratio. The cDNA was synthesized by reverse transcription using the EasyScript™ cDNA Synthesis kit (abm, Canada) with 2 µg of RNA as template and 1 µl of oligo dT primers. The cDNA was diluted five times,

and its quality was checked by amplifying the reference genes using end-point PCR and specific primers (Table 1), applying the Tucan taq kit (Corpogen, Colombia). The PCR reaction consisted of an initial pre-denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, an annealing step at 55°C for 30 seconds, an extension step at 72°C for 30 seconds and a final extension at 72°C for 7 minutes. A T100™ Thermal Cycler (Bio-Rad, USA) was used, and the amplification products were resolved in a 2% agarose gel using Hydragreen™ (ACTGene, USA) as DNA dye and the ENDURO™ GDS (Labnet International, USA).

Table 1
Primer sequences and amplification sizes of the seven candidate reference genes used for RT-qPCR in the *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep

Gene	Gene name	Accession number	Sequence	Amplicon size (bp)
ACTB	β-actin	NM_001009784.2	F- ACTGGGACGACATGGAGAAG R- GGGGTGTTGAAGGTCTCAAA	157
YWHAZ	3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_001267887.1	F- GCTGGTGATGACAAGAAAGG R- AGTTAAGGGCCAGACCCAGT	121
SDHA	Succinate dehydrogenase complex, subunit A	XM_012125144.1	F- ATGCTGGGGAAGAATCTGTC R- ATGGCTCTGCATCGACTTCT	107
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001190390.1	F- GGGTCATCATCTCTGCACCT R- GGTCATAAGTCCCTCCACGA	176
PGK1	Phosphoglycerate kinase I	NM_001142516.1	F- ACTCCTTGCAGCCAGTTGCT R- AGCACAAGCCTTCTCCACTTCT	101
TUBB2A	Tubulin β-2-A	XM_027958682.1	F- GAACACATTCAGCGTCATGC R- CAGGGTACGGAAGCAGATGT	154
B2M	β-2-microglobulin	XM_027971438.1	F- CCAGAAGATGGAAAGCCAAA R- AGCGTGGGACAGAAGGTAGA	159

Quantification of the expression of reference gene transcripts by RT-qPCR.

The genes β-actin (*ACTB*), tyrosine monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), flavoprotein A subunit succinate dehydrogenase complex (*SDHA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), phosphoglycerate 1 (*PGK1*), tubulin β-2-A (*TUBB2A*) and β-2-microglobulin (*B2M*) were selected based on reports of reference

genes in sheep (Peletto et al., 2011; Zang et al., 2011), beef cattle (Heinz, Mberema, & Sparagano, 2017), and swine (Niu, Yang, Zhang, & Hua, 2016). The primers (Table 1) were designed using the Primer3plus software according to Bustin et al. (2009). We used 1 µl of the cDNA synthesized from muscle as a template for each RT-qPCR reaction, with the specific primers of each gene in duplicate. The qPCR reaction was carried out using the Luna® Universal qPCR Master Mix kit (New England BioLabs, USA) in Fast ramp mode, and the PCR was performed using a StepOne™ Real-time PCR thermal cycler (Applied Biosystems, USA).

Stability of gene expression

An analysis of variance was carried out to observe differences in the values of the cycle threshold (Ct) as well as the coefficient of variation and the differences between means. The expression of the reference genes and their stability were determined using the computational algorithms geNorm version 3.5 (Vandesompele, Preter, Poppe, Roy, & Paepe, 2002), NormFinder version 0.953 and BestKeeper (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004). The results were integrated to obtain a consensus using the RefFinder software (Xie, Xiao, Chen, Xu, & Zhang, 2012). The Ct values were converted into relative quantities using the Δ-Ct formula $Q = 2^{\Delta\text{-}Ct}$, where Δ-Ct is the difference between the sample with the lowest Ct and the Ct value of the sample in question (Livak et al., 2001). The software geNorm was used to calculate the stability of gene expression (M value), which is based on paired variations of an individual reference gene relative to all other tested control genes. Genes with an M value below the threshold of 1.5 have stable expression.

NormFinder, on the other hand, uses an ANOVA-based model to identify the stability of the reference genes, calculating intragroup and intergroup variations. Genes with lower values indicate greater stability. The calculations made by the software BestKeeper are based on the standard deviation (SD) and the coefficient of variation (CV) determined by the Ct values of each reference gene. Genes with an SD value < 1.0 are considered stable, and the gene with the lowest SD and CV values was determined as the best reference gene, this software also allows to establish a pair-wise correlation coefficient, being more stable than those with a higher correlation.

Results and Discussion

Identifying reliable reference genes to normalize gene expression in the *Longissimus thoracis et lumborum* muscle of male and female Colombian Creole sheep would represent a valuable tool. This is because various treatments, such as administering diets or supplements, may allow the genetic potential of this breed to be exploited. Normalization is essential to obtain reliable relative quantification of gene expression by RT-qPCR, which demands a systematic validation of reference genes for different experimental conditions (Nolan, Hands, & Bustin, 2006).

In Figure 1, the Ct values discriminated by gender are shown: Males (A), Females (B) and the total Overall values (C). The *GAPDH* gene presented the lowest Ct value, similar to the values reported in the *Longissimus thoracis et lumborum* muscle of Lori-Bakhtiari Lambs by Aziziyan, Sadeghi, Ganjkhanlou and Bahnamiri. (2020) and in Jianyang big-eared goats in the same muscle (Zhu, Lin, Liao, &

Wang, 2015). The gene *TUBB2A* showed the highest Ct values according to Clark et al. (2017), and in the BioProject (PRJEB6169), it showed the lowest number of *TUBB2A* transcripts per million in the muscle of sheep.

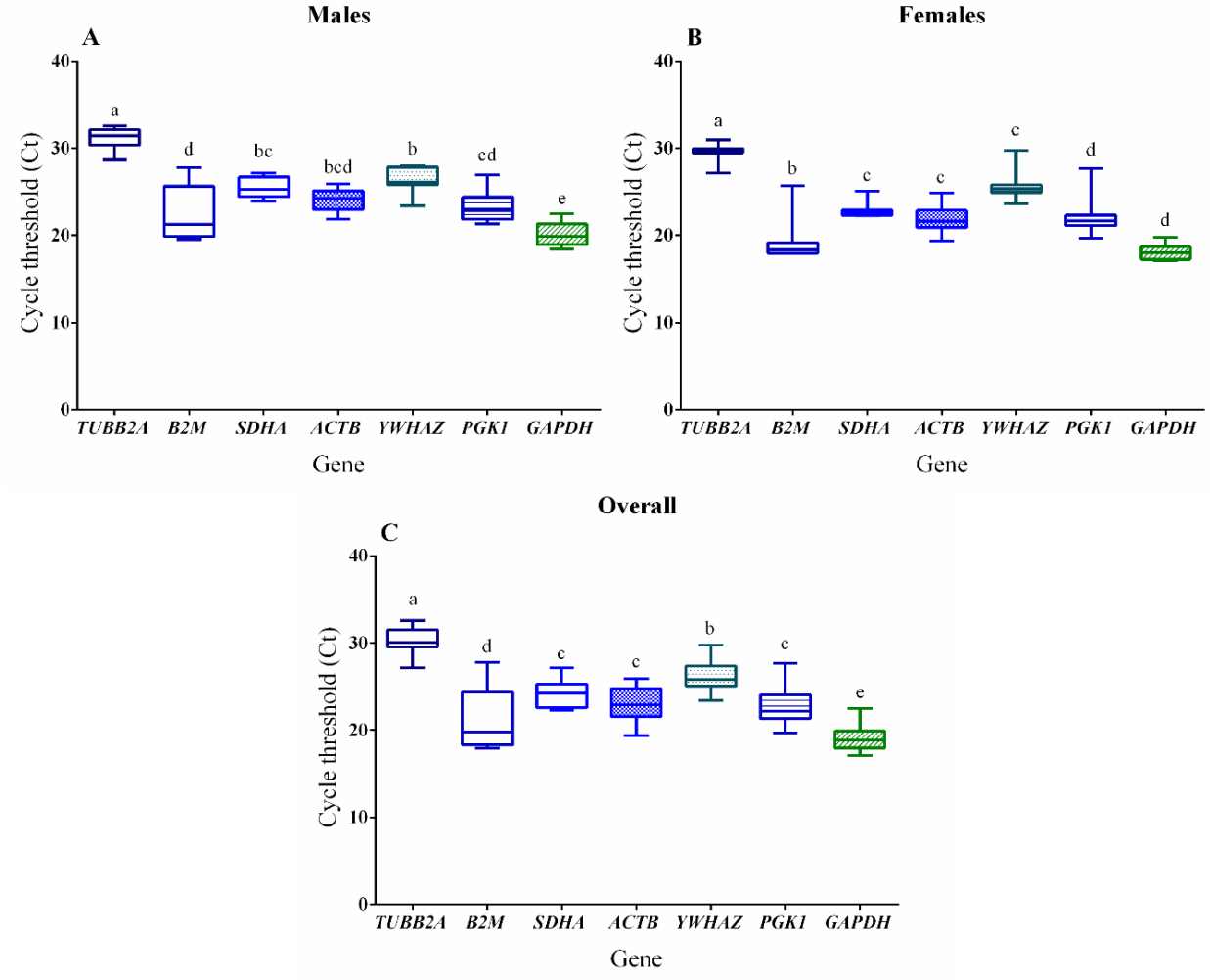

Figure 1. Ct values for seven reference genes in the *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep by gender. A. Male sheep, B. Female sheep, C. Overall. Box and whiskers plot; the mean is marked by a line inside the boxes.

Using the M index performed by the geNorm software (Figure 2), we observed that the *GAPDH* gene showed the lowest M value in Males and the lowest M value in the Overall group, being the most stable gene according to this software. In addition, the Normfinder software showed the *GAPDH* as the more stable gene in Males, whit a value of 0.519, and in the Overall group, with a value of 0.772 (Table 2 and Figure 3). Likewise, the *GAPDH* gene showed the highest correlation coefficient in the Males group (0.887), using the BestKeeper software (Table 3A). The *GAPDH* has been considered as a reliable reference gene and

has frequently been used for normalization in the biceps brachii muscle of small-tailed Han sheep and Dorper sheep (C. Zhang et al., 2014). In addition, this gene plays a role in the muscle growth and in horn cancer in zebu bulls (Tripathi et al., 2012), showing a stable expression. In the present study, although the diet and environmental conditions were the same for all Colombian Creole sheep used in the experiment, the gender of the animals was different, which could have maintained a constant expression of the *GAPDH* gene.

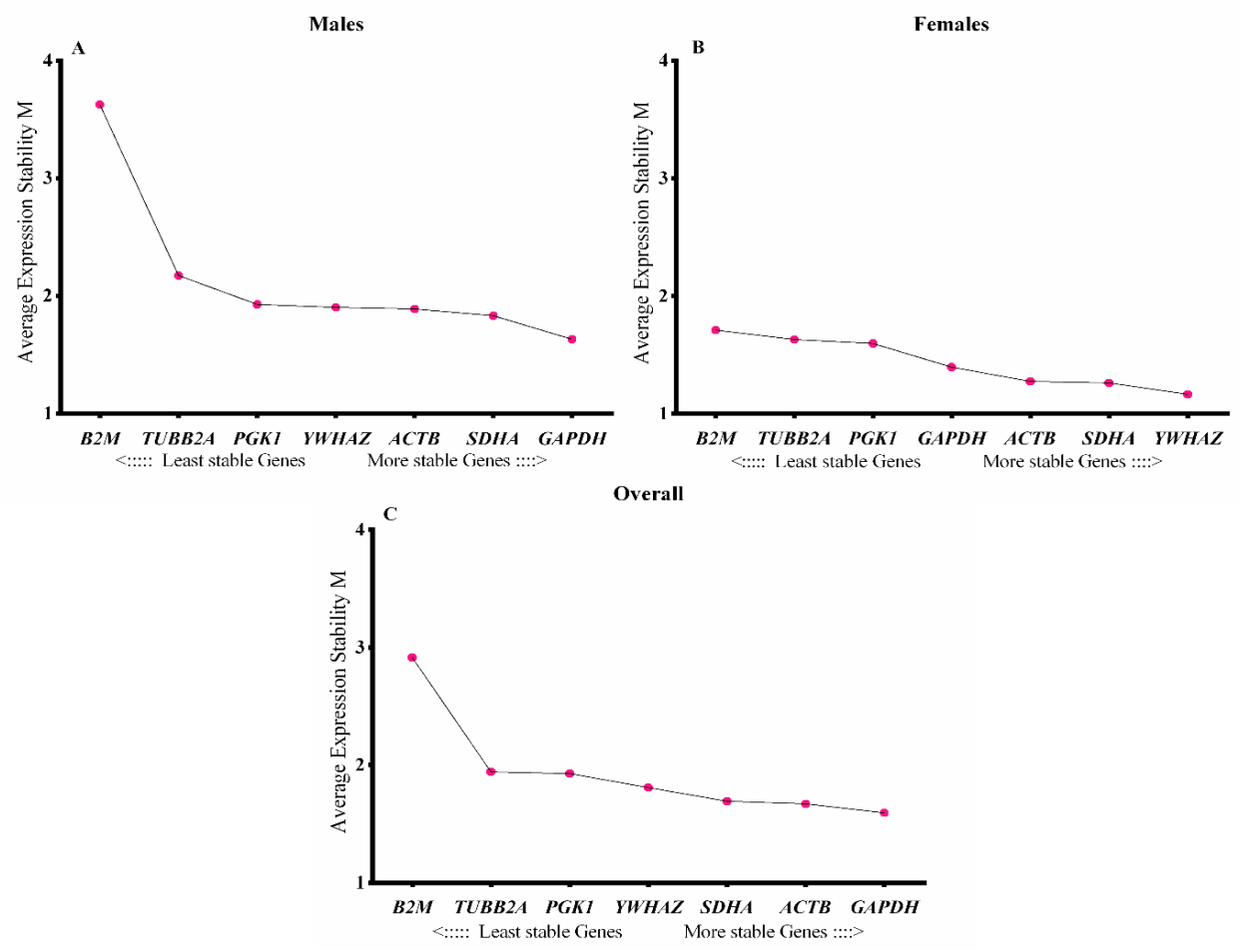

Figure 2. M stability index values of the expression of seven reference genes in the *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep by gender, using the geNorm software. The lower the M values, the greater the stability.

Table 2
Stability values of seven reference genes in *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep by gender, using the NormFinder software. The lower the values, the greater the stability

Gene	Group		
	Male	Female	Overall
ACTB	1.089	0.686	0.894
YWHAZ	1.202	0.242	1.202
SDHA	1.045	0.733	0.967
GAPDH	0.519	1.013	0.772
PGK1	1.384	1.328	1.404
TUBB2A	1.355	1.406	1.341
B2M	3.475	1.519	2.697

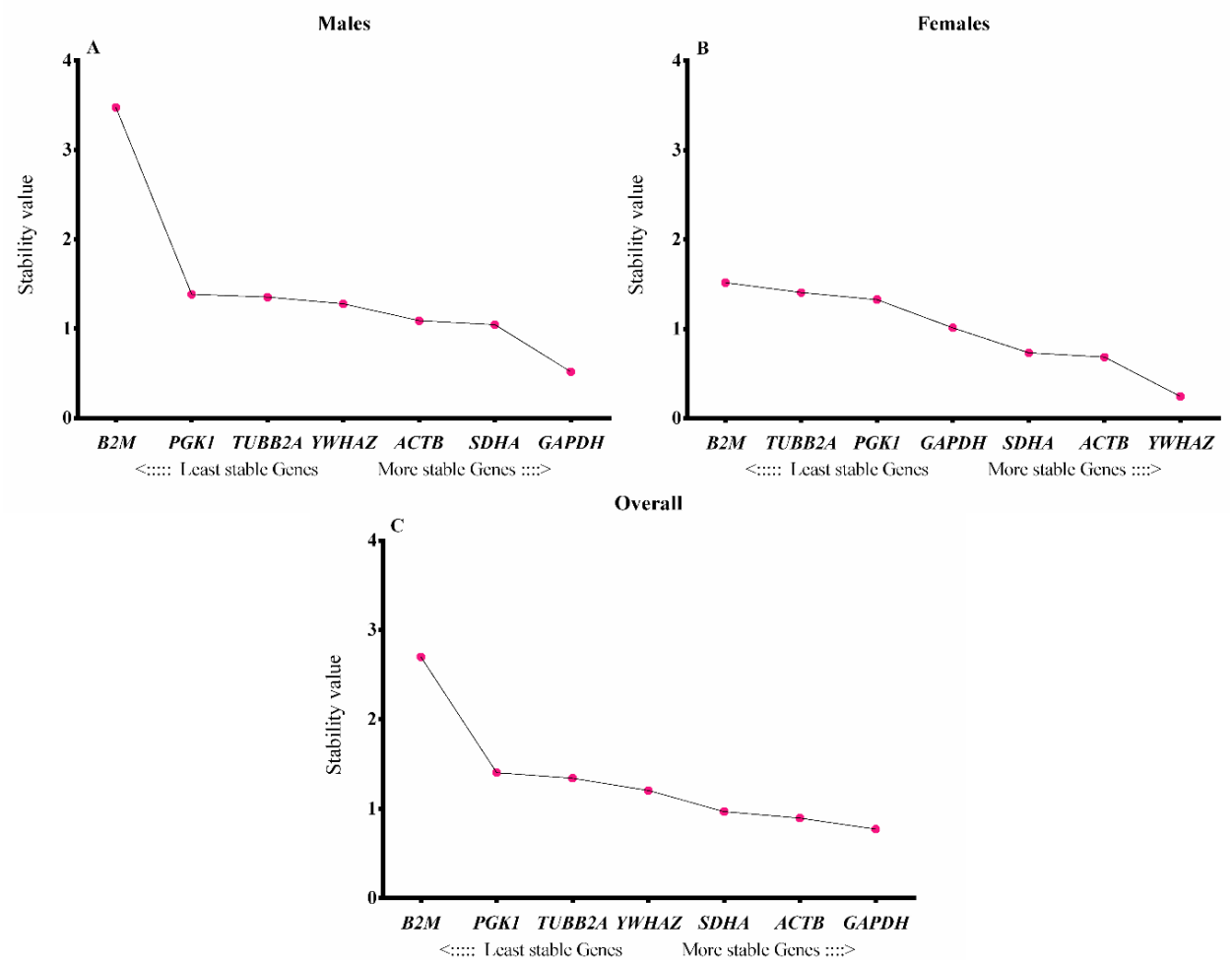

Figure 3. Stability values of the expression of seven reference genes in *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep by gender, using the Normfinder software. The lower the stability values, the greater the stability.

The NormFinder software also showed the *B2M* gene as the least stable one, with M values of 3.475 in Males, 1.519 in Females and 2.697 in the Overall group. The *B2M* gene encodes a component of class I major histocompatibility complex molecules (Williams, Barber, & Flavell, 1989), and was used as a reference gene to normalize the expression of genes associated with lipogenesis in the *Longissimus thoracis et lumborum* muscle of bovines, being a good candidate due to its stability and significance in lipid metabolism (Hiller, Herdmann, & Nuernberg, 2011). The *B2M* gene has also shown stable bone expression values in sheep osteoporosis processes (Schulze et al., 2017), in an Ischemic skin tissue (Ruedrich, Henzel, Hausman, & Bogie, 2013), and in adipose tissue of rats (An et al., 2012). In addition, it has been used as a candidate gene to predict neuronal injury and its progression in models of murine amyotrophic lateral sclerosis (Staats et al., 2013) and is one of the least stable genes in both equine adipose tissue (Nazari, Parham, & Maleki, 2015), and in canine muscular dystrophy (Hildyard et al., 2019). In the same way, in the *pectoralis mayor* muscle of chicken (*Gallus gallus*), the *B2M* gene has been ranked as the least stable one in different development periods (Nascimento et al., 2015). Furthermore, the concentration of *B2M* can change and is useful when distinguishing risk levels in patients with acute heart failure (Kawai et al., 2010) and as a renal function marker since it filters in the glomeruli and is absorbed by the tubular proximal cells, where it is metabolized (Argyropoulos et al., 2017). Therefore, its use as a reference gene may vary with different metabolic conditions.

The *YWHAZ* gene was the most stable one for Females, according to the geNorm software (Figure 2) and the Normfinder software, with a value of 0.242 (Table 2 and Figure 3). In a previous study, the *YWHAZ* gene has shown an increasing expression correlated whit the live weight in the loin muscle of Merino sheep (Kulichenko, Kovalev, Pisarenko, & Volynkina, 2016) and was less stable than *GAPDH, PGK1,* and *B2M* in rodent skeletal muscle (Gong et al., 2016). In addition, testosterone modulates gene expression pathways in semitendinosus and splenius muscles in Dorset rams (Mateescu & Thonney, 2002) and in the gastrocnemius muscle in mouse (Clark et al., 2017) and can affect the expression of *YWHAZ* in the prostate of Noble rats. Therefore, in our study, the stability of this gene in female sheep can be associated with the lower concentration of testosterone present in Colombian Creole female sheep. In previous studies, *YWHAZ* has been shown to be a stable transcript during heat stress in the whole blood of sheep (Peletto et al., 2011) and in neutrophils both from healthy sheep and from sheep with foot diseases (Vorachek, Bobe, & Hall, 2013a). Wu et al. (2020) have reported that the stability of Y*WHAZ* transcript in different muscles of yaks (*Bos grunniens*) was higher than that of *ACTB, SDHA, B2M,* and *GAPDH.*

Based on the SD obtained in the BestKeeper software, the gene *SDHA* was the most stable one in each gender group. It codes for a succinate dehydrogenase enzyme which is part of a protein complex that plays a role in the Krebs cycle and the electron transport chain (Hederstedt & Rutberg, 1981), and its transcript has been shown to be stable in sheep and bovine neutrophils (Vorachek et al., 2013a; Vorachek, Hugejiletu, Bobe, & Hall, 2013b).

According to the correlation coefficient shown by the BestKeeper software, the most stable gene for the Overall group was *ACTB,* with 0.868 (Table 3C). Similarly, the Normfinder software presented the *ACTB* gene as the most stable gene in the Overall group, with a value of 0.894 (Table 2 and Figure 3).

Nevertheless, this gene has been reported as one of the least reliable reference genes in the different tissues of Lanzhou fat-tailed sheep (Zang et al., 2011), in the development of the *Lonsgissimus dorsi* muscle of Landrace pigs (Niu et al., 2016), in muscle engineered from rat groin (An et al., 2012), and in healthy dogs and dogs with muscular dystrophy (Hildyard et al., 2019). It should be noted that the *ACTB* gene codes for beta actin, which is part of cytoskeletal actins (Garrels & Gibsont, 1976) involved in cell motility, structure, and integrity. Therefore, any modification in the structure or cell integrity and changes related to the growth of animals can generate differential expressions of *ACTB* and affect the results for different developmental stages in the muscles of these animals.

Table 3
Repeated pair-wise correlation analysis and BestKeeper consensus of seven reference genes in *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep by gender. The closer the value to 1, the higher the correlation

Males							
A	*ACTB*	*YWHAZ*	*SDHA*	*GAPDH*	*PGK1*	*TUBB2*	*B2M*
YWHAZ	0.234	-	-	-	-	-	-
SDHA	0.328	0.768	-	-	-	-	-
GAPDH	0.749	0.706	0.567	-	-	-	-
PGK1	0.445	0.822	0.668	0.863	-	-	-
TUBB2	0.345	-0.507	-0.484	0.087	-0.219	-	-
B2M	-0.277	-0.305	-0.457	-0.172	-0.363	0.582	-
BestKeeper	0.582	0.615	0.461	0.887	0.717	0.308	0.258
Females							
B	*ACTB*	*YWHAZ*	*SDHA*	*GAPDH*	*PGK1*	*TUBB2*	*B2M*
YWHAZ	0.919	-	-	-	-	-	-
SDHA	0.801	0.897	-	-	-	-	-
GAPDH	0.754	0.746	0.842	-	-	-	-
PGK1	0.767	0.899	0.885	0.858	-	-	-
TUBB2	0.797	0.666	0.432	0.249	0.377	-	-
B2M	0.789	0.944	0.935	0.784	0.967	0.455	-
BestKeeper	0.906	0.978	0.936	0.846	0.956	0.585	0.970
Overall							
C	*ACTB*	*YWHAZ*	*SDHA*	*GAPDH*	*PGK1*	*TUBB2*	*B2M*
YWHAZ	0.601	-	-	-	-	-	-
SDHA	0.739	0.617	-	-	-	-	-
GAPDH	0.818	0.603	0.825	-	-	-	-
PGK1	0.648	0.868	0.642	0.732	-	-	-
TUBB2	0.714	0.151	0.394	0.466	0.216	-	-
B2M	0.473	0.362	0.434	0.419	0.429	0.645	-
BestKeeper	0.868	0.753	0.818	0.855	0.825	0.637	0.744

Microtubules are involved in cellular functions such as neurodevelopment or chromatid separation in mitosis (Breuss & Keays, 2014). Gene *TUBB2A* encodes the β tubulin 2 alpha protein, which is an essential component of microtubules that is continuously incorporated and released (Gierke, Kumar, & Wittmann, 2010). Diseases such as diabetes or muscle hypertrophy can modulate the concentration of tubulins, including α and γ tubulin and the skeletal muscle of rodents (Aurélio et al., 2016). In this study, *TUBB2A* transcript presented the lowest correlation coefficient in the Females group (0.585) (Table 3B) and in the Overall group (0.637) (Table 3C) despite having the lowest standard deviation of the Overall group (Figure 4). These findings may be associated with the estrous cycle of female sheep, since they could be found in a different estradiol peak, and it has been proven that the dimers of estradiol inhibit the polymerization of tubulin and microtubule dynamics, affecting the regulation of the *TUBB2A* gene dependent on estradiol concentrations in females (Michal et al., 2018). On the other hand, Liu & Victor (2003) reported that estradiol and testosterone have opposite effects on microtubule polymerization, which could be associated with the low correlation coefficient in the Overall group.

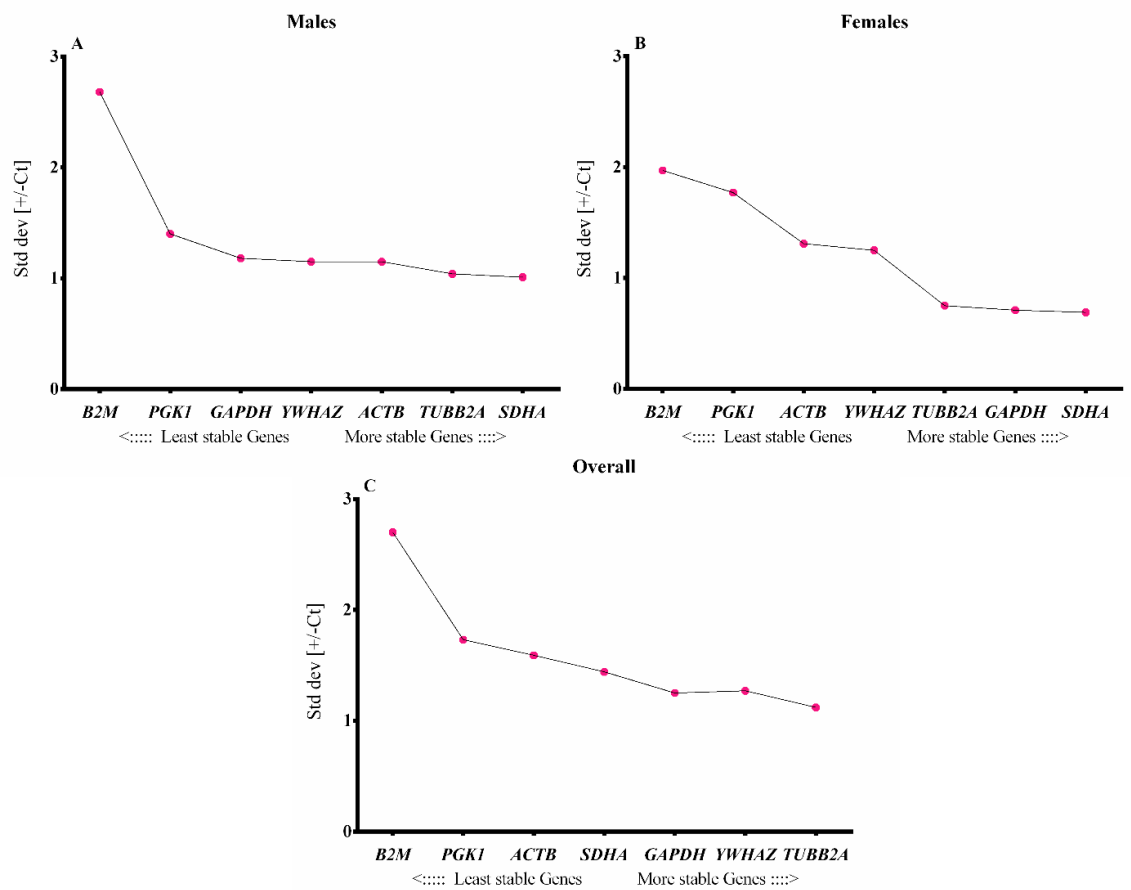

Figure 4. Standard deviation (SD) values of seven reference genes in *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep by gender, using the BestKeeper software. A lower SD indicates a more stable expression.

In this study, the stability order calculated by RefFinder was as follows: *GAPDH > SDHA > ACTB > TUBB2A > YWHAZ > PGK1 > B2M* (Figure 5). Similar trends have been reported by Zang et al. (2011) in fat-tailed sheep in liver and smooth muscle samples using the geNorm software (*GAPDH > TUBB > SDHA > YWHAZ > PGK1 > ACTB*). In addition, Peletto et al. (2011) observed that the stability order in Biellese sheep in whole blood subjected to heat stress was *YWHAZ > GAPDH > SDHA > ACTB > B2M.*

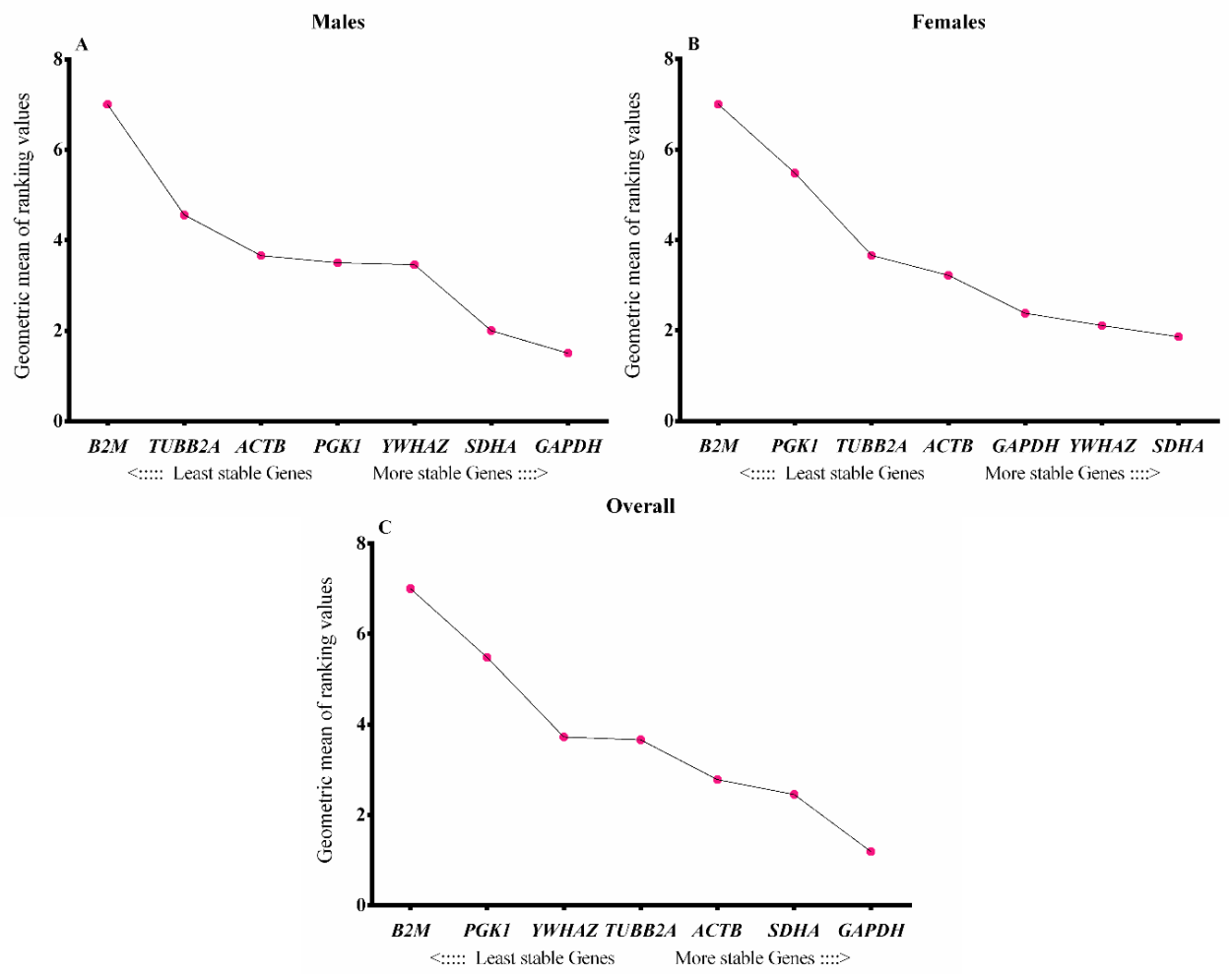

Figure 5. General consensus values of seven reference genes in *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep by gender using the RefFinder software. The ranking is based on the geometric mean of the ranges obtained in the three programs. A lower value indicates a more stable expression.

The *GAPDH* enzyme is involved in various metabolic processes such as stress response, cell dysfunction, cytotoxicity, or cell proliferation, making it essential in maintaining homeostasis and regulating cell death, with a constant gene expression (Tristan, Shahani, Sedlak, & Sawa, 2011). Similarly, it may be associated with the maintenance of meat color stability (Xin et al., 2018). In the present study, the expression of the *GAPDH* gene transcript was the most stable one according to geNorm, Normfinder, and Bestkeeper. However, these results were in contrast with those reported for rodent muscles with muscular dystrophy (Hildyard et al., 2019), Biellese sheep in whole blood at basal conditions (Peletto et al., 2011), Landrace pigs at different stages of embryonic development in *Longissimus thoracis et lumborum* muscle (Wang et al., 2017), and grouper fish (*Epinephelus akaara*) during different stages of gonadal development (Niu et al., 2016), where the *GAPDH* transcript was the least stable one in the three algorithms used.

Overexpression of the *GAPDH* gene has been observed in tumor cells obtained from human prostate cancer (Zhong & Simons, 1999; Mori, Wang; Danenberg, Pinski, & Danenberg, 2008), and human cervical cancer (Kim et al., 1998), which may be related to alterations in cell cycle regulation as well as an increase in glycolysis (Persons, Schek, Hall, & Finn, 1989; Tristan et al., 2011). J. Y. Zhang et al. (2015) have reported that GAPDH is implicated in diverse functions regardless of its role in energy metabolism. According to Nazari et al. (2015), the increased proliferative capacity of some cells in equines, such as adipose tissue mesenchymal stem cells, may have induced a low stability of its expression.

Gene *PGK1* encodes the glycolytic enzyme phosphoglycerate kinase 1, which is found in different tissues and catalyzes the reversible conversion of 1, 3 diphosphoglycerate to form an ATP molecule (Zerrad et al., 2011). In our study, the *PGK1* transcript was one of the least stable ones, together with *B2M,* in ovine *Longissimus thoracis et lumborum* muscle. This trend has also been reported by Peletto et al. (2011) in whole blood of sheep and in the muscle of rodents under heat stress, where PGK1 and B2M were less stable than *GAPDH, YWHAZ,* or *B2M* (Gong et al., 2016). However, in the healthy and fractured mandibular condyle of sheep, *PGK1* gene has been a reliable reference gene, along with *ACTB* (Jiang, Xue, Zhou, Li, & Zhang, 2015).

Conclusions

In the *Longissimus thoracis et lumborum* muscle of Colombian Creole sheep, the reference gene with better stability for grazing conditions was *GAPDH,* which can therefore be used for relative RT-qPCR quantifications in this breed. If both genders are used in the study groups, it is possible to use combinations of genes including *SDHA, ACTB,* and *GAPDH* to obtain more reliable normalizations. Our results suggest that YWHAZ transcript is a reliable reference gene in the *Longissimus thoracis et lumborum* muscle of female Colombian-Creole sheep.

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Bioethics and biosecurity committee approval

This study was approved by the Animal Use Ethics Committee of the University of Tolima, under license number 02/2017.

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