SELECTION OF NORMALIZERS FOR DIFFERENTIAL MIRNAS EXPRESSION IN DARK-CUTTING BEEF SAMPLES

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I. INTRODUCTION

MiRNAs are small (19-25 nucleotides) noncoding single-stranded RNA molecules that regulate gene expression in eukaryotic organisms at the post-transcriptional level as part of many physiological processes [1]. Several factors can alter their expression in the animal muscle cells, including different pre-slaughter stressors associated with quality defects such as dark, firm, and dry (DFD) beef, also known as dark-cutting beef [2]. The use of miRNAs as indirect indicators of abnormal muscle-to-meat conversion processes and meat quality is therefore of great interest. The quantification of miRNAs requires highly sensitive and specific techniques such as real-time quantitative PCR (RT-qPCR) and its normalization with muscle miRNAs, whose expression can be considered constant under the conditions of the study, to reduce experimental variations and improve the accuracy. Here, we propose a set of miRNAs candidates for RT-qPCR normalization in normal and DFD beef.

II. MATERIALS AND METHODS

Ten carcasses from Asturiana de los Valles yearling bulls of similar characteristics (farm, transport, weight) were selected, being 5 classified as DFD (pH_{24}≥6.2) and the other 5 as Control (normal pH_{24} from 5.4 to 5.6). Muscle samples (10 g) were collected from the *Longissimus thoracis et lumborum* at 24h *post-mortem*. Total RNA was isolated from 20-29 mg of muscle tissue using the miRNeasy Mini Kit (QIAGEN), followed by deep sequencing. Candidates for reference miRNAs that could be used as normalizers for RT-qPCR were pre-selected using the NormFinder algorithm, which calculates a stability value that combines intra and intergroup variation [3]. Afterwards, RT-qPCR was used to validate the candidates using an additional thirteen pairs of DFD and Control samples. A T-Student test of independent samples was performed to examine differences between miRNA levels in Control and DFD beef. The significant level was set at $P<0.05$. GeNorm was further used to evaluate the candidates after RT-qPCR [4].

III. RESULTS AND DISCUSSION

From the sequencing results, eight reference candidates that had high stability values given by the Normfinder algorithm were selected and tested by RT-qPCR in Control and DFD samples, finding no significant differences between groups (Table 1).

Table 1. Ranking of the most stable miRNAs according to the Normfinder algorithm.

miRNA	**C_t (control)** (mean ± SEM)	**C_t (DFD)** (mean ± SEM)	**p-value**
mmu-let-7d	28.55 ± 0.7	28.96 ± 0.8	0.313
hsa-miR-125b	24.79 ± 0.7	25.03 ± 0.9	0.580
bta-miR-425-5p	29.93 ± 0.8	30.25 ± 0.9	0.486
bta-miR-660	27.87 ± 0.8	28.22 ± 0.8	0.430
hsa-miR-148b	28.93 ± 0.6	29.02 ± 0.8	0.814
hsa-miR-151-3p	28.55 ± 0.7	28.96 ± 0.8	0.640
hsa-miR-10b	24.67 ± 0.7	25.04 ± 0.9	0.417
bta-miR-342	26.41 ± 0.6	26.76 ± 0.8	0.348

GeNorm algorithm was used to determine the candidates' potential contributions as normalizers. It generates a stability value M using the average pairwise variation between all tested genes, accompanied by stepwise exclusion to rank the candidates according to their expression stability. The lower the value of M, the most stable the expression of gene (Figure 1A). In this study, miR-let7d-5p, miR-125b and miR-10b showed the lowest M value, thus the most stable expression.

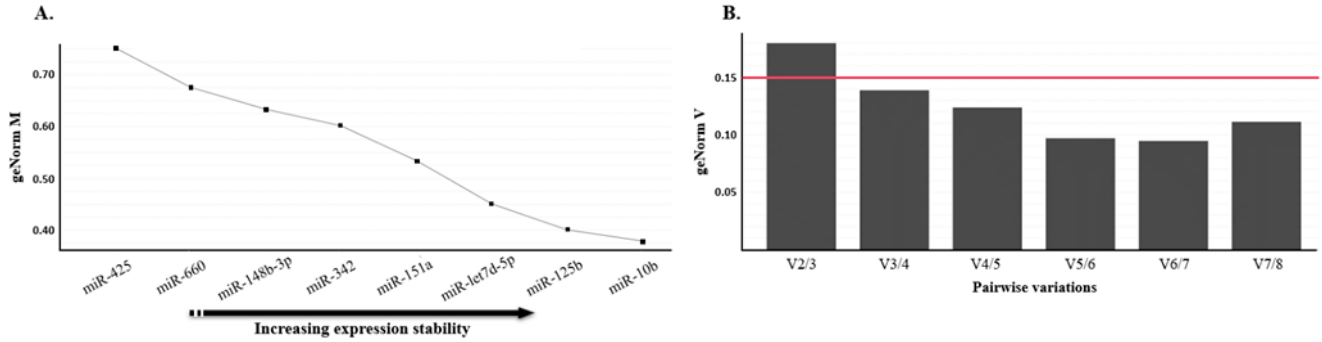

Figure 1. GeNorm analysis of RT-qPCR-based candidate reference genes. (A) GeNorm average expression stability value (M) for the eight most stable miRNAs according to NormFinder. (B) GeNorm normalization factor (V) (determined by pairwise variation of (n/n+1) being 'n' the minimum number of genes) for the optimal number of reference genes.

In addition, geNorm also calculates a normalization factor value (V), which determines the optical number of reference genes required for reliable normalization. A threshold of 0.15 V value was established for appropiate normalization and once this threshold has been reached, there is no need to include any additional reference genes [4]. Figure 1B shows that V3/4 was less than 0.15, indicating that the combination of these three miRNAs achieved the best reference normalization factor. MiR-10b, miR-125b and miR-let7d-5p are important myomiRNAs known to target several genes influencing skeletal muscle development and differentiation [5].

IV. CONCLUSION

The combination of three miRNAs (miR-let7d-5p, miR-125b and miR-10b) as normalizers of normal (Control) and dark-cutting (DFD) beef allows an accurate quantification of miRNAs when using RT-qPCR.

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