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13. Optimized RT-qPCR Detection

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Optimized RT-qPCR Detection of Hepatic Lipopolysaccharide-Binding Protein in Diet-Induced Obese Mice

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Abstract

High-throughput RT-qPCR results on hepatic lipopolysaccharide-binding protein (LBP) expression in obese subjects are essential, as they reveal the endotoxin's role in the development of obesity and non-communicable disease (NCD). This study aimed to optimize RT-qPCR detection of LBP in diet-induced obese mice. This study primarily focused on addressing high variability through reference gene normalization. A total of six male C57BL/6 mice aged 6 weeks were randomly allocated into two dietary treatments (n = 3), consisting of mice fed with the standard chow diet (SCD group) and mice fed with the high-fat and high sucrose diet (HFHS group) ad libitum for 8 weeks. Relative quantification strategies involving the standard $2^{-\Delta\Delta Ct}$ method (calibrator as mean) and the modified $2^{-\Delta\Delta Ct}$ method (calibrator as individual sample-matched biological replicates) were compared in terms of their variability. Obesity was successfully induced in the HFHS treatment group, as indicated by significantly higher body weight, calorie intake, and LBP relative expressions compared to the SCD group. In addition, a sample-specific calibrator approach using the modified $2^{-\Delta\Delta Ct}$ method resulted in lower variability in relative gene expression levels. A modified $2^{-\Delta\Delta Ct}$ method, which utilizes a sample-specific calibrator to counteract sample-specific variability, was successfully employed to address high variability in RT-qPCR results.

Keywords: lipopolysaccharide-binding protein, obesity, high-fat and high sucrose diet, RT-qPCR

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INTRODUCTION

Rising obesity prevalence has become a major public health concern, as it is one of the primary risk factors for non-communicable diseases, which are the leading cause of death worldwide (World Health Organization, 2023). Obesity is a major risk factor for metabolic dysfunction-associated steatotic liver disease (MASLD), the most frequent metabolic liver disease worldwide, with a global prevalence of 32% (Teng *et al*., 2023). The increasing prevalence of obesity and MASLD is closely associated with the societal dietary shift towards a Western diet characterized by high sugar and fat content, as well as low dietary fiber (McKeown *et al*., 2022).

LBP in obesity-related research is considered a surrogate marker for lipopolysaccharide (LPS), a Gram-negative bacterial product, indicating altered intestinal permeability (Seethaler *et al*., 2021). LBP is primarily expressed in hepatocytes, as it is an acute-phase protein and is regulated by the IL-6/JAK/STAT3 pathway (Ehlting *et al*., 2021; Serban *et al*., 2020). LBP plays a crucial role in the LPS transfer cascade to TLR4, as LBP introduces LPS to TLR4, thereby initiating the

downstream MYD88-dependent NF-κB pathway (Mohammad and Thiemermann, 2021; Page *et al*., 2022). NF-κB pathway activation leads to the release of pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α (Mohammad and Thiemermann, 2021). This particular inflammatory pathway underscores the significance of LBP as a marker of endotoxemia, which contributes to the development of diet-induced obesity.

Understanding the biological mechanisms that drive obesity and its complications is crucial for developing effective prevention strategies. In recent years, transcriptomic approaches have gained prominence in obesity research, offering insights into gene regulation patterns associated with metabolic and inflammatory responses (Tanaka *et al*., 2019). Gene expression analysis using RT-qPCR is considered accurate and specific, as it quantifies mRNA expression and predicts protein synthesis. It has become the gold standard in gene expression studies (Zhu *et al*., 2020).

However, the accuracy and reproducibility of RT-qPCR results depend on several critical technical factors. These include the quality and quantity of the isolated RNA, the design and specificity of primers, amplification optimization, and proper normalization using a stable reference gene (F. Zhao *et al*., 2021). In the context of obesity, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, ribosomal protein large P0 (RPLP0), 18S ribosomal RNA (18S), and TATA box-binding protein (TBP) were widely used as reference genes in obesity-related research (Fan *et al*., 2020). GAPDH has been demonstrated to be a relatively stable reference gene in immunological studies (Giri *et al*., 2022; Kwon *et al*., 2021). The normalization method for relatively quantifying mRNA target gene expression over that of a reference gene is carried out using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

This study aimed to analyze LBP expression using optimized RT-qPCR analysis in high-fat and high-sucrose (HFHS) -induced obese C57BL/6 mice. The gene of interest and reference gene Ct value were compared among different biological replicates (which were also conducted on different days) to determine biological and technical variations. The modified $2^{-\Delta\Delta Ct}$ method was used to normalize LBP expression over GAPDH at various concentrations to address this limitation.

MATERIALS AND METHODS

Ethical Approval

All experimental treatments were conducted according to the guidelines set by the Institutional Animal Care and Use Committee (IACUC) and approved by the Health Research Ethics Committee of Hang Tuah University Surabaya (approval no E/017/UHT.KEPK.03/IV/2024).

Study Period and Location

Animal acclimatization, treatment, termination, and organ extraction were conducted at the Animal Laboratory, Faculty of Pharmacy, Widya Mandala Catholic University, Surabaya. RNA isolation, reverse transcription, polymerase chain reaction, and data acquisition were performed at the Research Center for Vaccine Technology and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya. The study was carried out over three months, from July 1 to September 30, 2024.

Experimental Design

A total of six male C57BL/6 mice (17–25 g) were purchased from the Kemuning Animal Laboratory (Karanganyar, Indonesia) at six weeks of age. The required sample size was estimated using a priori analysis (Mean: difference between two independent means) with G*Power 3.1 Software (Düsseldorf, Germany) and the following parameters: effect size d = 5.00, α = 0.05, and a target power of 0.95 according to a previous study (Latorre *et al*., 2022). The mice were acclimatized for seven days with access to a standard chow diet and water ad libitum, housed in solid plastic cages with a controlled environment (12-hour light-dark cycle, room temperature of 22–25°C, and humidity levels of 50–60%). The summary of the experimental design is shown in Figure 1.

After acclimatization, mice were randomly allocated into two different groups (n = 3): the SCD group, fed a standard chow diet (10% kcal from fat) and water; and the HFHS group, fed a high-fat (45% kcal from fat) pellet and high-sucrose (20% w/v) drinking water. All diets were provided ad libitum for a period of eight weeks. The standard chow diet provides 3.8 kcal/g, with 69.5% of its energy derived from carbohydrates, 21% from protein, and 16.5% from fat, while the high-fat diet contains 5 kcal/g and consists of 39% kcal from carbohydrates, 16% from protein, and 45% from fat. Body weight was recorded weekly, and calorie intake was recorded daily. At the end of treatment, all mice were euthanized with intraperitoneal ketamine-xylazine injections for liver extraction.

Liver Tissue Extraction

Mice were macro-dissected under anesthesia. An incision was made from the thorax to the abdomen, and the rib cage was cut open to reveal the heart. Transcardial perfusion was performed to flush the remaining blood from the liver. This procedure was initiated by inserting a 26G needle into the left ventricle, preceded by an incision in the right atrium for drainage. Once the needle was inserted, a 0.9% NaCl solution was administered to flush the organ for 5–10 minutes or until the liver appeared pale. The liver and its lobes were completely excised from connective tissue and related vascular tissue. Biliary sacs were excised from the liver tissue.

RNA Isolation

Liver samples were snap-frozen in liquid nitrogen and stored at –80°C immediately after extraction. RNA isolation was conducted according to the protocols for RNAsimple Total RNA Kit (Tiangen Biotech, Beijing, China). All procedures were conducted on ice to maintain the freezing chain. The RNA isolate was either directly used for RT-qPCR or cryopreserved for future use. The RNA concentration of each sample was quantified using a Qubit™ 4 Fluorometer (Invitrogen™, USA) by analyzing 1 µL of each sample.

RT-qPCR

Complementary DNA (cDNA) was synthesized by reverse transcription of RNA using the FastKing gDNA Dispelling RT SuperMix (Tiangen Biotech, Beijing, China) with the MiniAmp™ Thermal Cycler (Applied Biosystems™, USA). Since this study utilized two-step RT-qPCR, cDNA could be cryopreserved for future use. Quantitative PCR was conducted using 2X Forget-Me-Not™ EvaGreen® qPCR Master Mix (Biotium, Singapore). All reaction mixes were prepared according to the manufacturer's standard protocol. Amplification using the QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, USA) was conducted with the following run parameters: denaturation at 95°C for 5 seconds; annealing at 60°C for 10 seconds; extension at 72°C for 10 seconds as recommended by the primer manufacturer.

Gene Relative Expression Analysis

Relative methods were used in this study, employing relative quantification normalized to a reference gene ($2^{-\Delta\Delta Ct}$), assuming an ideal PCR efficiency (100%). The standard $2^{-\Delta\Delta Ct}$ method was normalized to the overall mean value of the calibrator. However, the modified $2^{-\Delta\Delta Ct}$ was normalized to individual mean values of the calibrator, based on their respective concentrations. In this instance, the SCD group was considered the calibrator, and the HFHS group was considered the treatment. This modified version of the $2^{-\Delta\Delta Ct}$ method is a sample-specific variation of the Livak method (Livak and Schmittgen, 2001). This modification was adopted to overcome inter-run variations in RNA concentration and amplification efficiency, which can be particularly relevant in small animal models with high biological variability. Furthermore, considering the small sample size (n = 3 per group), this method enhances within-group resolution while maintaining analytical robustness.

Primer Design and Validation

The primers used in this study included LBP (NM_008489.2) as the gene of interest and

GAPDH (NM_001289726.2) as the reference gene. All primers were obtained from Macrogen (Singapore) and validated using OligoEvaluator™ (Sigma-Aldrich®) to confirm any secondary structures and primer dimers. All primers were negative for secondary structures and primer dimers. Primer sequence, Tm, and GC content are listed in Table 1. Melt temperatures (Tm) of PCR products were recorded for each biological group to detect any variance in thermal blocks and to further ensure amplification consistency, as shown in Table 2. The housekeeping gene GAPDH showed highly consistent Tm values across biological groups (SCD: 83.28 ± 0.18 °C; HFHS: 83.46 ± 0.21 °C), indicating negligible inter-run temperature variance. Similarly, LBP Tm values remained constant (SCD: 84.86 ± 0.05 °C; HFHS: 84.99 ± 0.05 °C), validating the specificity and repeatability of the amplification. The use of consistent melt temperature further supports the reliable detection of hepatic LBP, which is a clinically relevant biomarker in veterinary diagnostics for hepatic endotoxemia.

Data Analysis

Data are presented as mean ± standard error of the mean (SEM). Treatment differences were assessed using an independent T-test (with Welch's correction if data were distributed heterogeneously) or the Mann-Whitney test (if data violated the Gaussian distribution). The statistical analysis was conducted using GraphPad Prism (version 9.5.0, San Diego, CA, USA). Data were considered statistically significant if the $p < 0.05$.

RESULTS AND DISCUSSION

HFHS Diets Induce Obesity and Hypercaloric Intake in Mice

Body weights of each group were measured weekly during the 8-week treatment to illustrate body weight progression and at week 8 to determine obesity status, as presented in Figure 2A-B. Mice induced with HFHS diet showed a significantly higher body weight compared to standard-fed mice (43.87 ± 2.31 vs. 33.77 ± 0.28; $p = 0.023$) at the 8th week of treatment. This significant difference in body weight was first observed in the 4th week of treatment ($p = 0.038$). Weekly body weight details are listed in Table 3. In agreement, daily calorie intake was significantly higher in HFHS-fed mice compared to standard-fed mice (19.58 ± 1.70 vs. 10.88 ± 0.88; $p < 0.001$) as shown in Figure 2C.

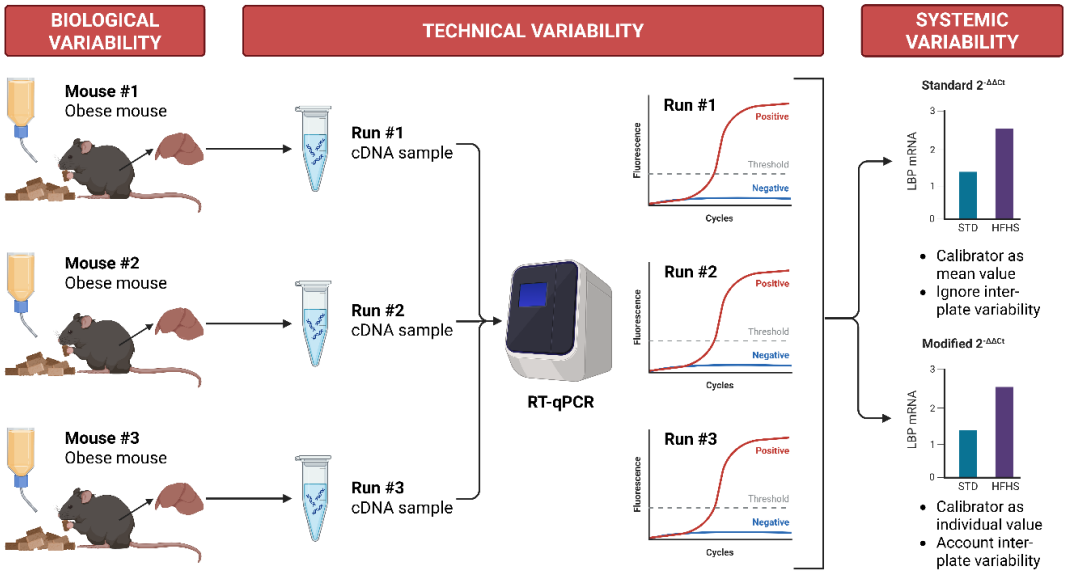

Figure 1. Experimental design to assess optimal LBP expression analysis in obese mice. The biological group consisted of mice fed a standard diet and mineral drinking water (SCD group, not shown) and mice fed an HF (45% calories from fat) and HS (20% sucrose in drinking water) diet (HFHS group) for 8 weeks. Created in BioRender (https://BioRender.com/amdte79).

Table 1. Primer identity

Primer	Sequence	Tm (°C)	GC (%)
GAPDH-F	5’ AGG TCG GTG TGA ACG GAT TTG 3’	62.8	52
GAPDH-R	5’ TGT AGA CCA TGT AGT TGA GGT CA 3’	59.4	43
LBP-F	5' GAT CAC CGA CAA GGG CCT G 3'	64.8	63
LBP-R	5' GGC TAT GAA ACT CGT ACT GCC 3'	59.6	52

Tm, annealing temperature; GC, guanine-cytosine content.

Table 2. Product melt temperature summary

Target gene	Biological group	Tm (°C)
GAPDH	SCD	83.28 ± 0.18
	HFHS	83.46 ± 0.21
LBP	SCD	84.86 ± 0.05
	HFHS	84.99 ± 0.05

Data presented as mean ± SEM. Tm, product melt temperature; SCD, standard chow diet; HFHS, high-fat high-sucrose.

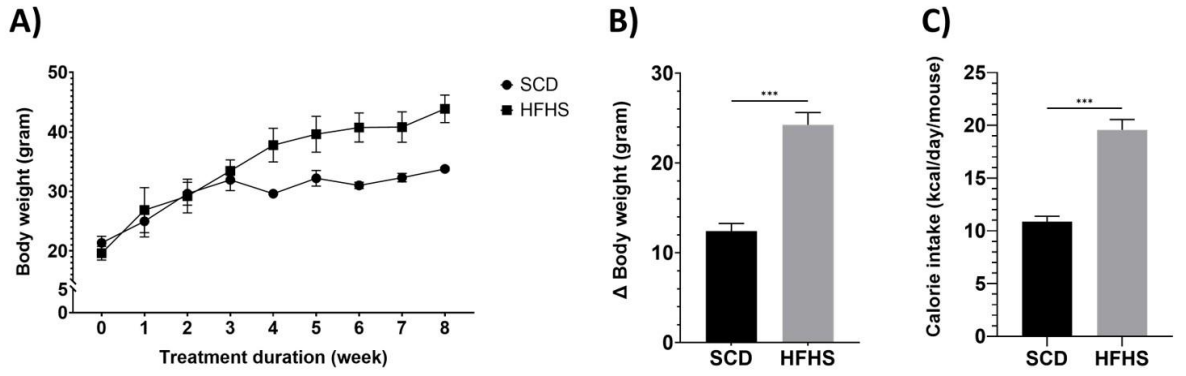

Figure 2. Anthropometric and nutritional status during the experiment. (A) Body weight progression during 8-week treatment. (B) Total body weight gain. (C) Daily calorie intake during the 8-week treatment. Data are presented as mean ± SEM (n = 3). Statistical significance was assessed using an independent t-test (Welch’s correction for Figure B): ***$p < 0.001$. SCD, standard chow diet group; HFHS, high-fat high-sucrose group.

Hepatic LBP Relative Expression

Hepatic LBP relative expression was estimated using the $2^{-\Delta\Delta Ct}$ (standard and modified) methods. Each biological replicate and STD-HFHS pair (n = 3; each pair) was conducted on a different day, thereby increasing the biological and technical variability. To address relative quantification analysis that minimizes variability-related bias, this study compared two relative quantification methods: the calibrator’s mean-based normalization (Figure 3B) and individualized sample calibrator-based normalization (Figure 3C). GAPDH and LBP Ct values were slightly increased in the HFHS group compared to the SCD group ($p = 0.129$; $p = 0.225$, respectively), as shown in Figure 3A and Table 4. However, overall LBP is significantly higher in the HFHS group compared to GAPDH despite the dietary differences (31.31 ± 1.20 vs. 26.71 ± 1.25; $p = 0.012$). The standard $2^{-\Delta\Delta Ct}$ method revealed that the HFHS group had increased LBP relative expression compared to the SCD group, although this difference was not significant (1.27 ± 0.54 vs. 2.17 ± 0.62; $p = 0.167$). However, the modified $2^{-\Delta\Delta Ct}$ method showed a significantly increased LBP relative expression in the HFHS group compared to the SCD group (1.00 ± 0.00 vs. 1.80 ± 0.24; $p = 0.032$). Taken into account, the variability of

LBP relative expression between the two methods was indicated by the standard deviation, standard error of the mean, and coefficient of variability (Table 5). Overall statistical measures of dispersion (SD, SEM, and %CV) revealed lower variability in the modified $2^{-\Delta\Delta Ct}$ method compared to the standard $2^{-\Delta\Delta Ct}$ method in this particular experimental design.

Diet-induced obesity was successfully developed in this study using a high-fat (HF, 45% kcal) and high-sucrose (HS, 20% [w/v] in drinking water) diet through ad libitum feeding for 8 weeks. All HFHS-fed mice exceeded the obesity threshold of 38.84 g (15% above the standard group) (de Moura e Dias *et al*., 2021). This result aligns with previous studies using the same HFHS diet composition for 60 days (Tjahjono *et al*., 2024). Furthermore, calorie intake was significantly higher in HFHS-fed mice compared to standard-fed mice, indicating a higher palatability of the HFHS diet (De Francesco *et al*., 2019).

Table 3. Weekly mice body weight

Biological group	Week								
	0	1	2	3	4	5	6	7	8
SCD	21.33 ± 1.12	24.97 ± 2.61	29.60 ± 1.91	31.93 ± 1.80	29.60 ± 0.10	32.20 ± 1.31	31.00 ± 0.53	32.30 ± 0.70	33.77 ± 0.28
HFHS	19.60 ± 1.15	26.83 ± 3.78	29.20 ± 2.82	33.43 ± 1.85	37.77 ± 2.83	39.60 ± 3.01	40.73 ± 2.44	40.80 ± 2.54	43.87 ± 2.31

Data presented as mean ± SEM. SCD, standard chow diet; HFHS, high-fat high-sucrose.

Table 4. Descriptive statistics comparing GAPDH and LBP Ct value among diet groups

Descriptive statistics	SCD		HFHS	
	GAPDH	LBP	GAPDH	LBP
Mean	25.17	30.28	28.25	32.35
SD	2.71	3.462	3.00	2.54
SEM	1.57	1.999	1.73	1.47
%CV	10.77%	11.44%	10.62%	7.86%

SD, standard deviation; SEM, standard error of mean; %CV, coefficient of variation; SCD, standard chow diet; HFHS, high-fat high-sucrose.

Table 5. Descriptive statistics comparing the two $2^{-\Delta\Delta Ct}$ methods

Descriptive statistics	Standard $2^{-\Delta\Delta Ct}$		Modified $2^{-\Delta\Delta Ct}$	
	SCD	HFHS	SCD	HFHS
Mean	1.27	2.17	1.00	1.80
SD	0.93	1.07	0.00	0.41
SEM	0.54	0.62	0.00	0.24
%CV	73.45%	49.16%	0.00%	22.98%

SD, standard deviation; SEM, standard error of mean; %CV, coefficient of variation; SCD, standard chow diet; HFHS, high-fat high-sucrose.

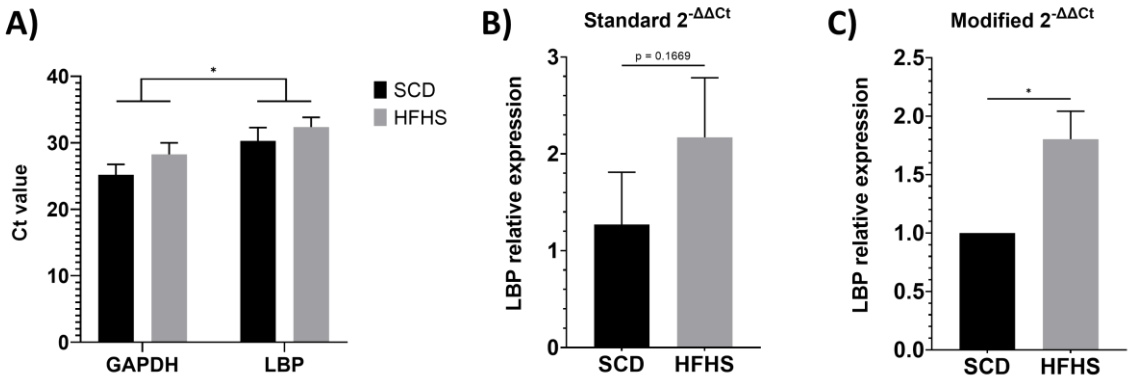

Figure 3. Hepatic LBP relative expression was analyzed using two different $2^{-\Delta\Delta Ct}$ methods. (A) Ct value of GAPDH and LBP among dietary treatment, (B) Standard $2^{-\Delta\Delta Ct}$ method, and (C) Modified $2^{-\Delta\Delta Ct}$ method. Data are presented as mean ± SEM (n = 3). Statistical significance was assessed using an independent t-test (Figures A and B) and a Mann-Whitney test (Figure C): $*p < 0.05$. SCD, standard chow diet group; HFHS, high-fat high-sucrose group.

Obesity is associated with gut dysbiosis and increased gut permeability, which leads to subsequent metabolic endotoxemia (Clemente-Postigo *et al*., 2019; Suryadiningrat *et al*., 2021). LBP, as a surrogate marker for LPS, is measured through gene expression analysis to assess endotoxemia status in obese mice. However, it is known that diet-induced obesity can alter gene expression, including that of the reference gene GAPDH (Fan *et al*., 2020). Therefore, this study analyzes the Ct value of each gene in each diet treatment. There is no significant difference in GAPDH or LBP Ct values among dietary treatments. However, this is not easily evident, as GAPDH was steadily expressed in various dietary challenges. There is no other reference gene measured. Therefore, no further validation is available for the GAPDH expression stability. Overall, the comparison of LBP and GAPDH Ct values showed significant results, suggesting that GAPDH abundance is higher than that of LBP in the context of the cumulative dietary effect.

Further analysis suggests that the standard $2^{-\Delta\Delta Ct}$ method (normalized to the calibrator's mean) showed no significant differences in LBP relative expression between dietary treatments, with high statistical variability. This suggests that the standard $2^{-\Delta\Delta Ct}$ method is not suitable for normalizing LBP expression across different concentrations and biological replicates, contributing to inter-plate variability. There is limited research that explicitly analyzes inter-plate variability in the standard $2^{-\Delta\Delta Ct}$ method. The modified $2^{-\Delta\Delta Ct}$ yields a significantly higher LBP expression in the HFHS group compared to the SCD group, with lower statistical variability compared to the standard $2^{-\Delta\Delta Ct}$ method. In line with this result, a previous study demonstrated that sample-specific calibration showed minimal expression variability, not only in RT-qPCR but also in Western blot (Hawkins and Storey, 2017; Regier and Frey, 2010; R. Zhao *et al*., 2019). This suggests that the individual calibrator approach, which matches the concentration and plate of the treatment group to the respective concentration and plate of the calibrator sample, is a reliable and rational method for overcoming sample-specific variability.

Using the modified $2^{-\Delta\Delta Ct}$ method, LBP expression was found to be upregulated, LBP expression was found to be upregulated in the HFHS group relative to the standard group. While this finding supports the hypothesis of increased LPS exposure in diet-induced obesity, it is essential to acknowledge that this result is based solely on gene expression data. No corresponding protein-level quantification (e.g., serum LBP or LPS concentration) or downstream inflammatory markers (e.g., IL-1, IL-6, or TNF-α) were measured in this study. Therefore, the functional and systemic implications of the increased hepatic LBP expression remain speculative. Moreover, the relatively small sample size (n = 3 per group) may limit the statistical power and

generalizability of the findings. Future studies incorporating larger sample sizes, protein-level assays, and inflammatory profiling are necessary to confirm whether hepatic LBP elevation translates into systemic endotoxemia and inflammation.

CONCLUSION

This study successfully developed diet-induced obesity and LBP upregulation in C57BL/6 mice through HFHS treatment. The analysis of LBP relative expression was conducted using an optimized RT-qPCR protocol, particularly using sample-individualized relative quantification as a modification of the $2^{-\Delta\Delta Ct}$ method. The modified $2^{-\Delta\Delta Ct}$ method showed minimal variability in target gene relative expression compared to the standard counterpart. Addressing variability in RT-qPCR with the correct analysis method reduces systematic error and improves the reliability of Ct data.

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AUTHORS' CONTRIBUTIONS

YT and BDN contributed to the conceptualization of the study. Methodology was developed by YT, BDN, HW, EIS, and SK. IGPAW, APH, SD, SW, and ME carried out the formal analysis and investigation. YT, BDN, and IGPAW prepared the original draft of the manuscript, while BDN was responsible for review and editing. Visualization was conducted by YT and IGPAW. Funding acquisition was supported by EIS and PT. All authors read and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

Clemente-Postigo, M., Oliva-Olivera, W., Coin-Aragüez, L., Ramos-Molina, B., María Giraldez-Perez, R., Lhamyani, S., Alcaide-Torres, J., Perez-Martinez, P., El Bekay, R., Cardona, F., Tinahones, F. J., & Bekay, E. R. (2019). Metabolic endotoxemia promotes adipose dysfunction and inflammation in human obesity. *American Journal of Physiology Endocrinology Metabolism*, 316, 319–332.

De Francesco, P. N., Cornejo, M. P., Barrile, F., García Romero, G., Valdivia, S., Andreoli, M. F., & Perello, M. (2019). Inter-individual variability for high fat diet consumption in inbred C57BL/6 mice. *Frontiers in Nutrition*, 6, 67.

de Moura e Dias, M., dos Reis, S. A., da Conceição, L. L., Sediyama, C. M. N. de O., Pereira, S. S., de Oliveira, L. L., Gouveia Peluzio, M. do C., Martinez, J. A., & Milagro, F. I. (2021). Diet-induced obesity in animal models: points to consider and influence on metabolic markers. *Diabetology and Metabolic Syndrome*, 13(1), 32.

Ehlting, C., Wolf, S. D., & Bode, J. G. (2021). Acute-phase protein synthesis: A key feature of innate immune functions of the liver. *Biological Chemistry*, 402(9), 1129–1145.

Fan, X., Yao, H., Liu, X., Shi, Q., Lv, L., Li, P., Wang, R., Tang, T., & Qi, K. (2020). High-Fat Diet alters the Expression of Reference Genes in Male Mice. *Frontiers in Nutrition*, 7, 589771.

Giri, P. S., Bharti, A. H., & Dwivedi, M. (2022). Decreased GZMB, NRP1, ITPR1, and SERPINB9 Transcripts Lead to Reduced Regulatory T Cells Suppressive Capacity in

Generalized Vitiligo Patients. *Journal of Immunology Research*, 2022, 3426717.

Hawkins, L. J., & Storey, K. B. (2017). Improved high-throughput quantification of luminescent microplate assays using a common Western-blot imaging system. *MethodsX*, 4, 413–422.

Kwon, N., Lee, K. E., Singh, M., & Kang, S. G. (2021). Suitable primers for GAPDH reference gene amplification in quantitative RT-PCR analysis of human gene expression. *Gene Reports*, 24, 101272.

Latorre, J., Díaz-Trelles, R., Comas, F., Gavaldà-Navarro, A., Milbank, E., Dragano, N., Morón-Ros, S., Mukthavaram, R., Ortega, F., Castells-Nobau, A., Oliveras-Cañellas, N., Ricart, W., Karmali, P. P., Tachikawa, K., Chivukula, P., Villarroya, F., López, M., Giralt, M., Fernández-Real, J. M., & Moreno-Navarrete, J. M. (2022). Downregulation of hepatic lipopolysaccharide binding protein improves lipogenesis-induced liver lipid accumulation. *Molecular Therapy Nucleic Acids*, 29, 599–613.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, 25(4), 402–408.

McKeown, N. M., Fahey, G. C., Slavin, J., & Van Der Kamp, J. W. (2022). Fibre intake for optimal health: how can healthcare professionals support people to reach dietary recommendations? *The BMJ*, 11(1), e054370.

Mohammad, S., & Thiemermann, C. (2021). Role of Metabolic Endotoxemia in Systemic Inflammation and Potential Interventions. *Frontiers in Immunology*, 11(2), 594150.

Page, M. J., Kell, D. B., & Pretorius, E. (2022). The Role of Lipopolysaccharide-Induced Cell Signalling in Chronic Inflammation. *Chronic Stress*, 6(2), 24705470221076390.

Regier, N., & Frey, B. (2010). Experimental comparison of relative RT-qPCR quantification approaches for gene expression studies in poplar. http://www.biomedcentral.com/1471-2199/11/57

Seethaler, B., Basrai, M., Neyrinck, A. M., Nazare, J. A., Walter, J., Delzenne, N. M., & Bischoff, S. C. (2021). Biomarkers for assessment of intestinal permeability in clinical practice. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 321(1), G11–G17.

Serban, D., Mihai Branescu, C., Cristian, D. A., & Dascalu, A. M. (2020). Bilirubin, Interleukin 6 (IL 6) and Lipopolysaccharide-Binding Protein (LBP)-Biomarkers of Sepsis in Appendicular Peritonitis. *Review Chim*, 71(7), 444–454.

Suryadiningrat, M., Kurniawati, D. Y., Mujiburrahman, A., & Purnama, M. T. E. (2021). Dietary polyvinyl alcohol and alginate nanofibers ameliorate hyperglycemia by reducing insulin and glucose-metabolizing enzyme levels in rats with streptozotocin-induced diabetes. *Veterinary World*, 14(4), 847–853.

Tanaka, N., Kimura, T., Fujimori, N., Nagaya, T., Komatsu, M., & Tanaka, E. (2019). Current status, problems, and perspectives of non-alcoholic fatty liver disease research. *World Journal of Gastroenterology*, 25(2), 163–177.

Teng, M. L. P., Ng, C. H., Huang, D. Q., Chan, K. E., Tan, D. J. H., Lim, W. H., Yang, J. D., Tan, E., & Muthiah, M. D. (2023). Global incidence and prevalence of nonalcoholic fatty liver disease. In *Clinical and Molecular Hepatology*, 29(1), 32–42.

Tjahjono, Y., Foe, K., Ratnasari Wilianto, Y., Christianto Khudrati, W., Yesery Esar, S., Jafet, N., Made Andika Bara Kusuma, I., Ade, L., Dian Novita, B., Wihadmadyatami, H., Handayani Tjan, L., Nugraha, J., Santoso, S., & Wijaya, H. (2024). HP inulin-MCT dietary fiber improves lipid metabolism and prevents non-alcoholic steatohepatitis in obese mice. *Journal of Functional Foods*, 120, 106367.

World Health Organization. (2023). World Health Statistics 2023: Monitoring health for the SDGs, Sustainable Development Goals.

Zhao, F., Maren, N. A., Kosentka, P. Z., Liao, Y. Y., Lu, H., Duduit, J. R., Huang, D., Ashrafi, H., Zhao, T., Huerta, A. I., Ranney, T. G., & Liu, W. (2021). An optimized protocol for stepwise optimization of real-time RT-PCR analysis. *Horticulture Research*, 8(1), 179.

Zhao, R., Khafipour, E., Sepehri, S., Huang, F., Beta, T., & Shen, G. X. (2019). Impact of Saskatoon berry powder on insulin resistance and relationship with intestinal microbiota in high fat–high sucrose diet-induced obese mice. *Journal of Nutritional Biochemistry*, 69, 130–138.

Zhu, H., Zhang, H., Xu, Y., Laššáková, S., Korabečná, M., & Neužil, P. (2020). PCR past, present and future. *BioTechniques*, 69(4), 317–325.

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