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Characterization and expression of phenylalanine hydroxylase in rabbits under different feeding conditions of faba bean (*Vicia faba* L.)

Hanan I. Sayed Ahmed[1]*, Ghada Shebl[1], Mohamed Hamza[2], Asraf Haider[1]

[1]Botany Department, Faculty of Science, Tanta University, Tanta, Egypt.
[2]Faculty of Medicine, Tanta University, Tanta, Egypt.

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ABSTRACT

Phenylalanine hydroxylase (PAH) enzyme plays a key role in dopamine biosynthesis in humans, rabbits, and most mammals. Rabbits, as an ideal model, were used to study the expression of the PAH gene under different feeding conditions of faba bean (*Vicia faba* L., Sakha 3). PAH genes (≈1,400 bp) in control and faba bean-fed rabbits were PCR amplified, sequenced, and aligned with the reference gene (acc. no. 013672). The first 320 pb of PAH sequences representing the N-terminal of the gene was almost identical. High genetic similarity values were detected in PAH gene sequences for both control and faba bean-fed rabbits with the reference gene (99.80%). The results indicated very little sequence variations, which have no effect on the enzyme activity in both control and faba bean-fed rabbits. Real Time quantitative Polymerase Chain Reaction (RT-qPCR) analysis showed that the PAH gene was overexpressed after feeding on dry faba bean form compared with feeding on the fresh form. Furthermore, Western blotting results reflected superior PAH protein due to the direct influence of feeding on dry faba bean. In conclusion, our findings indicated the direct effect of fresh and dry faba bean diet on increasing phenylalanine amino acid in rabbits' blood, which consequently increases the expression of PAH gene, thus improving the quality of life for humans.

1. INTRODUCTION

Phenylalanine hydroxylase (PAH) enzyme is the main factor for dopamine biosynthesis which is responsible for converting phenylalanine to tyrosine and then to other essential components like dopamine, melanin, adrenaline, and noradrenaline with the same pathway in plants, animals, and humans [1–3]. Previous studies have indicated the importance of this enzyme with satisfying levels in the body of humans and animals [4–8].

Liver and kidney are the main organs that excrete the PAH enzyme [9,10]. The deficiency of PAH enzyme leads to the accumulation of phenylalanine (Phe) amino acid in the blood, causing phenylketonuria (PKU), a disease that causes mental retardation in infants and children [11–21]. Moreover, phenylalanine deficiency in the blood leads to low dopamine levels in the body, which is the only reason for Parkinson's disease and L- dihydroxyphenylalanine (L-DOPA)-induced dyskinesia (LID) [7,22,23]. Therefore, to sustain normal health, the PAH enzyme must be found in its active form and normal ratio in humans, animals, and plants.

For animals, normal phenylalanine level is vital due to its responsibility in the secretion of different essential hormones like prolactin [24,25]. Also, the direct influence of phenylalanine on animal behavior was cleared. For example, in different types of locusts where phenylalanine is the basic molecule in the defending cycle against predatory birds, phenylacetonitrile, a compound synthesized from phenylalanine, plays an important role by converting into hydrogen cyanide, which is toxic to predatory birds [26].

Additionally, phenylalanine is a precursor of many plant phenolic compounds, especially in faba bean [27]. Many studies have indicated that Sakha 3 faba bean genotype has the highest amount

*Corresponding Author
Hanan I. Sayed Ahmed, Botany Department, Faculty of Science, Tanta University, Tanta, Egypt. E-mail: hanan.ahmed @ science.tanta.edu.eg

of phenylalanine [28–31]. Direct diet was applied to increase phenylalanine levels in rabbits' blood which reflected a significant increase in PAH gene expression [32–34],

Our investigation aimed to evaluate the influence of a direct diet with fresh and dry forms of faba bean (Sakha 3 genotype) on PAH enzyme levels in rabbits. PAH genes of rabbits were PCR amplified, sequenced, and aligned with the reference gene in the GenBank.

2. MATERIALS AND METHODS

2.1. Plant Materials

Faba bean (*Vicia faba* L., Sakha 3 genotype) seeds were obtained from Sakha Agricultural Research Station and planted for a whole season during September 2018–March 2019 in suitable soil with recommended agriculture conditions. Plants were used as rabbit food in two forms (dry and fresh) with different quantities, as shown in Table 1.

2.2. Animal Samples and Feeding

New Zealand male rabbits (*Oryctolagus cuniculus*), 6 months old, were used in this study. Rabbits were grown and treated in the laboratories of the Faculty of Science, Tanta University, according to the regulations and rules of scientific ethical research with license no. IACUC-SCI-TU-0126. Rabbits were fed fresh and dry beans as a supplemented diet in different quantities for a period of 6 months, as shown in Table 1. Control rabbits were fed a normal diet without faba bean. After the feeding period, blood samples were collected from the ear vein, after sterilization with 70% ethyl alcohol. Blood samples were drawn using a medical syringe and collected in separate tubes. Rabbits were sacrificed and their livers were collected and stored for further work.

2.3. Quantification of Phenylalanine Amino Acid

Phenylalanine assay kit (Abcam, ab83376) was used according to manufacturer's protocol for direct quantification of phenylalanine amino acid levels in rabbit blood samples. The results were recorded as an average of three sample rabbit replicates.

Table 1: Faba bean feeding experiment design.

Rabbits	Faba bean feeding type	Feeding period	Feeding dose (g)
1	Control	6 months	150
2	Fresh		50
3	Fresh		100
4	Fresh		150
5	Dry		50
6	Dry		100
7	Dry		150

2.4. PCR Amplification of PAH Genes

PAH gene fragments of rabbits (*O. cuniculus*) were amplified, eluted, and sequenced. Nucleotide sequences of treated rabbit samples were aligned and compared with control as well as with reference sequence in the GenBank.

Total genomic DNAs were purified according to the manufacturer's protocol of the GeneJET Genomic DNA purification kit (K0721/ Thermo Fisher). Forward and reverse primers specific to the PAH gene (accession number 013672) were designed using the Primer-BLAST tool (www.ncbi.nlm.nih.gov/tools/primer-blast/primertool), as shown in Table 2. Then, Dream *Taq* PCR Master Mix (2×) was used to amplify gene fragments.

Thermal cycler (CreaCon, Holland) was used with the following conditions: 40 cycles; each cycle consisted of denaturation at 95°C for 30 seconds, followed by annealing at 37°C for 1 minute and extension at 72°C for 2 minutes. There was an initial delay for 15 minutes at 95°C at the beginning of the first cycle and a 10 minutes delay at 72°C at the end of the last cycle as a postextension step. Agarose gel (1.5%) was applied to migrate amplicons via MultiSUB Mini Horizontal Electrophoresis System with Power Pro 300 Power supply (Cleaver Scientific, UK) and imaging with gel documentation system (OmniDOC, Cleaver Scientific, UK). Data analysis was carried out using TotalLab analysis software (ww.totallab.com, ver.1.0.1).

2.5. Sequencing and Alignment of PAH Genes

Amplified PCR fragments were purified with E.Z.N.A.®Gel Extraction Kit (D2500-01, Omega BIO-TEK, USA). Samples were sent to Micron-Corp., Korea, for sequencing with ABI PRISM® 3100 Genetic Analyzer. Aligned PAH sequences were analyzed on the NCBI website (http://www.ncbi.nlm.nih.gov/website) using BLAST to confirm their identity. Genetic distances and multialignments were computed by the pairwise distance method using Clustal Omega software analysis (https://www.ebi.ac.uk/Tools/msa/clustalo/). The nucleotide sequences were also compared with the highest homology sequences available in the GenBank.

2.6. Quantitative RT-qPCR Analysis of PAH Genes

Quantitative RT-qPCR was applied to detect PAH gene expression after feeding rabbits (*O. cuniculus*) on dry and fresh beans. Gene expression was determined using Maxima SYBR Green/ Fluorescein qPCR Master Mix by Rotor-Gene Q (Qiagen, USA) using a two-step cycling protocol and glyceraldehyde-3-phosphate as a housekeeping gene. TRIzols reagent (15596026, Life Technologies, USA) was applied for total RNA purification from liver samples. Yield and quality of total RNA were determined spectrophotometrically at 260 and 260/280 nm ratios, respectively.

Table 2: Sequence features of specific PAH primers.

Primer	Sequence	Tm	GC content
Forward	5′- ATG TCG GCT GTG GTC CTA GAA AAT GG -3′	60.7ºC	50%
Reverse	5′- TCA GCA ATG GTC AGT TGA CAG ACC -3′	59.2ºC	50%

2.7. Western Blot Analysis of PAH Protein

Western blot technique was applied to evaluate the PAH protein expression. Total soluble proteins were extracted and separated via the SDS-PAGE technique using OmniPage. A vertical protein electrophoresis system with PowerPro 300 power supply (Cleaver Scientific, UK) was conducted according to Laemmli [35] and Brunelle *et al.* [36]. After documentation with OmniDOC (Cleaver Scientific, UK) gel documentation system, PAH protein bands were detected with their specific molecular weight. Protein bands were transferred to Amersham Hybond-N+ (GE Healthcare, USA) via electroBLOT Blotter (Cleaver Scientific, UK) and hybridization was carried out against the anti-PAH antibody (Abcam, ab178430) according to the manufacturer's protocol. Finally, PAH bands were evaluated via the chemiPRO Western blot imaging system [37].

3. RESULTS

3.1. Levels of Phenylalanine Amino Acid in Blood

Phenylalanine content was estimated in rabbits' blood to evaluate the effect of the faba bean-feeding diet. Data presented are the average of three replicates of rabbit samples. Generally, a positive correlation was found between faba bean dose and phenylalanine levels in the blood. As shown in Figure 1 and Table 3, rabbits fed a high dose (150 g) of dry bean showed the greatest increase in phenylalanine level (311 nmol/l), whereas rabbits fed a low dose (50 g) showed a low level of phenylalanine (120 nmol/l). Similarly, rabbits fed a high dose (150 g) of fresh faba bean had high levels of phenylalanine (98 nmol/l), whereas rabbits fed a low dose (50 g) had low levels of phenylalanine (77 nmol/).

3.2 PAH Gene Sequence

PAH gene fragments (≈1,400 bp) were amplified and cleared on agarose gel (Fig. 2). PAH genes of control and the six faba bean-fed rabbits were sequenced and aligned with the reference gene (accession number 013672).

The first 320 pb of PAH sequences representing the N-terminal of the gene was almost identical as shown in sequence alignment (Fig. 3). High genetic similarity values were detected in PAH gene sequences for both control and faba bean-fed rabbits with the reference gene sequence (Table 4). PAH gene sequence for the control sample and rabbits fed 100 g of dry faba bean reflected the highest genetic similarity with the reference sequence (99.80%). The lowest genetic similarity (97.40%) was recorded for rabbits fed 150 g of fresh faba bean.

3.3. Expression of PAH Gene

Quantitative RT-qPCR was used to evaluate the effect of feeding by fresh and dry faba bean on PAH gene expression levels. As shown in Table 5, rabbits fed 150 g of dry faba bean reflected a superior increase in gene expression level, almost threefold, compared with those fed 150 g of fresh faba bean which increased gene expression level with twofold the gene expression of control. Our findings indicated overexpression of the PAH gene as a direct influence of feeding on dry faba beans.

3.4. Activity of PAH Enzyme

Western blot analysis was used to detect the effect of the faba bean diet on PAH protein in rabbits. As shown in Figure 4, total proteins from rabbit samples were fractionated and compared with control. A specific PAH protein band between 50 and 60 kDa was detected for all samples (Fig. 4A). Rabbits fed 150 g of dry beans reflected a remarkable increase in PAH protein expression level compared with controls and rabbits fed 150 g of fresh faba bean (Fig. 4B). These results indicate a clear positive correlation between feeding on dry faba bean and overexpression of PAH enzyme.

4. DISCUSSION

In this investigation, rabbits (*O. cuniculus*), as a biological model, were used for studying PAH gene sequence, expression, and enzyme activity. Perfect matching dopamine biosynthesis pathway between rabbits and humans and high similarity of PAH gene sequence between rabbits and humans were reported [38,39].

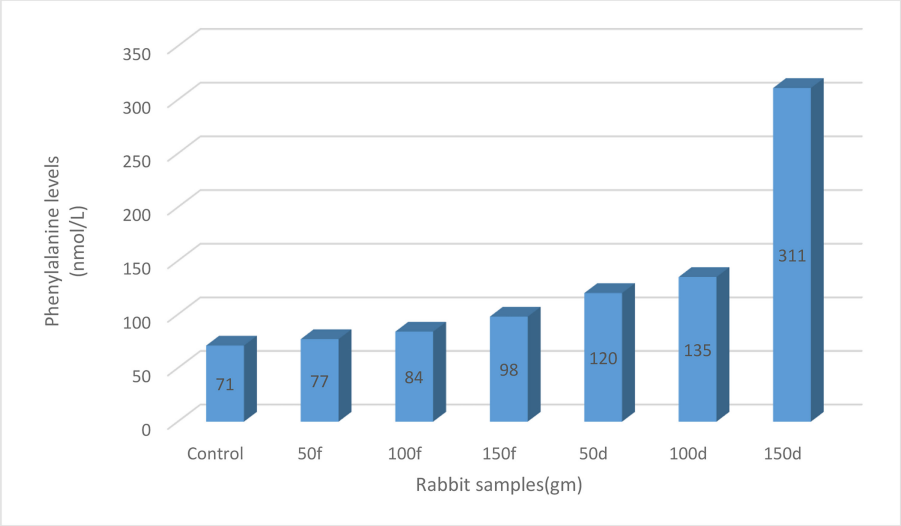

Figure 1: Phenylalanine levels (nmol/l) for control rabbits (lane 1), fresh beans-fed rabbits (lanes 2–4), and dry beans-fed rabbits (5–7).

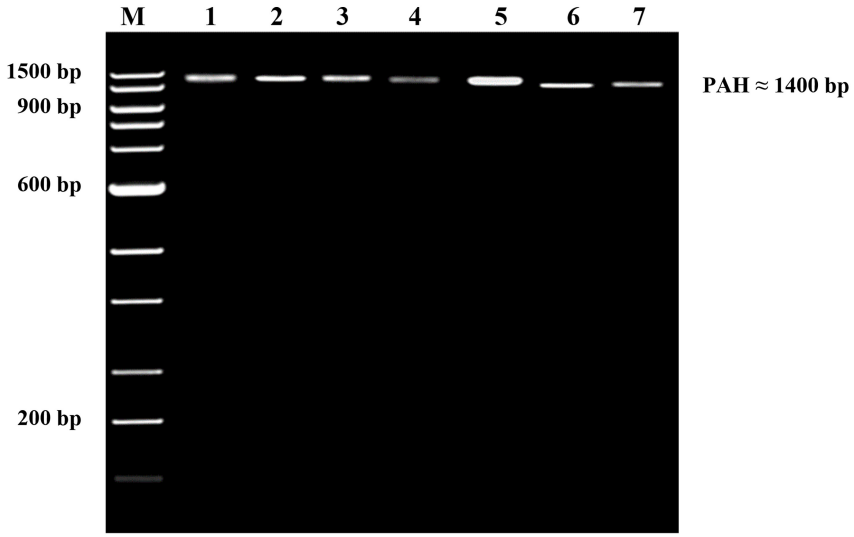

Figure 2: PCR genomic products of PAH gene with ≈1400 bp for control rabbits (lane 1), fresh beans-fed rabbits (lanes 2–4), and dry beans-fed rabbits (lanes 5–7).

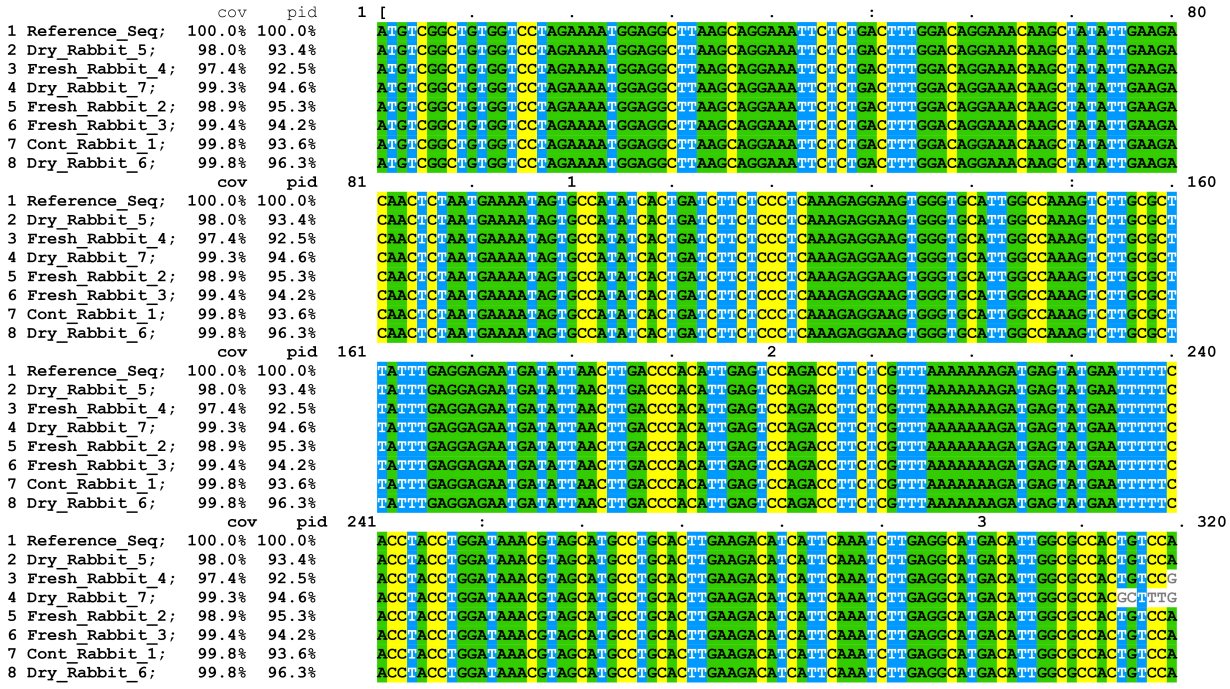

Figure 3: Nucleotide sequence alignment of N-terminal (320 bp) PAH gene fragment amplified from control and six rabbit samples with the reference sequence (accession number 013672) in the GenBank.

Faba bean (*Vicia faba* L.) Sakha 3 genotype was chosen as a diet rich in phenylalanine to study the effect of different feeding conditions on PAH expression and enzyme activity. Previous studies showed that faba bean plants contain high amounts of phenylalanine and phenolic amino acids [28,40,41].

The results of the present study indicated an increase of phenylalanine level in rabbit blood after feeding on faba bean. Our results indicated a direct correlation between increasing the amount of faba bean diet rich in phenylalanine and increasing the level of phenylalanine amino acid in rabbits' blood, which enhances PAH gene expression and enzyme activity. This is in accordance with the study by Shebl *et al.* [31], who indicated the direct influence of diet rich in phenylalanine as faba bean Sakha 3 genotype on increasing phenylalanine in rabbits' blood.

Our findings obtained by quantitative RT-qPCR reflected a high increase in PAH gene expression level in rabbits fed fresh and dry faba bean (twofold and threefold, resp.). These results are confirmed by Western blotting analysis, which indicated overexpression of PAH enzyme. Fitzpatrick [42] indicated that PAH enzyme activity in the liver responds in a cooperative fashion to phenylalanine concentrations,

Table 3: Phenylalanine levels in blood for control and six treated rabbits.

	Rabbit samples						
	Control	Fresh beans			Dry beans		
		50 g	100 g	150 g	50 g	100 g	150 g
Phenylalanine levels (nmol/l)	71	77	84	98	120	135	311

Table 4: Sequence similarity of PAH gene from rabbit samples with Reference gene (Acc. No. 013672) from GenBank.

Code no	PAH gene sequence	Similarity%
Control_rabbit_1	Control Rabbits	99.80 %
Fresh_rabbit_2	Rabbits fed on (50 g) of fresh faba bean.	98.90 %
Fresh_rabbit_3	Rabbits fed on (100 g) of fresh faba bean.	99.40%
Fresh_rabbit_4	Rabbits fed on (150 g) of fresh faba bean.	97.40 %
Dry_rabbit_5	Rabbits fed on (50 g) of dry faba bean.	98.00 %
Dry_rabbit_6	Rabbits fed on (100 g) of dry faba bean.	99.80 %
Dry_rabbit_7	Rabbits fed on (150 g) of dry faba bean.	99.30 %

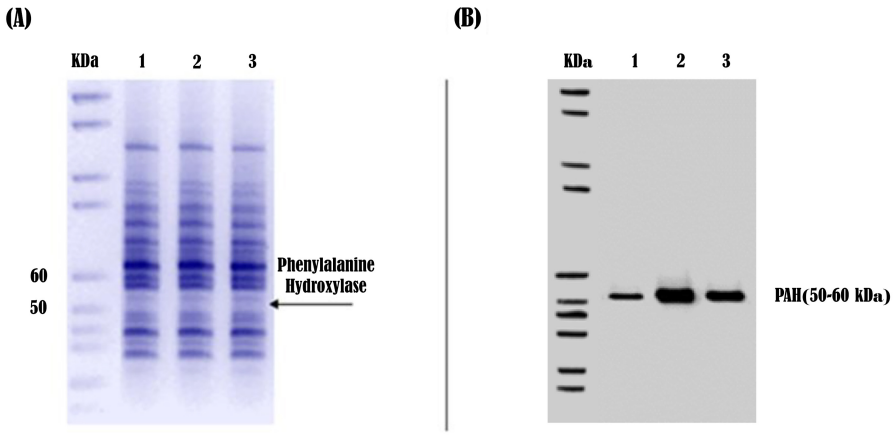

Figure 4: Electrophoretic protein patterns (A) and PAH Western blot (B) for three rabbit samples. 1: control, 2: rabbit fed 150 g of dry beans, and 3: rabbit fed 150 g of fresh beans.

with low activity at basal concentrations of phenylalanine and increased activity when the concentration of phenylalanine in the blood rises. However, Lartey and Austic [43] indicated that levels of PAH mRNA were not affected by the dietary of IAA-Phe mixture to the basal diet of chicken.

Western blotting analysis indicated a clear positive correlation between feeding rabbits on dry faba bean and overexpression of PAH enzyme. Our results are in agreement with those of Lartey and Austic [43], who reflected the positive influence of the addition of the IAA-Phe mixture on the basal diet of chicken with an increase in PAH level in blood. Our findings for using a phenylalanine-rich diet as a helpful tool to improve PAH levels are in agreement with the study by Cao *et al.* [44]. They indicated the effect of dietary supplementation with leucine and phenylalanine on pancreas development, PAH enzyme activity, and related gene expression in male Holstein calves. A positive correlation was detected between supplementation of phenylalanine and an increase in the number of pancreatic cells. In accordance with our results for use of Western blotting analysis to evaluate PAH activity level, Gunasekera and Hyland [45] applied this technique using the PAH 8 antibody to record and monitor the total PAH enzyme concentration level in mice. Furthermore, the applied PAH antibody in our study was supported by Silva *et al.* [46]. They used a monoclonal antibody raised against monkey liver PAH to detect this protein in *Drosophila melanogaster*.

In this investigation, PAH gene fragments were PCR amplified, sequenced, and aligned with the reference gene. High genetic similarity values were detected in PAH gene sequences for both control and faba bean-fed rabbits with the reference gene. The results indicated very little sequence variations, which have no effect on the enzyme activity in both control and faba bean-fed rabbits. Previous studies indicated that PAH genes exhibit natural mutations between different species and even among individuals of the same species. Some of these mutations are mild and do not affect the enzyme activity; however, severe mutations can occur, resulting in serious diseases such as phenylketonuria in humans [15,19,20].

Table 5: PAH gene expression folds in control and faba bean fed rabbits.

Samples			Gene being Tested Experimental (TE)	Gene being Tested Control (TC)	Housekeeping Gene Experimental (HE)	Housekeeping Gene Control (HC)	ΔCt values for the experimental (ΔCTE)	ΔCt values for the control (ΔCTC)	Delta Ct Value (ΔΔCt)	2^-ΔΔCt (expression fold change)-fold Expression level in the experimental condition the expression as in the control condition
Control			24	21.5	20	19.2	4	2.3	1.7	0.3
Treatments	dry beans	150 g	23.5	21.5	21.2	19.2	2.3	2.3	0	1.0
	fresh beans	150 g	24.3	21.5	21.3	19.2	3	2.3	0.7	0.6

ΔCt values (Experimental) = Ct (Tested Exp) – Ct (Housekeeping Exp).
ΔCt values (Control) = Ct (Tested Control) – Ct (Housekeeping Control).
ΔΔCt values = ΔCt values (Experimental) − ΔCt values (Control).
Relative gene expression fold change = 2^−ΔΔCt.

Our results agree with those of Mitchell *et al.* [12] who detected duplications and deletions in PAH gene sequences with no mutations of the enzyme activity. However, on the other hand, Güttler and Guldberg [11] and Vockley *et al.* [13] identified causative mutations in 686 PAH deficiency patients and indicated that the PAH mutation genotype is determinant in most patients.

Sequence analyses of mutant PAH enzymes established that the N-terminal domains are responsible for the divergent regulatory properties of the enzymes, while the C-terminal catalytic domains are responsible for substrate specificity [47]. Our results shown in Figure 3 indicated 100% sequence similarity in the first 320 pb of PAH sequences, representing the N-terminal of the gene.

5. CONCLUSION

In conclusion, our findings indicated the direct effect of fresh and dry faba bean diet on increasing phenylalanine amino acid in rabbits' blood, which consequently increases the expression of PAH gene, thus improving the quality of life for humans.

6. DISCLOSURES

6.1. Authorship contribution

All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements.

6.2. Funding

There is no funding to report.

6.3. Conflicts of interest

The authors report no financial or any other conflicts of interest in this work.

6.4. Publisher's note

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