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Impact of Polyherbal Formulation on Transcriptome Profiling of Chicken Breast Muscle: Elucidation of Molecular Mechanisms for the Enhanced Cellular Feed Efficiency in Broiler Chickens

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ABSTRACT

To elucidate the cellular mechanisms underlying feed efficiency (FE) enhanced by polyherbal formulation (PHF), a combination of *Andrographis paniculata*, *Punica granatum*, and *Emblica officinalis*, the microarray technology was used to identify the genetic pathways related to feed utilization through genomical profiling of breast muscle in Cobb broiler chickens supplemented with or without PHF. 300 male day-old chicks were randomly distributed into two groups of 6 replicates with 25 each, including control (basal-diet) and PHF (Basal-diet+PHF-400g/ton), and their impact on performance parameters was assessed on day 42. Similarly, the global gene expression of breast muscles collected in each group was profiled using Agilent chicken whole genome microarray technology. Further, the Database for Annotation Visualization and Integrated Discovery (DAVID) Bioinformatics Resources and gene ontology (GO) analysis were used to identify the functional clustering of differentially expressed genes (DEGs) and gene network pathways associated with FE. RT-qPCR was subsequently conducted to cross-validate the expression of genes identified by DAVID. The PHF supplementation significantly improved the body weight gain (BWG) and feed conversion ratio in broilers compared to the control. Totally, 1338 DEGs (756 up-regulated and 582 down-regulated) were identified, of which 732 DEGs of PHF were significantly different from the control group. However, bioinformatics analysis revealed a significant modulation of 198 DEGs (94 up-regulated and 104 down-regulated) after hierarchical clustering, whose collective expression indicates significant enrichment of FE-related biological processes in the PHF-treated group. Further, a deeper understanding of the following DEGs (*ND1, ND2, ND3, ND4, ND4L, ND5, ND6 CYTB, COX1, COX2, COX3, ATP6, PGC1-α, PPAR, MEF2, PARK2, Wnt3A, Wnt11, Golgb1*, and *IGF1*) was established by mapping with Kyoto-Encylopedia-of-Genes-and-Genomes (KEGG) pathway in relation to mitochondria cellular respiration, mitochondrial biogenesis, mitophagy, energy metabolism and muscle growth in the breast muscle. Moreover, validation of microarray analysis of selected genes using RT-qPCR showed that the genes (*ND1, ND2, ND3, ND5, CYTB, ATP6, PGC1-α,* and *Wnt11)* were expressed in the same direction as that of GO analysis. In conclusion, supplementation of PHF resulted in transcriptional modulation in the mitochondrial functions, which was correlated to the improvements of corresponding phenotypic traits (FE and BWG) in broiler chickens.

Keywords: Differentially expressed gene, Feed efficiency, Genome microarray, Mitochondrial respiration

INTRODUCTION

Since the global population keeps increasing from 700 crores in 2011 to 800 crores in 2030 and 900 crores in 2050, the requirement for high-quality animal protein on the earth will be doubled by 2050 (Tian et al., 2016). Thus, improving the efficiency and sustainability of animal production systems depends heavily on features like feed efficiency (FE). Conventionally, the inclusion of antibiotics at subtherapeutic levels in a nutritionally balanced poultry diet maximizes the genetic potential for growth and FE in broilers (Basit et al., 2020). However, the feed grade antibiotic growth promoters were banned by the European Union due to the risk of possible multiple drug-resistance of human pathogenic bacteria and the issue

of drug residues in meat products. In addition, the cost factor, possibility of the evolution of antibiotic-resistant microbes and condemnation by consumer associations as well as by scientists over antibiotic growth promoters enforced the poultry researchers and feed industries to find an alternative to antibiotics, which resulted in the evolution of probiotics, prebiotics, and organic acids supplements (Rahman et al., 2022). The feed manufacturers have recently adopted a new type of natural feed additives that are the result of contemporary science. These additives include botanicals, such as suitable blends of herbs or plant extracts, to maintain the health and good production of livestock that feed humanity (İpçak and Alçiçek, 2018; Opoola et al., 2019). Despite marked improvements in growth and FE using these feed additives (Havenstein et al., 2003), there remains a significant within and between-strain variation of these traits in broilers (Connor, 2015). This variation in relation to performance could be enlightened by recent findings of several authors (Ojano-Dirain et al., 2004), who proved that mitochondrial function and biochemistry were entangled with FE in broilers. Mitochondria, a well-defined cytoplasmic organelle of the cell, generate adenosine triphosphate (ATP) from the diet through a process called oxidative phosphorylation using oxygen and simple sugars (Picard et al., 2018). Being a powerhouse of the cell, mitochondria produce 90% of the energy utilized by the cell; an association of mitochondrial dysfunction with oxidative phosphorylation was presumed to have a substantial effect on the phenotypic expression of FE in animals. This was supported by Bottje et al. (2006), who demonstrated that the breast muscle of high FE birds showed higher activities of mitochondrial complexes and the respiratory control ratio when compared to low FE birds. However, there has been very scant information about specific genes or gene networks, as well as a very limited study of global gene expression profiling that underlies the interaction of mitochondrial function and FE, modulated by natural feed additives (Kong et al., 2011).

Polyherbal formulation (PHF), presumed to enhance cellular feed efficiency, is a combination of *Andrographis paniculata, Punica granatum,* and *Emblica officinalis* belonging to the families of Acanthaceae, Lythraceae, and Phyllanthaceae, respectively, in poultry (Mathivanan et al., 2008; Patel et al., 2016; Gosai et al., 2023; Jahja et al., 2023). These plants are well-known for betterment of feed conversion ratio (FCR), augmentation of body weight, and control of mortality rate in chickens (Tipakorn, 2002; Danet et al., 2013; Patel et al., 2016; Saleh et al., 2017). However, to date, there is no scientific data available on the effect of PHF on global gene expression that demonstrates the enhancing effect of FE in broiler chickens.

Nutrigenomics, a novel research field, is used to elucidate the cellular basis of the interaction between nutrients and other dietary bioactives using the genetic material (Ismail and Ergören, 2023). It is widely known that any change in nutrition impacts the patterns of genomic profiling (Lee et al., 2015; Resnyk et al., 2017; Martínez-Martínez et al., 2020), hence, these modified genomes can be targeted and identified to understand the impact of nutritional variations on the whole genome. Microarray is one of the most recent advances in technology in analysing the entire transcriptome that can be used to uncover the pathways and networks underlying feed efficiency. Therefore, the present study sought to elucidate the molecular basis for PHF effectiveness (growth and FE in broiler chickens) with the help of microarray technologies and two bioinformatics tools with Database for Annotation, Visualization and Integrated Discovery (DAVID) and gene ontology (GO), followed by further validation of target genes using RT-qPCR assay.

MATERIALS AND METHODS

Ethical approval

Institutional ethical committee approval (No. AHS/PR/03/2016) was obtained before the conduct of study and it was conducted by authorised, qualified and trained veterinarians, scientists and technicians in compliance with the guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Polyherbal formulation

The PHF is crude powder, indexed as Feed-X, which contains *Andrographis paniculata, Punica granatum,* and *Emblica officinalis,* and is manufactured by Natural Remedies Pvt., Ltd., Bengaluru, India. The PHF was supplemented with the diet at a dose of 400g/ton of feed.

Experimental design and inclusion level

Three-hundred-day-old Cobb-430 chicks, with an average weight of 45g, were randomly distributed into two groups of 150 each, including control (basal-diet [BD]) and PHF (BD+PHF-400g/ton), with six replicates of twenty-five birds each. Both the groups were raised on a

basal diet (ME – 3100 kcal/kg and CP – 22, 20 and 18% in starter, grower and finisher phases, NRC, 1994) and the management practices were carried out as described by Selvam et al. (2017), except for the floor space. Birds were provided 0.027m^2 from day 1-21 and 0.055m^2 per bird from day 22-41. The performance parameters, including body weight gain (g), FCR (Marimuthu and D'Souza, 2019), and mortality (%), were assessed on day 39.

Sample collection

Following weighing on day 41, six chickens were chosen at random from each group, sacrificed by jugular vein exsanguination, and their breast muscles were sampled. After removing about 0.5 g of breast muscle, it was rinsed with prechilled phosphate-buffered saline (PBS), chopped into 5 x 5 mm pieces, and immediately stored in RNAlater® solution (Sigma-Aldrich, Canada) for 12 hours. It was then kept at -80°C until the RNA was extracted for transcriptome analysis. In addition, a piece of breast muscle (n = 10/group) was rapidly frozen using liquid nitrogen after prechilled PBS washing, and then stored at -80°C until avian uncoupling protein (avUCP) and protein carbonyl analysis were performed.

Preparation of tissue homogenate

A weighed quantity of breast muscle was added to four parts of PBS and homogenized using a tissue T18 digital ultra-turrax® homogenizer (IKA, Staufen, Germany). Then, it was centrifuged at 13800 RPM for one minute at 4℃, followed by sonication for 30 seconds. An aliquot was collected and stored at -20℃ for further analysis.

Chicken uncoupling protein assy

An uncoupling protein (UCP) level was measured using Nori® chicken avUCP ELISA kit (Genorise Scientific, Inc., USA). Briefly, breast muscle homogenate (1:4 dilution) was allowed to bind with immobilized chicken-specific (UCP2) antibody at room temperature for 2 hours. Then the samples were incubated with the detection antibody (specific for chicken UCP2) and detection reagent solution, followed by the addition of substrate solution for colour development, and its intensity was measured at 540 nm using Versamax Tunable Microplate Reader (BN02314 Model) purchased from Molecular Devices, LLC, USA.

Protein carbonyl assay

The protein carbonyl level was assessed by the protein carbonyl colorimetric assay kit (Cayman Chemical USA). Briefly, the breast muscle homogenate (1:5 dilution) was derivatized with carbonyl residues of dinitrophenylhydrazine, precipitated the derivatized protein using trichloroacetic acid, and washed with a 1:1 ethanol: Ethyl acetate mixture. Then it was resuspended in guanidine hydrochloride and measured spectrophotometrically at 370 nm using a Versamax Tunable Microplate Reader (BN02314 Model) obtained from Molecular Devices, LLC, US.

RNA isolation

Breast muscle samples were homogenized in QIAzol® Lysis Reagent Reagent, (Qiagen, USA) using a Smasher™ homogenizer (BioMerieux, USA), and isolated the total RNA with the help of RNeasy® Lipid Tissue Kit (Qiagen, Valencia, CA) method, and estimated the total RNA concentration of all 12 samples at 260/280 nm ratio using ND-2000 spectrophotometer (Nano Drop Technologies Inc., Wilmington, Delaware). Then it was subjected to RNA integrity assessment using Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), and subsequently used for microarray analysis.

cRNA generation, probe-labelling, and microarray-hybridization

The methodology used to synthesize cRNA, label it, and array hybridize it was followed as described by D'Souza et al. (2019).

Assemblage of microarray data and scrutiny

The schematic representation of the microarray scrutiny workflow is shown in Figure 1, and carried out as described in our previous work reported by D'Souza et al. (2019). GSE114769 could be used for the accession of microarray data from respective web sources.

Identification and hierarchical clustering

The data was normalized using logarithmic transformation, and the differentially expressed genes (DEGs) were selected using ± 0.6 as the cut-off criterion. Then the DEGs with similar expression profiles were clustered, which permits the user to form a group with the same profiles (Marimuthu et al. 2022).

Bioinformatics

The DAVID 6.7 database was used to identify the gene ontology (GO) terms related to the location and function of the significant DEGs, and the association of GO terms with other pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways was also studied.

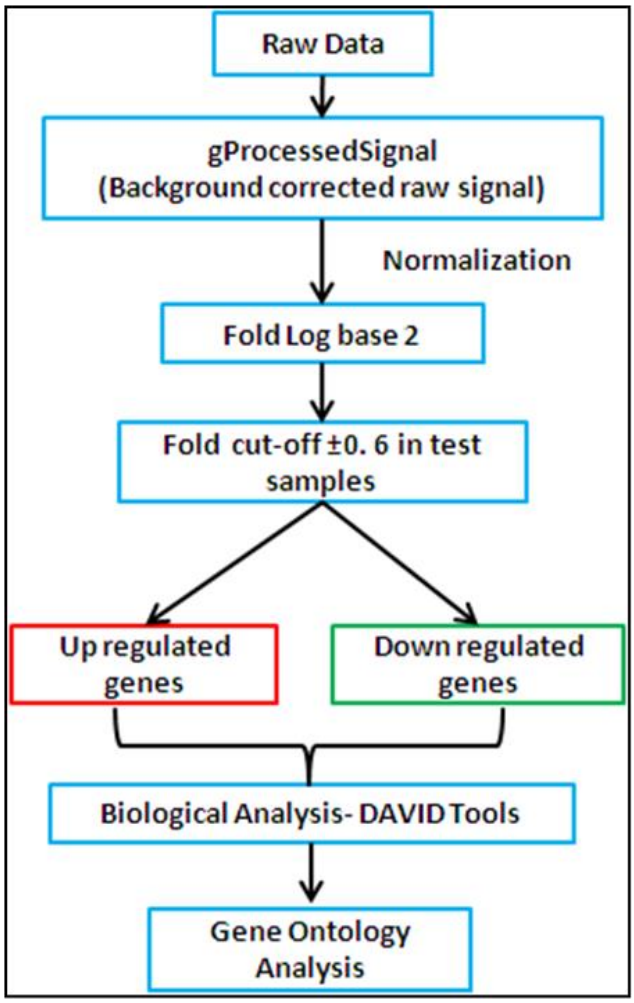

Figure 1. Schematic representation of the microarray analysis of breast muscle workflow

Quantitative Real-time PCR

The expression of selected genes involved in FE was reconfirmed using RT-qPCR. Three micrograms of total RNA from each sample of individual RNA from the control and test groups were used. The primers (Wang et al., 2019) were designed for selected DEGs using Primer 3 Plus online primer design software considering the exon and coding region of the transcripts. The total RNA was converted into cDNA using Affinity Script qPCR cDNA synthesis kit (Agilent Technologies, USA) as per the manufacturer`s protocol. In brief, approximately 1000 ng of RNA from each sample was taken for cDNA synthesis, and the first-strand cDNA was synthesized using universal oligo dT primers. Then, the expression levels of selected DEGs were analyzed using SYBR Green chemistry (Brilliant II SYBR Green qPCR master mix, Agilent Technologies, USA) in a Stratagene MX3005P instrument (Agilent Technologies, USA).

Statistical analysis

The significant DEGs with a false discovery rate (Benjamini–Hochberg test) of $p < 0.05$ were identified using unpaired t-test of GeneSpring version 7.2 (Agilent Technologies) software and the significance of growth performance was tested using unpaired t-test procedure of SPSS (version 9.3, SAS Institute Inc., Cary, NC, USA). The data were expressed as mean ± Standard Deviation (SD), and statistical significance was declared at $p < 0.05$.

RESULTS

Phenotypic traits

Following supplementation of PHF, there was a significant augmentation in body weight and improvement in FCR observed on day 42 when compared with the control group ($p < 0.05$, Table 1).

Assessment of avUCP and protein carbonyl levels

The results indicated that PHF inclusion showed a significant ($p < 0.05$) improvement in avUCP and protein carbonyl levels as compared to the control group (Figures 2 and 3).

Identification of differentially expressed genes

The genome-wide expression profiling of each probe was assigned to compare the PHF and control groups. The raw data files were intra-array normalized and then validated using GenespringGX software. A total of 756 genes were overexpressed and 582 genes were down-regulated (Table 2). Among them, 732 genes were modulated significantly at a threshold of $p < 0.05$, wherein 500 and 232 genes were expressed progressively higher and lower, respectively, when compared to the control group. Those identified DEGs differentiated the 2 experimental groups when represented on a heat map scale, in a hierarchical clustering analysis (Figure 4).

Bioinformatics

The PHF supplementation significantly modulated certain genes of breast muscle, and further, the biological complexity of gene expression was delineated using the DAVID 6.7 online tool, which is a GO term enrichment analysis software that highlights the most relevant GO terms associated with a given gene list ($p < 0.05$). There are three components to a GO annotation including cellular, biological process, and molecular function. In the entire data set, some of the mitochondrial DEGs were identified whose collective expression indicates a significant enrichment in mitochondria biological processes which includes system development, electron transport chain (ETC), multicellular organismal development, anatomical structure development, cellular

developmental process, response to estrogen stimulus, cell differentiation, and nucleoside binding response in PHF treated group. The associated expressed and repressed genes with significant GO terms were tabulated in Tables 3 and 4, and were also represented as a heat-map in Figures 5 and 6. In addition, a deeper insight was made with respect to selective up-regulated DEGs (*ND1, ND2, ND3, ND4, ND4L, ND5, ND6 CYTB, COX1, COX2, COX3, ATP6, PGC1-α, PPAR, MEF2, PARK2, Wnt3A, Wnt11, Golgb1 and IGF1*) involved in mitochondrial respiratory chain, mitochondrial biogenesis, mitophagy, energy metabolism and muscle growth using KEGG database (Table 5).

Examination of specific differentially expressed genes involved in the electron transport chain with RT-qPCR

The profile of specific DEGs involved in mitochondrial biogenesis and ETC signalling pathway in the mitochondria of breast muscle, as well as in structural development, as demonstrated by DAVID, was further reconfirmed using qPCR assays. The qPCR values of *ND1*, *ND2*, *ND3*, *ND5*, *CYTB*, *ATP6*, *WNT11*, and *golgB1* genes in the tissues were almost similar to those of the microarray values (Table 6). Thus, the qPCR data basically substantiates the results of the GO analysis of the selected genes.

Table 1. Outcome of polyherbal formulation on performance parameters in broiler chickens aged 42 days

Parameters	Day	Control	PHF (400 g/ton)
BWG (g) (n=148-150)	42	1845.47 ± 20.73	***1968.87 ± 48.35
FCR (n=6)	42	1.722 ± 0.01	***1.649 ± 0.024
Mortality (%)		1.33	0.00
Breast muscle (g)		441.50 ± 13.91	**513.33 ± 34.24

Values are represented as mean ± SD, **p < 0.01 and *** p < 0.001 indicate the significant difference between the two groups; BWG: Body weight gain; FCR: Feed conversion ratio; PHF: Polyherbal formulation.

Table 2. Differentially expressed breast muscle genes of broiler chickens

Group	Up	Down
Control (breast muscle)	-	-
PHF (breast muscle)	756	582

PHF: Polyherbal formulation

Table 3. Up-regulated genes and associated significant gene ontology (GO) terms

GO Term	Count	P-Value	Genes
GO:0048731~system development	28	0.0005	*DRD1, PTGS2, MYBPC3, UCHL1, CLU, XRCC6, NINJ2, DAB1, MEIS2, GBX2, PRL, SPP1, TCF7, SMAD9, GSC, MYO6, IKZF1, LDB1, TBX5, MET, IGF1, EPHA2, ANXA2, SLIT3, HOPX, COL1A2, SEMA4D, BMP5*
GO:0022900~electron transport chain	6	0.0009	*ND1, ND5, ND2, ND3, CYTB, PPARGC1A*
GO:0007275~ multicellular organismal development	32	0.0015	*DRD1, PTGS2, UCHL1, CLU, XRCC6, MYBPC3, NINJ2, MEIS2, DAB1, GBX2, PRL, SPP1, DVL3, TCF7, MYO6, SMAD9, GSC, IKZF1, LDB1, TBX5, MET, IGF1, EPHA2, ANXA2, SLIT3, DKK3, ISL2, WNT9B, HOPX, COL1A2, SEMA4D, BMP5*
GO:0048856~ anatomical structure development	28	0.0016	*DRD1, PTGS2, MYBPC3, UCHL1, CLU, XRCC6, NINJ2, DAB1, MEIS2, GBX2, PRL, SPP1, TCF7, SMAD9, GSC, MYO6, IKZF1, LDB1, TBX5, MET, IGF1, EPHA2, ANXA2, SLIT3, HOPX, COL1A2, SEMA4D, BMP5*

Table 4. Down-regulated genes and associated significant gene ontology (GO) terms

GO Term	Count	P Value	Genes
GO:0048869~ cellular developmental process	27	0.0009	*RTN4, CDX2, FOXA2, ERBB4, MITF, NEO1, IL15, PIWIL1, POU2F1, AGRN, FGF1, EGR1, KIF3A, CREB1, SLIT1, STAT3, SLIT2, LAMA1, KRT19, LHFPL5, RGS2, KRT14, MYH11, GHRL, ADAM17, PBX3, DBN1*
GO:0043627~ response to estrogen stimulus	4	0.0011	*KRT19, SOCS2, GHRL, STAT3*
GO:0030154~cell differentiation	26	0.0013	*RTN4, CDX2, ERBB4, FOXA2, MITF, IL15, NEO1, PIWIL1, POU2F1, AGRN, FGF1, EGR1, CREB1, SLIT1, SLIT2, STAT3, LAMA1, KRT19, LHFPL5, RGS2, KRT14, MYH11, ADAM17, GHRL, PBX3, DBN1*
GO:0001883~ purine nucleoside binding	37	0.0054	*IFIH1, ERBB4, ASS1, TBK1, STK17B, BRSK2, MKNK1, ASNS, ITM2B, CAMKK2, RRAGC, GSS, GSR, NOS2, KIF3A, MAK, SWAP70, MAP2K3, MSH4, ACACA, RPS6KC1, MAPK11, ACLY, MCM3, SRPK1, MCM5, GART, GRK6, MYH11, ABCC3, ABCC4, MAPK8, ACAD11, AACS, KALRN, ATAD2B, DDX51*
GO:0001882~ nucleoside binding	37	0.0060	*IFIH1, ERBB4, ASS1, TBK1, STK17B, BRSK2, MKNK1, ASNS, ITM2B, CAMKK2, RRAGC, GSS, GSR, NOS2, KIF3A, MAK, SWAP70, MAP2K3, MSH4, ACACA, RPS6KC1, MAPK11, ACLY, MCM3, SRPK1, MCM5, GART, GRK6, MYH11, ABCC3, ABCC4, MAPK8, ACAD11, AACS, KALRN, ATAD2B, DDX51*

Table 5. Results of microarray for up-regulated genes in response to polyherbal formulation treatment in broiler chickens on day 41

Genes	Microarray fold change	p value	Gene Ensemble ID
ND1	1.7	0.0004	ENSGALG00000042750
ND2	1.6	0.0014	ENSGALG00000043768
ND3	1.7	0.0010	ENSGALG00000030436
ND4	1.5	0.0007	
ND4L	1.5	0.0066	
ND5	1.5	0.0049	ENSGALG00000029500
ND6	1.4	0.0058	
CYTB	1.6	0.0027	ENSGALG00000032079
COX1	1.4	0.1191	
COX2	1.3	0.0329	
COX3	1.1	0.0509	ENSGALG00000015591
ATP6	1.53	0.0104	ENSGALG00000015618
PGC1-α	1.9	0.0056	
PARK2	1.25	0.0380	
PPAR	1.3	0.028	
Wnt3A	1.5	0.033	ENSGALG00000042657
Wnt11	1.59	0.011	
GOLGB1	2.94	0.048	ENSGALG00000009783
IGF1	1.77	0.094	ENSGALG00000012755
WFIKKN2	1.32	0.5808	
MSTN	1.24	0.1252	
IGF1R	0.92	0.7742	

PHF: Polyherbal formulation

Table 6. Comparison of the results of microarray and qPCR analyses of selected genes in response to polyherbal formulation treatment in broiler chickens on day 41

Genes	qPCR log fold change (Log2)	Microarray log fold change (Log2)
ATP6	0.58	0.77
WNT11	0.44	0.84
Cyt-b	0.73	0.87
NAD1	0.45	0.79
NAD2	0.59	0.73
NAD3	0.81	0.87

PHF: Polyherbal formulation

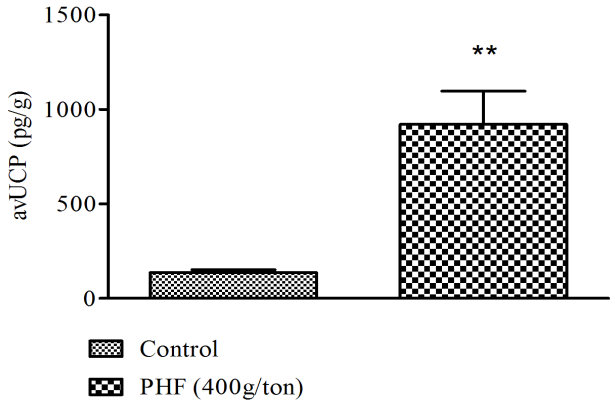

Figure 2. Effect of polyherbal formulation on avian uncoupling protein (avUCP). **p < 0.01 indicates the significant difference between the two groups; PHF: Polyherbal formulation.

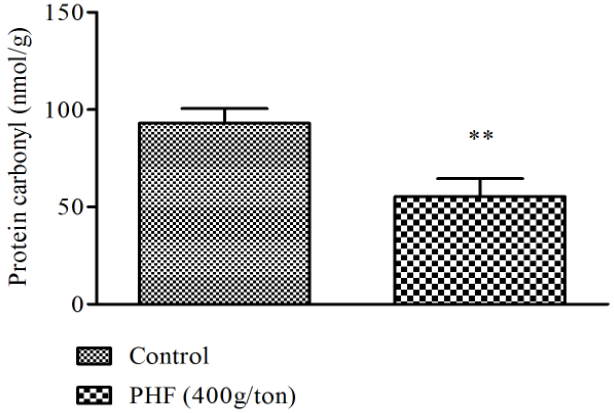

Figure 3. Effect of polyherbal formulation on protein carbonyl. ** indicates significant difference between the two groups (p < 0.01); PHF: Polyherbal formulation.

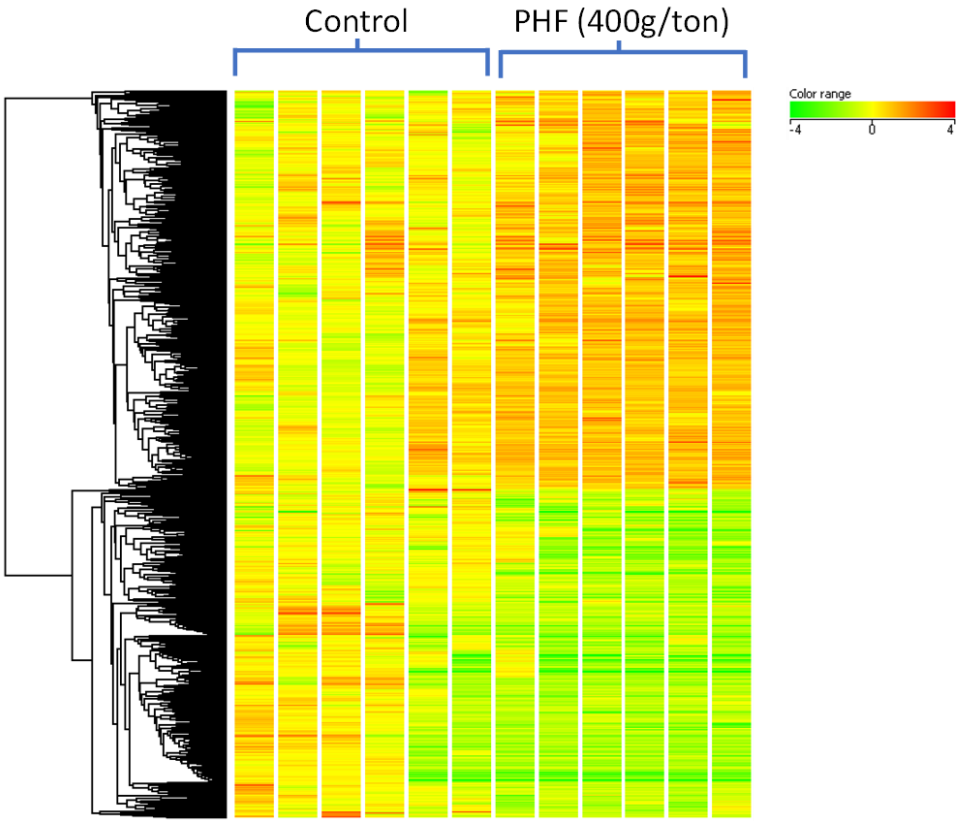

Figure 4. Cluster of differentially regulated genes. Up and down-regulated genes are represented by red and green colors, respectively, while yellow represents no change in fold expression values; PHF: Polyherbal formulation.

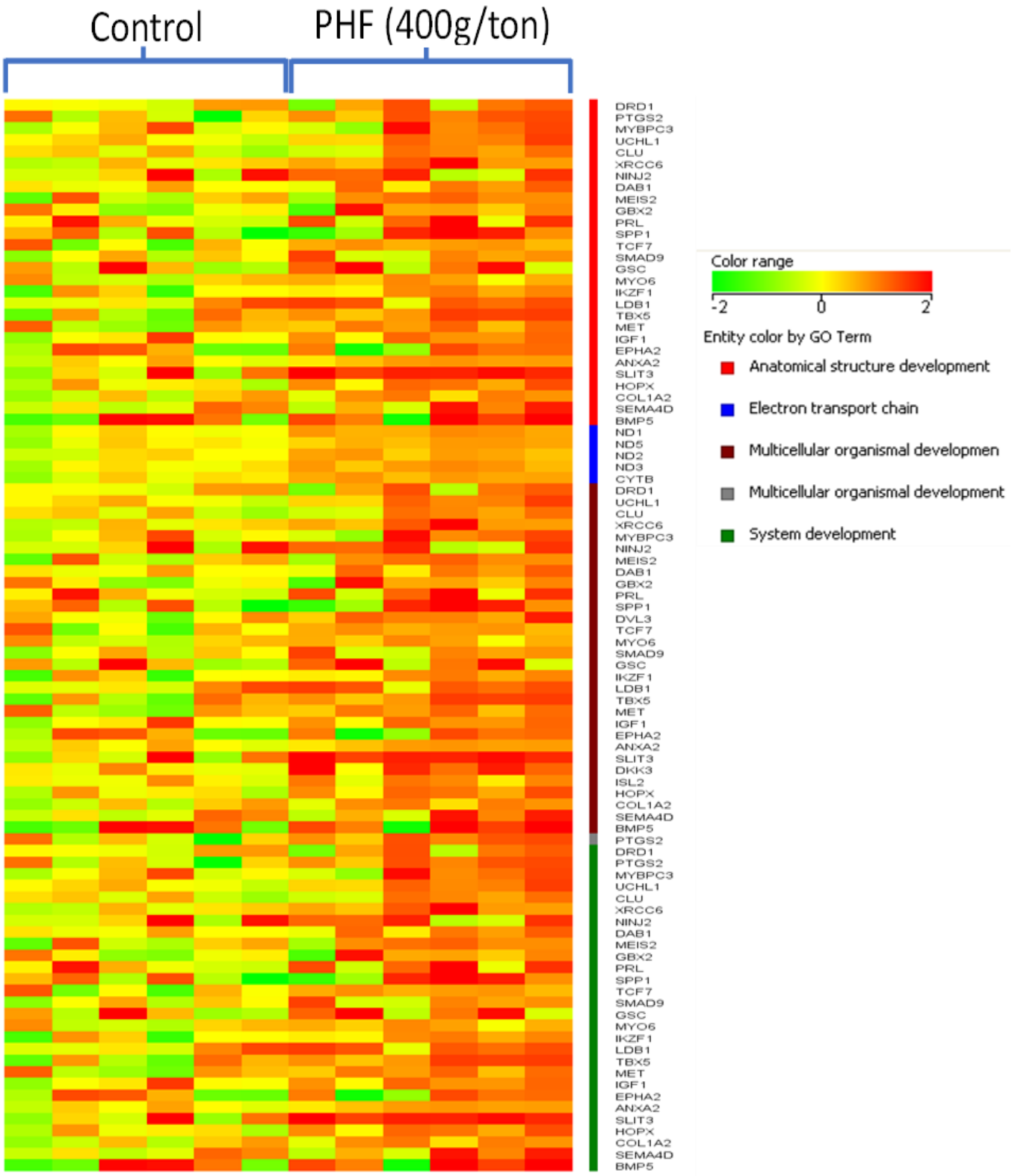

Figure 5. Heatmap showing expressed genes associated with the significant gene ontology terms. Up and down-regulated genes are represented by red and green colors, respectively, while yellow represents no change in fold expression values; PHF: Polyherbal formulation.

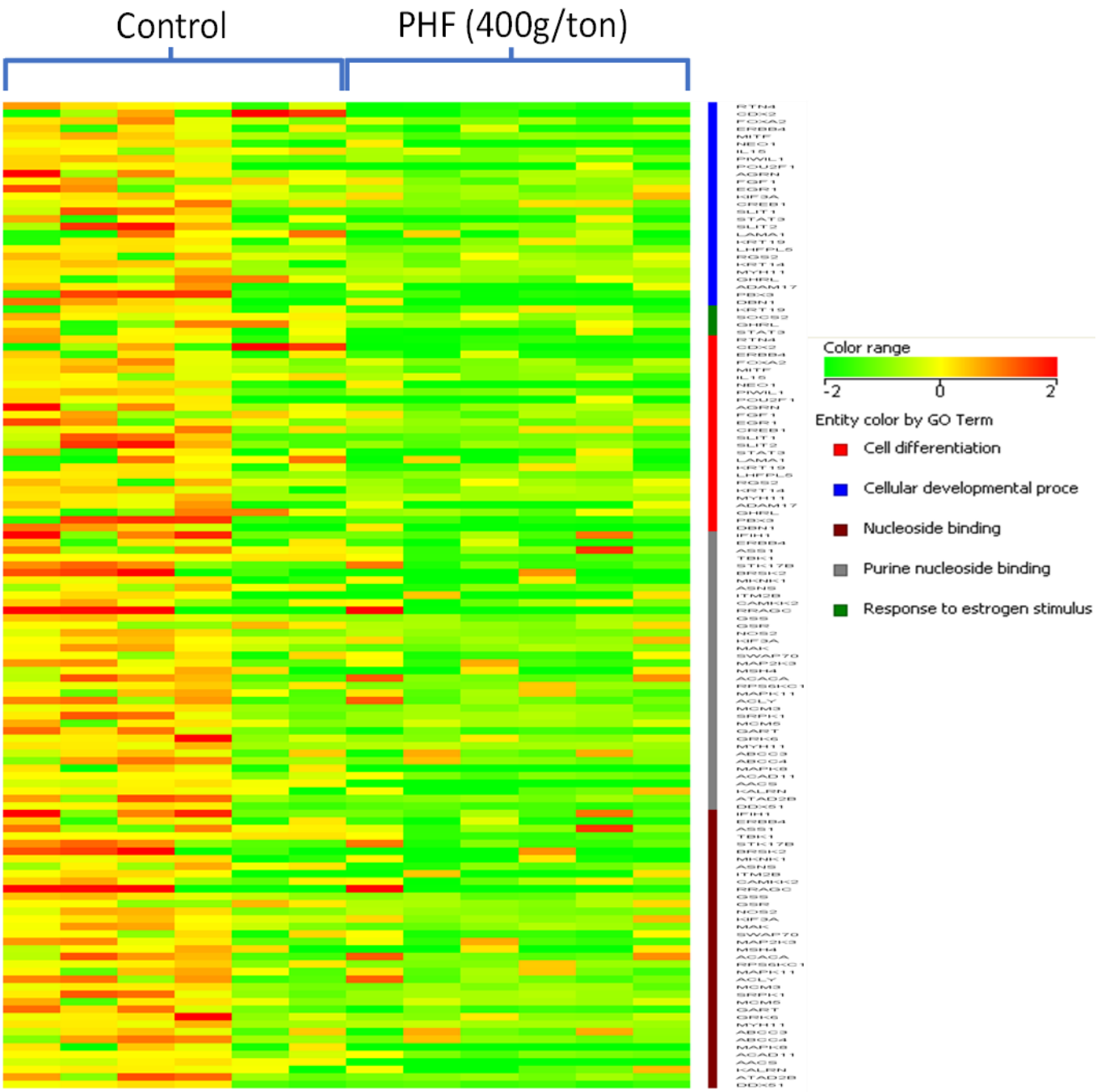

Figure 6. Heatmap showing repressed genes associated with the significant GO terms**.** Up and down-regulated genes are represented by red and green colors, respectively, while yellow represents no change in fold expression values; PHF: Polyherbal formulation

DISCUSSION

This study demonstrated that dietary inclusion of PHF enhanced the performance traits of broiler chickens. Microarray analysis indicated that PHF-mediated transcriptional changes associated with ETC, mitochondrial biogenesis, mitophagy, and structural development were reflected by enhanced FE in chickens. FE is a compound trait affected by two complex traits, such as feed intake and weight gain, which means the genomic profile of FE phenotypes may be different among the populations. Recent studies have proved that high feed efficiency (FE) broilers moderate their nutrient utilization and body energy expenditure based on the amount of gene products responsible for the function of the inner mitochondrial membrane protein. Correspondingly, the

negative performance of low FE broilers was the outcome of disturbances in the electrons or protons gradient, leading to mitochondrial dysfunction, through the reduction of mitochondrial ATP synthesis and generation of higher reactive oxygen species (Iqbal et al., 2005; Krueger et al., 2008; Bottje and Carstens, 2009).

In addition, low FE may be related not only to the expression of genes that encode proteins of ETCs, but also to the lowered activity of the respiratory-chain complexes (Bottje and Carstens, 2009). Furthermore, Ojano-Dirain et al. (2007) discovered variations in the expression of mRNA encoding for mitochondrial transcription factors, proteins, and duodenal tissue between low and high FE broilers by analyzing the expression patterns of the genes involved in mitochondrial energy metabolism (Ojano-Dirain et al., 2007). The above observation demonstrated that FE of birds largely depends on mitochondrial function; however, generating a comprehensive picture of gene expression patterns involved in FE using a gene-by-gene approach would be difficult and time-consuming. Hence, the present study provides, apparently for the first time, a comprehensive analysis of the chicken breast muscle, supplemented with PHF, gene expression profile using Agilent microarray. This microarray technology was used to understand the mitochondrial physiology and phenotypic expression of FE in broilers supplemented with PHF by comparing thousands of mRNAs from a given tissue, and simultaneously provides a wide-ranging assessment of expression levels (Kong et al., 2011; Bottje et al., 2012; D'Souza et al., 2019).

There are four multi-protein complexes (I to IV) and adenosine triphosphate (ATP) synthase (Complex V) in the respiratory chain/oxidative phosphorylation system of the inner mitochondrial membrane. The complex I consists of *ND1, ND2, ND3, ND4, ND4L, ND5, and ND6* parts, which are a large enzyme (NADH dehydrogenase) complex and are responsible for the first step in the electron transport process. The *CYTB* gene encodes for a protein called cytochrome b, which is one of 11 components of a group of proteins called complex III. *COX1*, *COX2*, and *COX3* are the subunits and functional core of the enzyme Cytochrome c oxidase (complex IV) that catalyzes the reduction of oxygen to water (Braun, 2020). *ATP6* gene encodes for subunit A of the F0 functional domain that forms one part (subunit) of a large enzyme called ATP synthase (complex V), which is responsible for the last step of oxidative phosphorylation (Jonckheere et al., 2012). In the mitochondrial respiratory chain, the movement of electrons starts from the complexes I and II through the NADH- or FADH-linked substrates such as glutamate and succinate, respectively, and then travels down the respiratory chain from ubiquinone to the terminal electron acceptor, oxygen (O_2). During this electron transport, a proton (H_2) is also pumped from the matrix to the intermembrane space, which generates the proton-motive force. The resulting force gives the energy for ATP synthesis [from adenosine diphosphate (ADP) and Pi by the F0F1 complex] as protons move back through ATP synthase (Schormann et al., 2019). These explanations demonstrate the relationship of ETC with mitochondrial efficiency in ATP production, which is associated with converting nutrients into body components in birds supplemented with PHF. The current results of microarray analysis revealed that the expression levels of some important genes, including *ND1, ND2, ND3, ND4, ND4L, ND5, ND6, CYTB, COX1, COX2, COX3*, and *ATP6*, implicated in mitochondrial respiratory chain/oxidative phosphorylation, were significantly affected by PHF supplementation. It was corroborated by other studies, which showed that modulation of genes involved in ETC leads to alterations in the utilization of nutrients (Iqbal et al., 2005; Ojano-Dirain et al., 2007; Brito et al., 2016). Nevertheless, any leakage of electrons due to genetic alterations in the mitochondrial DNA causes partial reduction of oxygen to reactive oxygen species (ROS) such as superoxide (O^{2-}) and H_2O_2 (Nolfi-Donegan et al., 2020; Sies et al., 2022). This ROS creates oxidative stress, if not metabolized by antioxidants, and oxidizes the critical biomolecules (e.g., lipids, proteins, and DNA) in the cell, which leads to further mitochondrial DNA modifications and inefficiencies that accentuate additional ROS production. In these conditions, uncoupling protein (UCP) mediated proton conductance gets activated as a negative feedback loop to reduce the ROS generation in the respiratory chain complexes, which results in mild uncoupling (Brand et al., 2004). Interestingly, high avUCP and low protein carbonyl levels were observed in the current study, further signifying the better efficiency of mitochondria in the breast muscle of PHF supplemented birds. This was supported by Raimbault et al. (2001) and Bottje et al. (2006), who observed avUCP gene expression of high FE broilers was higher (Raimbault et al., 2001; Bottje et al., 2006). Similarly, low FE broilers had high protein carbonyl levels, which indicates protein oxidation due to greater oxidative stress in low FE mitochondria (Stadtman and Levine, 2000). Additionally, upregulation of the *Park2* gene in breast muscle substantiates the better

mitochondrial function in the PHF treated group. This gene encodes one protein required for the PINK1/Parkin pathway of mitophagy (macroautophagy or bulk autophagy) that eliminates the damaged or superfluous mitochondria and preserves the population of healthy mitochondria. Greene et al. (2003) and Park et al. (2006) reported that loss of either mitochondrial PINK1/Parkin protein in Drosophila leads to mitochondrial respiratory chain dysfunction, which results in deterioration of flight muscles and dopaminergic neurons (Greene et al., 2003; Park et al., 2006). Surprisingly, the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1-α) gene, peroxisome proliferator-activated receptor (PPAR), and myocyte enhancer factor (MEF2) were also found to be up-regulated in the current study, indicating the enhancement of mitochondrial biogenesis and mitochondrial energy metabolism by PHF supplementation (Dorji et al., 2021). A key mechanism of cell adaptation and repair, mitochondrial biogenesis depends on transcriptional regulation controlled by multiple nuclear-encoded genes (PGC1-α) (Scarpulla, 2008; Piantadosi and Suliman, 2012). This was supported by Kong et al. (2011) and Bottje et al. (2012), who found that mitochondrial biogenesis was enhanced in birds with higher FE. PGC1-α also exerts strong effects on mitochondrial energy metabolism by coactivating both PPAR (nuclear receptor target) and MEF2 genes, which in turn regulate the mitochondrial fatty acid oxidation pathway (Vega et al., 2000) and glucose transport outside the mitochondria, respectively (Michael et al., 2001).

Furthermore, up-regulation of Golgin B1 (GOLGB1), WntA, Wnt11, and Insulin-like Growth Factors (IGF1) genes signifies that the protein accretion and muscle growth were improved in the breast muscle of PHF treated broilers. GOLGB1 is the Golgi integral membrane protein (gigantin), essential for the organization and retention of proteins in the Golgi apparatus (Linstedt and Hauri, 1993). This was supported by the results of Kong et al. (2011), who demonstrated that the up-regulation of GOLGB1 facilitates optimal packaging and transport of proteins to specific cell sites in high FE birds (Kong et al., 2011). Wnt signaling pathway is the positive regulator of myogenesis and is crucial for muscle, adipocytes, and bone development (Duprez, 2002; Yavropoulou and Yovos, 2007; Christodoulides et al., 2009). This was evidenced from the reports of Yue et al. (2011) who suggested that 7 genes (RHOA, CHP, Wnt3A, RAC1, Wnt11, Wnt9A, and MAPK9) on Wnt pathway were significantly associated with broiler weight gain and carcass paramaeters mainly by influencing muscle development rather than preadipocytes differentiation (Yue et al., 2011). IGF1 exerts a general effect on overall weight gain (Yoshida and Delafontaine, 2020), and overexpression in the muscle tissue leads to enhanced muscle growth in chicken (Mitchell et al., 2002). It was supported by Scanes et al. (1989), who stated that IGF-1 levels were significantly reduced in the low growth group in comparison to the high growth group at seven weeks of age.

The above discussions specify that PHF supplementation significantly improved the mitochondrial function in breast muscle as evidenced by the upregulation of molecular-function genes responsible for ETC, mitochondrial biogenesis, and mitochondrial energy metabolism. The PHF not only upregulates the genes at the nuclear level, but also influences the genes at the biological function level, including anatomical structure development (GO:0048856), multicellular organismal development (GO:0007275), and system development (GO:0048731). Interestingly, alteration in the expression of certain specific genes was cross-validated using RT-qPCR and substantiated by corresponding phenotypic traits such as feed efficiency and body weight gain improvement in broilers supplemented with PHF.

PHF also downregulates the genes involved in the apoptosis biological process that includes nucleoside binding (GO:0001882), purine nucleoside binding (GO:0001883), response to estrogen stimulus (GO:0043627), cell differentiation (GO:0030154), and cellular developmental process (GO:0048869).

CONCLUSION

In summary, whole genomic profiling of breast muscle demonstrated a significant modulation of genes involved in enhancing the mitochondrial cellular respiration for ATP generation, mitochondrial biogenesis, mitophagy, and energy metabolism in broiler chickens supplemented with PHF (400g/ton). This was supported by corresponding phenotypic traits such as FE and body weight gain improvement in chickens. These research findings confirm that PHF could be used as a natural cellular feed efficiency enhancer in poultry diets.

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Authors` contributions

Saravanakumar Marimuthu, Subramaniyam Suresh, and Prashanth D'Souza participated in sample collection. Subramaniyam Suresh drafted the manuscript. Saravanakumar Marimuthu revised the manuscript. Prashanth D'Souza designed the study and analyzed the data. All authors read and approved the final manuscript.

Availability of data and materials

The published research paper included all the data collected and analyzed during the conduct of the study.

Ethical considerations

This manuscript has not been published, accepted for publication, or is undergoing editorial review elsewhere. It also does not contain any sentences that have been plagiarized.

Conflict of interests

All authors declare that there is no conflict of interest.

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