

## Original Research Article

# Modulatory effect of Kolin Plus™, a polyherbal formulation on muscle growth development in choline deficient diet model of broilers: a microarray approach

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## ABSTRACT

**Background:** The present study was conducted to elucidate the genes and its associated pathways delineating the growth-promoting potential of polyherbal formulation (PHF), Kolin Plus™ using microarray in Cobb 430 broiler chickens.

**Methods:** Microarray was performed for four groups, namely, normal diet (ND) as G1, choline chloride deficient (CCD) diet as G2, choline chloride (CCL, 400 g/ton) as G3 and PHF (400 g/ton) as G4. Breast muscle samples were collected, and the growth-related gene expression profile was measured using the Agilent microarray platform.

**Results:** Totally 2900 differentially expressed genes (DEGs) in muscle tissue sample was revealed using hierarchical clustering based on the similarity of their expression profiles, which further allows the user to pick out groups of similar genes. Among them, 1000, 364 and 481 genes were significantly upregulated and 244, 485 and 326 genes were significantly downregulated between ND and CCD, CCL and CCD, PHF and CCD respectively. Furthermore, some of the focused genes (CSRP3, SOX10, BCO1, CALB1, LMOD2, KLF15, CTHRC1, PHGDH, UTS2R, and ANKRD2) were significantly ( $p < 0.05$ ) modulated by PHF (400 g/ton) supplementation in birds fed with CCD diet. These genes play an essential role in protein translation, energy metabolism, and muscle growth promotion.

**Conclusions:** It may be concluded that supplementation of PHF at 400 g/ton of feed could positively influence the certain focused genes associated with muscle growth promotion, which favours the productive phenotypic response in broiler chickens fed with CCD diet.

**Keywords:** Choline chloride, Muscle growth, Broilers, Polyherbal formulation, Microarray, Differentially expressed genes

## INTRODUCTION

Nutrition plays a critical role in capitalizing the productivity of livestock farming, as it can regulate the systemic energy homeostasis by controlling metabolic processes in the body.<sup>1,2</sup> Diets with the proper balance of

energy, protein, minerals, vitamins and essential fatty acids are vital to achieving the optimum growth performance in broiler chickens.<sup>3</sup> Moreover, skeletal muscle, an exquisitely sensitive tissue, plays a pivotal role in the growth of animals due to its significant mass that largely contributes to the whole-body energy

balance, and is also capable of increasing its mass in response to nutrient enriched feed supplementation.<sup>4,5</sup> However, only part of the nutrient requirement is met by feedstuff, therefore it is necessary to include additional nutrients in feed formulation.

Among the other additives, choline is one of the important water-soluble vitamin-like compounds as it can improve the production performance in broiler chickens.<sup>6</sup> Choline is a quaternary ammonium salt and an essential component of membrane phospholipids that contributes to the structural integrity and porosity of cell membranes.<sup>7</sup> In addition, choline and its derived metabolites such as acetylcholine and sphingomyelin serves as a precursor for neurotransmission and signal transduction mechanism, respectively.<sup>8</sup> Furthermore, choline is oxidized to betaine, an essential intracellular organic osmoregulator involved in methylation reaction, and regulation of gene expression through transcription factor activation in chickens.<sup>9</sup> Specifically, the biosynthesis of phospholipids plays an important role in the cell cycle as the membrane expansion is required for the doubling of cells during mitosis.<sup>10</sup> Evidence from several studies shows that the fatty acid component of phospholipids regulates the skeletal muscle mass and function.<sup>11</sup> Moreover, consumption of alpha-linolenic acid enriched diet improved the muscle morphology and function, including enlarged myofibres in the dystrophic hamsters.<sup>12</sup> Furthermore, the young chicks need more choline to prevent the growth retardation and perosis due to its inability to synthesize at a sufficient rate. Therefore, the choline or conjugated choline could be considered as an essential component to be added in broiler ration for better growth performance. We had earlier demonstrated the performance-enhanced effects of polyherbal formulation (PHF), Kolin Plus™ in broiler chickens fed with choline chloride deficient (CCD) diet.<sup>13</sup>

To the best of our knowledge, there are no scientific reports unravelling the genes and pathways influenced by choline deficiency in broiler chickens. In the current study, we used gene expression profiling by microarray to test our hypothesis that the increase in muscle growth due to supplementation of PHF is accompanied by regulation of differentially expressed genes (DEGs) in broiler chickens. Several reports suggested that microarray analysis of DEGs during skeletal muscle growth and development in broiler chickens. As moreover, DEGs related to growth and development was identified in skeletal muscle of broiler chickens using microarray analysis.<sup>14</sup> Therefore, the present study was planned to delineate the various underlying genes and pathways related to muscle growth in the tissue samples using microarray technology and two separate bioinformatics analysis systems, namely, Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 and Gene Ontology (GO).

## METHODS

### *Polyherbal formulation*

Kolin Plus™ is a proprietary PHF developed by M/s. Natural Remedies Private Limited, Bengaluru, India, containing *Acacia nilotica* and *Curcuma longa* plant parts.

### *Ethical approval and experimental design*

The approval was taken from the Institutional Ethics Committee (AHS/PR/01/2016) and all the animal experiments were performed at the poultry research station recognised by the Department of Scientific and Industrial Research, India; DSIR Reg. No.: TU/IV-RD/2000/2016 (situated in Tamil Nadu, India) for a period of 43 days. All birds were reared for 43 days under recommended managerial conditions according to manual of Cobb 430 broiler management. The detailed procedures related to groups allocation and methods used for rearing the broilers were reported previously.<sup>13</sup> Briefly, 900 one-day-old Cobb 430 broiler chicks were randomly assigned to six groups (5 replicates/group; 30 birds/replicate) consisting of a normal diet (ND) group as G1, CCD diet group as G2, choline chloride (CCL, 400 g/ton) group as G3 and three treatments of PHF (200, 400 and 500 g/ton) as G4, G5 and G6 respectively. CCD diet was employed to induce choline deficiency in CCD, CCL and PHF groups using 75% soya bean meal (SBM) and 25% soy protein isolate as a source of protein. However, PHF at 400 g/ton (G5) showed optimum performance when compared to other groups, which indicates that it could effectively replace the role of CCL in broiler chickens. Based on the outcome of the phenotypic response, four groups [ND, CCD, CCL (400 g/ton) and PHF (400 g/ton)] were selected for microarray hybridization, microarray data collection and analysis.

### *Isolation of RNA from muscle tissue*

RNA was isolated from breast muscle tissues of selected broilers using TRIzol reagent (Invitrogen, USA) in a frozen state. The quantity and purity of RNA were determined by NanoDrop ND-2000 spectrophotometer at 260/230 and 260/280 nm ratio (NanoDrop Technologies, Wilmington, Delaware). As the RNA quality was essential for microarray experiments, the integrity of total RNA was assessed with an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). RNA samples with RIN values of 6 or higher, were selected and used for further analysis.

### *cRNA preparation and probe labelling*

The labelled complementary RNA (cRNA) probes were generated by One Color RNA Spike-In Kit along with Agilent Quick Amp Kit, One-Color (Agilent Technologies, Palo Alto, CA) following the manufacturer's instructions. Briefly, double-stranded

complementary DNA (cDNA) was synthesized from 200 ng total RNA using MMLV-RT with T7 promoter primer. Cyanine-labelled cRNA targets were then transcribed *in vitro* using T7 RNA polymerase. Then the labelled cRNA probes were purified using the Qiagen RNeasy® Mini Kit (Qiagen Inc., Valencia, CA), and the concentration and quality of labelled cRNA probes were determined using a Nano-drop (ND-1000) spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). The labelling efficiency was determined using the yield and specific activity. The specific activity less than 6 and total yield less than 600ng of cRNA were considered as failed and repeated till the QC parameters were optimal.

### **Microarray hybridization**

In the current study, three biological pooled samples per group (2 samples per pool in equimolar concentrations) made from each tissue was considered to evaluate the gene expression variation between the control and treatment groups. The chicken gene expression array was designed using 28078 target mRNA sequences downloaded from NCBI's blast database. The array was designed in 4x44 K format with 45220 features. Of these 43803 features were available for custom probes and 1417 features were filled by Agilent control probes. Around 43154 probes were designed using Agilent's E-array portal and were included in the array and the blank features were filled by replicates.

Six hundred nanograms of labelled cRNA was fragmented at 60°C for 30 minutes in a fragmentation mixture containing 5 µl Agilent Blocking Agent (10x), 1 µl fragmentation buffer and 25 µl nuclease-free water, and the fragmentation was stopped by adding of 2x hybridization buffer. Then the final hybridization mixture for the 4x44 K Whole chicken genome microarrays (8 arrays/slide; total volume 50 µl–each 25 µl) was prepared by adding cRNA from fragmentation mixture to Agilent hybridization buffer (2x Lot # 6282962), (2xGE, HI-RPM). The sample was spun down for 1 minute at room temperature (RT) and immediately loaded onto the Agilent gasket slide. Hybridization on microarray slides (Agilent) was then carried out at 65 °C for 16 hrs using an Agilent sureHyb chamber and an Agilent hybridization oven. Slides were washed in Gene Expression wash buffer I (Agilent) at RT for one minute and in Gene Expression wash buffer II (Agilent, pre-warmed to 37 °C) for one more minute. Then all the slides were dried and arranged into an appropriate slide holder for scanning.

### **Microarray data collection and analysis**

Raw data text files were created using the Agilent's Feature Extraction software, containing the probe sequences (60 base pair length), the chromosome coordinates, gene profile, systematic name, description for the genes and the raw signal intensity for the probes.

The reproducibility of the data derived from microarray analysis was determined after normalizing the raw data files using 75<sup>th</sup> percentile shift method. To execute the normalization of intra-array signal intensity, processed signal (dye normalized background subtracted signal intensity) was log-transformed and then 75<sup>th</sup> percentile value was calculated separately for each array. In each breast muscle sample, the log-transformed intensity values for each probe was subtracted by the calculated 75<sup>th</sup> percentile value of the respective array and expression values were obtained.

### **Identification and hierarchical clustering of DEGs**

The log-transformed normalized data were further interrogated for the DEGs. The cut-off criteria for selecting the DEGs was  $\pm 0.6$  and the student t-test was applied to generate the p value (<0.05) for the similar fold change between the treatment and control samples in replicates.

### **Bioinformatics**

The DEGs were studied with pathway and function analysis tools to correlate with already existing biological pathways using multiple public databases. To understand the dynamics of gene expression, we applied DAVID 6.7 online analysis tool which provides typical batch annotation and GO term enrichment analysis to emphasize the biologically important GO terms associated with muscle growth and development.

## **RESULTS**

### **Phenotypic traits**

All chicks were healthy during the entire course of the experiment. The phenotypic response was significantly worsened in chickens fed with CCD diet as compared to ND (bodyweight- 2,051 g vs 2,134 g; feed conversion ratio- 1.71 vs 1.67). However, supplementation of PHF (400 g/ton) improved the performance parameters when compared to CCD group (bodyweight- 2,135 g vs 2,051 g; feed conversion ratio- 1.64 vs 1.71) and CCL (400 g/ton) group (bodyweight- 2,135 g vs 2,092 g; feed conversion ratio- 1.64 vs 1.68).<sup>13</sup>

### **Identification of DEGs**

The raw data files were intra-array normalized and then validated using gene spring GX software. A comparative study was conducted by comparing gene expression profiles in each tissue between treatment, ND and CCD respectively. A total of 2900 DEGs in muscle tissue sample was revealed using hierarchical clustering based on the similarity of their expression profiles, which further allows the user to pick out groups of similar genes. Among them, 1000, 364 and 481 genes were significantly upregulated and 244, 485 and 326 genes were significantly downregulated between ND and CCD,

CCL and CCD, PHF and CCD respectively in breast muscle tissue samples.

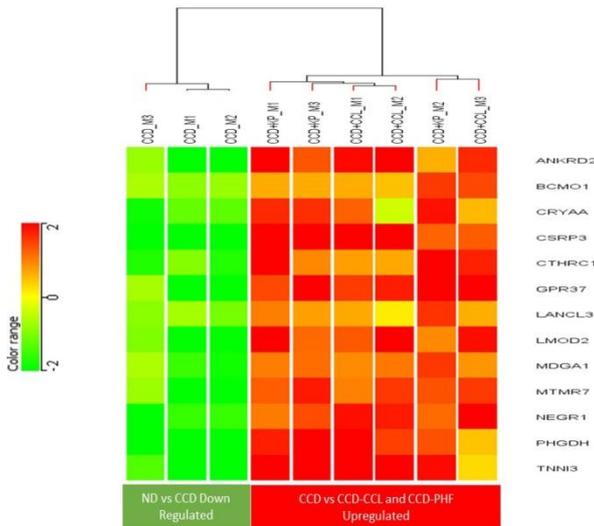


Figure 1: Heat map represents the up regulated genes of PHF and CCL and down regulated genes of CCD.

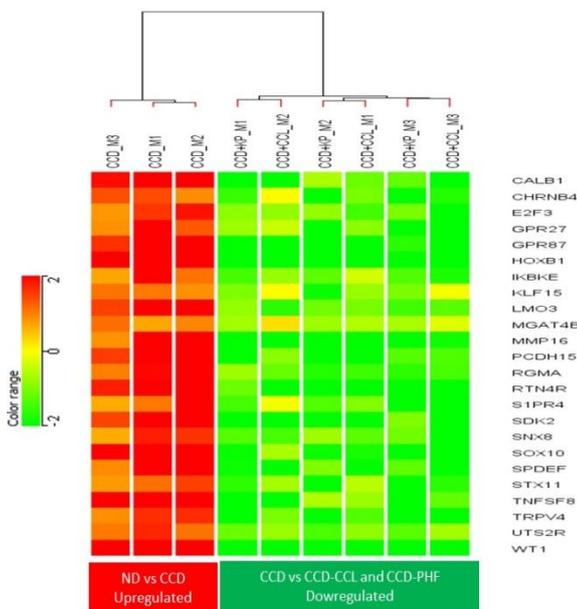


Figure 2: Heat map represents the up regulated genes of CCD and down regulated genes of PHF and CCL.

**Biological function clustering**

GO terms were annotated for all the DEGs whose expression were significantly enriched by CCL/PHF supplementation by performing the functional analysis (biological processes). Cellular component, biological process and molecular function are the three main components used for GO annotation. However, biological processes may arguably be the more relevant aspect of GO in relation to this study, therefore, only functional clusters belonging to this component have been presented. CCD and supplementation of PHF induced a

wide range of biological events in muscle tissues, including muscle contraction and development, plasma membrane development, growth and tissue development, neurogenesis and synapse function, neurotransmission, metabolic pathway and cell adhesion, and lipid metabolism. The reduced expression of genes from muscle tissues in the CCD group (vs ND group) was comparatively upregulated in PHF and CCL supplemented groups (Figure 1). Similarly, upregulated genes from muscle tissues in the CCD group (vs ND) were downregulated in PHF and CCL supplemented groups (Figure 2). These genes seem to play an essential role in protein translation, energy metabolism, and muscle growth promotion. Furthermore, the fold change of some focused DEGs genes relevant to muscle development were represented in Table 1.

Table 1: Representation of selected gene expression fold change.

Genes	Fold change		
	ND vs CCD	PHF vs CCD	CCL vs CCD
CALB1	2.895	-1.698	-2.392
SOX10	2.213	-2.148	-2.084
KLF15	0.999	-1.329	-0.290
UTS2R	1.281	-1.295	-0.797
CSRP3	-4.748	2.345	3.271
BCO1	-0.789	0.952	0.854
LMOD2	-1.849	1.568	1.774
CTHRC1	-1.482	2.549	1.060
PHGDH	-2.515	1.927	1.379
ANKRD2	-1.798	1.579	2.264

Gene fold change are provided in terms of log base 2 and are expressed as mean of 3 replicates.

**DISCUSSION**

Agricultural scientists and farmers have long been concerned with the improvement to the muscle mass of meat-producing livestock.<sup>15,16</sup> Poultry is one of the fastest-growing livestock sectors in developing countries. In poultry farming, growth performance plays a critical role in deciding the profitability. There is always a need for a product to improve the performance without disturbing birds health.<sup>17</sup> However, the inability to synthesize adequate quantity of choline leads to nutrient deficiency mediated growth retardation, fatty liver and perosis in young growing chicks.<sup>13,18</sup> Moreover, synthetic choline chloride encountered several drawbacks such as less bioavailability and hygroscopicity. Due to the threat of using synthetic products, there is always a search for herbal replacer.

In our previous report, we investigated the choline-like effect of PHF using CCD diet model developed by the substitution of intact SBM by soy protein isolate (defatted SBM).<sup>13</sup> Growth performance, FCR and breast yield were improved by PHF supplementation. The

productivity of farm animals is directly related to the cellular and molecular mechanisms regulating skeletal muscle growth and development.<sup>5</sup> Here, we hypothesised that the modulation of certain gene expression would influence muscle growth and differentiation. Hence, the current study was carried out to delineate the effects of PHF on growth-related genes in the muscle of the broilers fed with CCD diet. Genetic analyses were used to complement morphological changes and better understand the physiological events during muscle development. Microarray techniques have made it possible to relate the physiological state of the cell to different gene expression profile for studying large scale analysis of DNA sequences on a small surface.

CCD diet upregulated the SOX10, CALB1, KLF15 and UTS2R genes as compared to the ND group. Upon dietary supplementation of PHF and CCL to birds significantly downregulated the SOX10, CALB1, KLF15 and UTS2R genes when compared to the CCD group. Sry-related HMg-Box gene 10 (SOX10) regulates the embryonic developmental process and provides the survival cues for skeletal muscle progenitor cells as well.<sup>19</sup> Schmidt et al suggested that upregulation of SOX subgroup E (SOX9 and SOX10) proteins serve as inhibitors of skeletal muscle development and its decreased expression was noticed in late embryogenesis or during myogenic differentiation in the C2C12 system.<sup>20</sup> Our findings are in good agreement with previous reports that modulation of SOX10 gene expression may interfere with the expression of transcription factors required for myogenic differentiation.<sup>19,20</sup> Calbindin 1 protein encoded by CALB1 gene belongs to calcium-binding protein superfamily that includes calmodulin and troponin C. In addition to Yoshizawa et al reported that CALB1 gene is strongly associated with the degeneration of skeletal muscle in chickens.<sup>21</sup> Moreover, Mehedint et al also reported that mice fed with choline-deficient diet and choline-rich diet caused increased and decreased expression of CALB1 gene respectively.<sup>22</sup> Kruppel like Factor 15 (KLF15) regulates the skeletal muscle lipid utilization and physiologic performance in response to nutrient deprivation.<sup>23</sup> As the CCD birds were deficient in choline, the upregulation of KLF15 has occurred to compensate for the lipid utilization. Higher expression of KLF15 gene leads to activation of muscle atrophy-related proteins, which in turn promotes the loss of skeletal muscle mass in mice.<sup>24</sup> Urotensin 2 Receptor (UTS2R) gene actively involved in the regulation of skeletal muscle fat accumulation and fatty acid metabolism in cattle.<sup>25</sup> Pan et al also demonstrated that upregulation of UTS2 expression in skeletal muscle tissue was accompanied by reduced skeletal muscle weight and leads to muscle atrophy in mice.<sup>26</sup> The above literature substantiates our findings that dietary supplementation of PHF and CCL might contribute to the rapid growth of breast muscle and development through modulating the expression of SOX10, CALB1, KLF15 and UTS2R genes in broilers fed with CCD diet.

CCD diet downregulated the CSRP3, BCO1, CTHRC1, LMOD2, PHGDH and ANKRD2 genes as compared to the ND group. At the same time, high-level expression of CSRP3, BCO1, LMOD2, CTHRC1, PHGDH and ANKRD2 genes was noticed in birds fed with PHF and CCL diet. Cysteine and glycine rich protein (CSRP3) is a microtubule-related protein which was upregulated in the breast muscles of broilers.<sup>27</sup> Overexpression of CSRP3 in C2C12 cells enhanced the myogenic differentiation, whereas inhibition of CSRP3 gene expression blocked the myogenic differentiation of myoblast into skeletal muscle.<sup>28</sup> Beta-carotene oxygenase 1 (BCO1) is a cytoplasmic protein that acts as a carotenoid-cleaving enzyme in the  $\beta$ -carotene metabolism and converts it into retinoid.<sup>29</sup> Genetic disruption of BCO1 expression due to nutrient-deficient diet promotes the  $\beta$ -carotene accumulation leading to vitamin A deficiency and affecting the myoblast differentiation in broilers.<sup>30</sup> Collagen triple helix repeat containing 1 (CTHRC1) protein is expressed in neonatal smooth muscle cells and its upregulation correlates with muscle growth.<sup>31</sup> Duarte et al also observed that overexpression of CTHRC1 gene in skeletal muscle.<sup>32</sup> Muscle-specific isoform of leiomodulin (LMOD2) gene is associated with actin-myosin binding and muscle contraction. LMOD2 deficient mice showed disrupted sarcomeres assembly and up-regulation of LMOD2 expression results in elongation of sarcomere assembly in myocytes.<sup>33</sup> Kong et al reported that upregulation of LMOD2 gene responsible for skeletal muscle growth in broiler chickens.<sup>34</sup> Phosphoglycerate dehydrogenase (PHGDH) is a key metabolic enzyme in de novo serine biosynthesis.<sup>35</sup> Brown et al suggested that increased expression of PHGDH genes enhanced the skeletal muscle growth in ractopamine treated pigs.<sup>36</sup> Furthermore, upregulation of PHGDH gene was correlated with higher muscle content in broilers.<sup>37</sup> Ankyrin Repeat Domain 2 (ANKRD2) prominently expressed in skeletal than cardiac muscle, which played an intriguing role in skeletal muscle plasticity.<sup>38</sup> Accumulating evidence revealed that ANKRD2 gene expression is downregulated in patients with muscular dystrophy.<sup>39</sup> Furthermore, Chaillou et al reported that increased expression of ANKRD2 gene is responsible for the muscle growth and development in mice.<sup>40</sup> Thus, increased expression of CSRP3, BCO1, LMOD2, CTHRC1, PHGDH and ANKRD2 gene substantiate its involvement in muscle mass and weight gain of broilers fed with PHF and CCL diet.

## CONCLUSION

We report for the first time that dietary supplementation of PHF improved the muscle growth by downregulating (SOX10, CALB1, KLF15 and UTS2R) and upregulating (CSRP3, BCO1, CTHRC1, LMOD2, PHGDH and ANKRD2) genes of birds fed with CCD. We, therefore, speculated that supplementation of PHF was most likely to promote the muscle gain, which is ultimately reflected from better growth performance in CCD diet fed broiler chickens. The phytoconstituents present in the PHF

could be the reason for improved growth performance. Thus, it can be concluded that PHF can replace synthetic choline chloride as evident by the upregulation of muscle growth promoting genes in broilers.

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