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Assessment of Different Tannin Extracts on Avian Pathogenic *Escherichia coli* Metabolites Using Nuclear Magnetic Resonance

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A B S T R A C T

Tannins have been demonstrated to inhibit the growth of several chicken illnesses in vitro. The complex compositions of tannins make it difficult for microorganisms to develop bacterial resistance. This study aimed to evaluate the effect of condensed tannins (CT) extracts on metabolic profile of Avian Pathogenic *Escherichia coli* (APEC) using Nuclear Magnetic Resonance (^{1}H-NMR). The experimental groups were divided into three groups: control (no CT added), high in procyanidins (PC-CT) group, and high in prodelphinidins (PD-CT) group, with exposure times of 0, 10, and 24 h. APEC was observed to respond to CT extracted from Tilia flowers (high PC-CT) and black locust leaves (high PD-CT). The levels of amino acids including lysine, leucine, glutamate, phenylalanine, and pyroglutamate were increased with the high PD-CT treatment; however, no significant differences were observed between the PC-CT group and the control. Treatment of APEC culture with high PD-CT also led to a significant decrease in the level of lactate. Thus, high PD-CT affected these metabolisms and could be exploited to control the proliferation of APEC in poultry, thereby improving their health and performance.

Keywords: condensed tannins, ^{1}H-NMR, avian pathogenic *E. coli*, amino acids, fatty acids

INTRODUCTION

Plant extracts have been utilized as supplements in animal feeding due to their antibacterial activities. These extracts have shown potential to inhibit the pathogenic effects of various unwanted intestinal bacteria, thereby improving animal health (1). Consequently, numerous studies have investigated the phytochemical composition, biological activities, and antimicrobial properties of these plant extracts (2, 3).

Tannins, categorized as water-soluble polyphenolic substances with varying molecular masses, are commonly found in plants. Among them, condensed tannins (CT) represent a significant subset present in different plant species. These CTs include forms like procyanidins (PC) and prodelphinidins (PD), which exist as small oligomers or large chains and exhibit flavan-3-ol subunit geometries (4). The antimicrobial potential of CTs has been recognized, as their incorporation into animal diets has been linked to improved animal health and performance (2).

Animal productivity can be compromised by various harmful microorganisms (5). Notably, Avian pathogenic *Escherichia coli* (APEC) strains have been isolated from the

intestines of infected chickens, with some APEC strains causing colibacillosis in birds and posing a potential foodborne zoonotic risk. Research has explored control strategies, antibiotic resistance, and vaccine development against APEC. Additionally, factors like metabolism play a role in APEC's pathogenesis (6). CTs, specifically, have demonstrated the ability to suppress the growth of both Gram-negative and Gram-positive pathogenic bacteria (1). Phenolic compounds, based on evidence, hinder the attachment of bacteria such as *E. coli* to the gut epithelium and other organs, thereby preventing infections. Some phenolic compounds also exhibit bacteriostatic properties that impede infection development. Although research has primarily focused on phenolic compounds and their effects on various harmful microorganisms, including *E. coli*, differences in antimicrobial activities have been observed among different phenolic acids (7).

Nuclear Magnetic Resonance (NMR) spectroscopy, particularly ^{1}H-NMR, presents a rapid and efficient method for analyzing metabolites with minimal sample preparation. This technique involves subjecting atom centers to strong magnetic fields and radio-frequency pulses. Nuclei with irregular atomic ratios, or NMR-active nuclei, respond to the electric field and exhibit nuclear spin. Upon receiving these pulses, nuclei transition from low-energy to higher-energy spin states, subsequently emitting energy during a relaxation phase. This process allows for the detection and characterization of metabolites within a sample and is particularly useful for identifying multiple compounds simultaneously and quantifying changes in their concentrations (8).

The aim of this study was to examine how different CT compositions affected the metabolic profile of APEC cultures. This data could then be used to evaluate the potential of using these compounds as natural antimicrobials against APEC infections in poultry.

MATERIALS AND METHODS

Preparation of Condensed Tannins

Black locust leaves (*Robinia pseudoacacia*) and tilia flower (*Tilia* L.), these samples were purchased from Reading University, UK; and were oven-dried at 70 °C (Agilent Technologies, Waldbronn, Germany); then samples were ground by an impeller SM1 cutting mill (Retsch, Haan, Germany) to pass through a 1 mm sieve. These samples have a high CT concentration after being examined for total CT using the HCl-butanol-acetone technique (9). The samples were then purified using aqueous acetone (20/80 v/v) on the Sephadex LH-20 column as described by (10). The composition and concentration of different CT within the samples were then determined using the Thiolytic technique and HPLC–MS (11). Then, the tannin samples were kept in glass tubes at -20 °C.

Preparation of Samples with Bacterial Cultures

Three subtypes of APEC-O78 were grown at 37 °C in Luria Bertani (LB) medium on a shaker at a speed of 100 rpm, either in the presence or absence of CT, after being isolated from sick hens (12). These three tubes for each treatment were replicated six times for technical accuracy. In each tube, 50 mL of LB medium containing PD-CT or PC-CT was prepared with CT levels adjusted to 0.6 mg/mL, and 10 mL of APEC media were added. Control tubes were prepared using only APEC-LB broth. As mentioned earlier, this experiment was conducted at a pH of 6.5 and a temperature of 37 °C (13). Under aerobic conditions, the test bottles and their contents were incubated for 24 h. At 0, 10, and 24 h, 6 mL of incubation broth was extracted from each sample. To prepare for NMR research, these samples were preserved at -80 °C and stored in liquid nitrogen (14).

Sample Preparation for NMR

Incubation media specimens were enclosed in tubes, which were thawed in warm water at 25 °C. 400 µL of every collection was then transferred to a sterile microfuge vessel, where it was combined with 200 µL of phosphate buffer. The supernatants from these samples were placed into the NMR vials after being centrifuged at 10000 g for 10 min and then vortexed for 5 min. The supplies and apparatus for the prior experiment were identically prepared by 15, and the data were analyzed in the same way as described by (16).

Analysis of Processing Data

As outlined in reference (17), all spectra were acquired using MestReNova (Version 11.0.3). The results underwent filtering and processing to adjust the baseline and align the spectrum phasing measurement to TSP at (δ 0.00). For the purpose of eliminating extraneous information and mitigating data bias (between δ 0.5 and after δ 9.5), the acquired spectra were subsequently imported into MATLAB (Version R2017a). Moreover, the region containing the water peaks (at δ 4.8 and δ 5.0) was also excluded (18).

Statistical Analysis

By combining MATLAB with the Korrigan Toolbox (version 0.1, UK), a multiplex analytical method was used to assess the differences between these treatments. Principal Component Analysis (PCA) was applied, and probable outliers were discovered after the investigation. The effects of time and treatment were examined through the use of orthogonal projections to latent structure discrimination (OPLS). The R^2Y values were near 1 and the Q^2Y values were positive. An overestimate of less than 50% was considered to be a sign of an accurate system (19).

RESULTS AND DISCUSSION

Tilia flowers had a molar percentage of the PD to PC ratio of 3.0 (96.0%), whereas black locust leaves had an average polymerization of 9.8 and the molar level of the PD to PC ratio was 74.0 (25.0%). However, the CT contents reached 1 g CT/100 g extracts (purity of 90%).

The combined effect of intervention and duration on the metabolites is shown in Figure 1. The peak colors which range from blue to red, represent the relationship of the coefficient's magnitude. The outcome revealed various constituent amounts between the treated and untreated. Nevertheless, this graph demonstrates many levels associated with energy metabolic by-products such as acetate, succinate, and lactate that were noticeably changed in APEC strains treated with CT, particularly PD-CT. Acetate is a by-product of anaerobic fermentation, whereas succinate is an intermediate synthetic output of the TCA phase, which is an aerobic process (20).

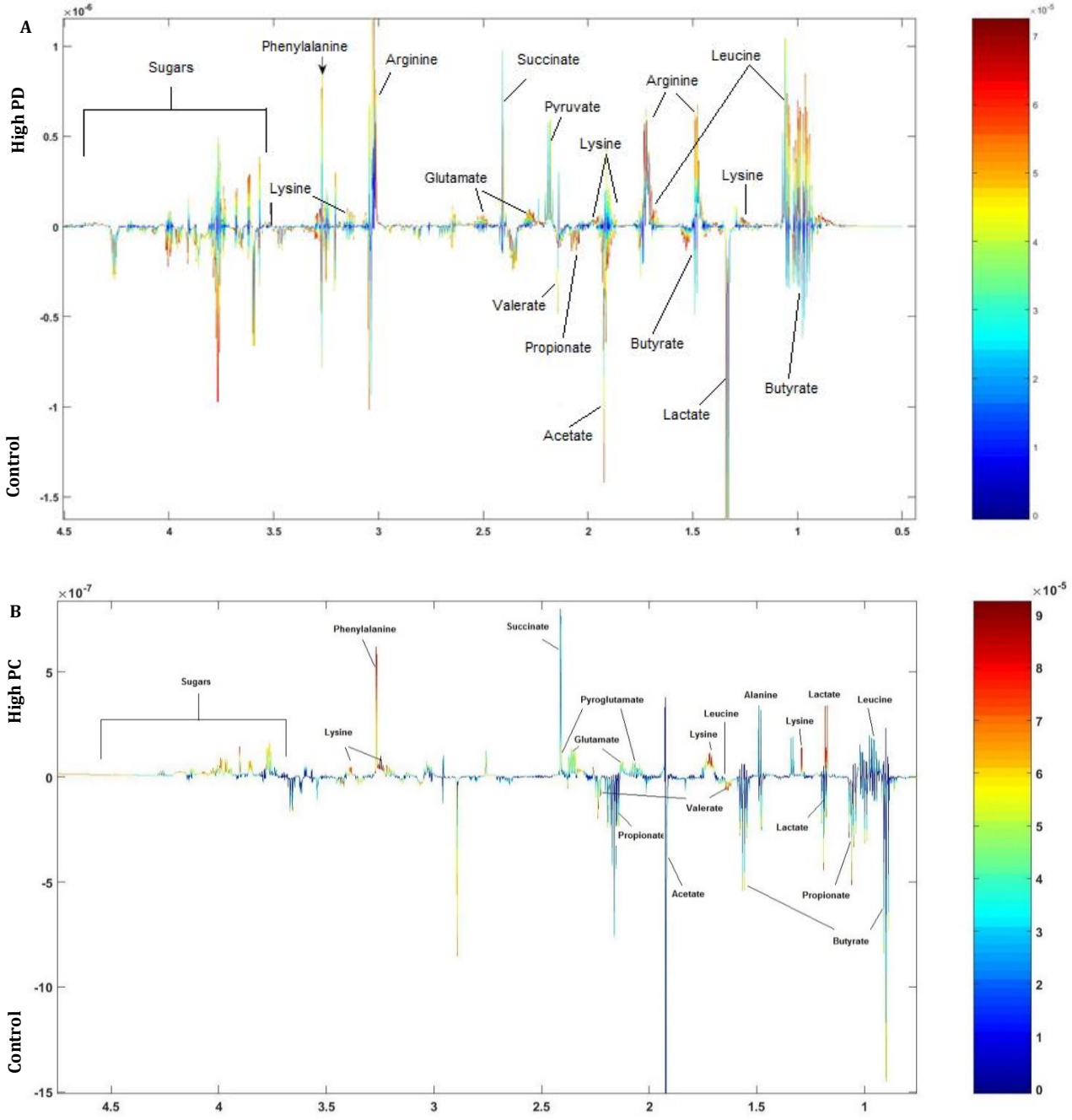

Figure 1. ^{1}H-NMR spectra colour plot shows the most important metabolites of APEC after 24 h between controls (bottom) and treated CTs (top) either **(A)** black locust (high PD at 0.6 mg/mL) or **(B)** tilia flowers (high PC at 0.6 mg/mL)

According to the present research, APEC exchanged with oxygen to generate the expected energy sources digestion by-products, yet once CT was administered, these fatty acid processes were blocked. The levels of carbohydrates were also not significantly affected by either approach or duration, but mannitol and fructose both rose with exposure (21) and the levels were higher when APEC was cultured into PD-CT. However, there was no meaningful interplay between the therapy and the passage of the period. Accordingly, when PD-CT was added to the APEC media, some metabolites were produced such as fructose, mannitol, glutamate, pyroglutamate, lysine, and phenylalanine; further, the synthesis of lactate and acetate was inhibited (22). The existence of CT in the group's given treatment was shown by signals >5.5 ppm in the CT peaks that were present in the aromatic region of the spectra (14). After 24 h, some of these peaks vanished, indicating that some of the CT had likely been metabolized by microbes as a source of nutrition. As anticipated, the control showed no signs of any CT peak areas (Figure 2).

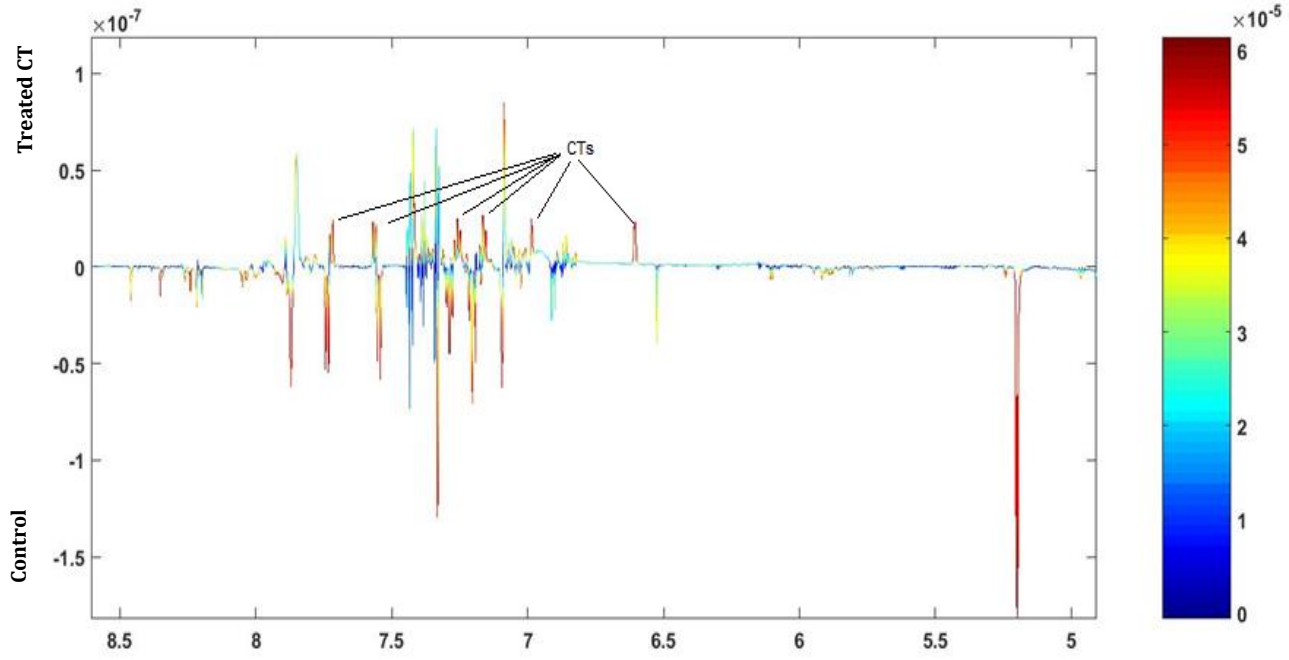

Figure 2. The color plot of ^{1}H-NMR spectra shows the aromatic region (> 5.5 ppm) in APEC culture after 24 h with or without CT compositions

On the other hand, Figures 3 and 4 show the results of a category system that looked at the uniformity between examples (16), and the PCA coupled with orthogonal partial OPLS. A color plot that depicts these data and explains the change in metabolism products with the various therapies at the different time segments yielded R^2 and Q^2 values of 65% for the first two variables (0, 10, and 24 h). At time points 10 h and 24 h, which are equally reported by (15), the OPLS plot distinguished among the control and treated CT groups, which is based on the first main element. The current graphs demonstrated that the concentrations of molecules in the treatment and control groups differed by $P<0.05$. Additionally, at various periods, these variations have been mapped in various colors (18).

Therefore, concentrations of these metabolites not only changed with time but were also affected by the different CTs, with PD having a much greater impact on APEC metabolism than PC. These findings matched with (14), who investigated in vitro effect of CT extracts on the major metabolites in the caecal chickens. As a result, the recent results might offer insight into the immunological and digestive conditions of poultry and serve as a foundation for more immunization studies, as (23) previously noted.

The apparent results of this research were the inhibition of APEC growth by both CT types. The PD-CT resulted in a much more pronounced suppression, which had an impact on both protein and energy consumption. In an in vitro investigation, CT with high molar fractions of PD-CT significantly inhibited APEC fermentation, which interfered with the development of this pathogen. This impact was larger than that of PC-CT.

Moreover, different amino acids such as lysine, glutamate, pyroglutamate, leucine, and phenylalanine were significantly increased that collaborated with PD-CT, as well as this treatment led to a significant decrease in the level of lactate; in contrast, no significant differences were recorded between the PC-CT and the control. In general, PD-CT showed significant impacts on these metabolisms and could be exploited to control the proliferation of APEC in poultry.

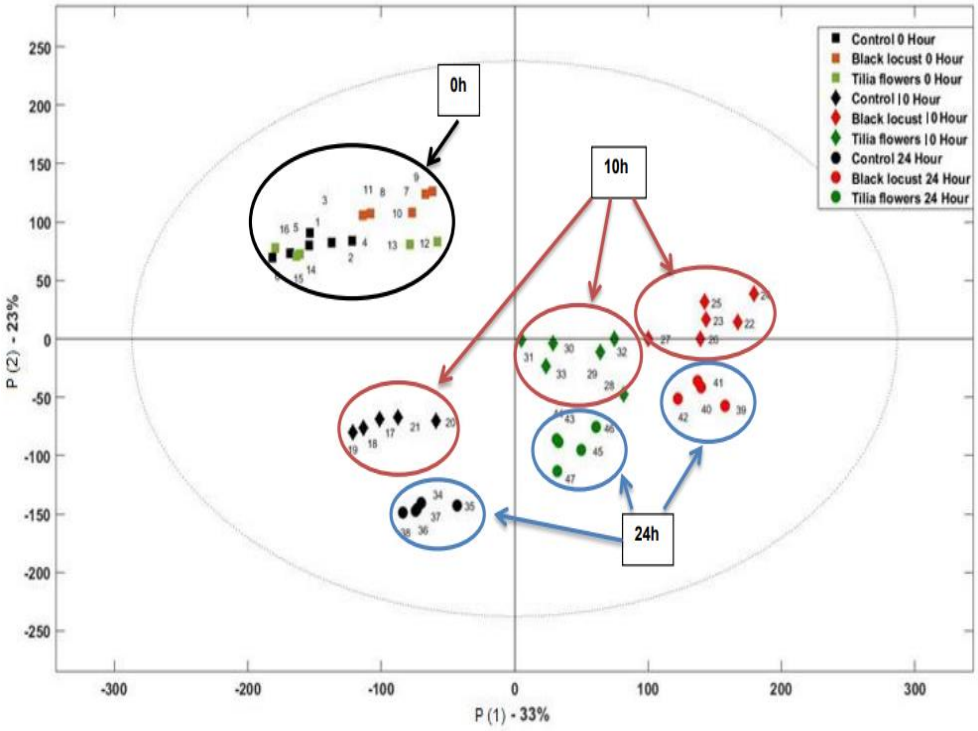

Figure 3. The APEC treated with high PD-CT or PC-CT for various time-points were used to create a principal component analysis (PCA) - ^{1}H-NMR spectra. $R^2 = 0.52$; $Q^2 = 0.42$; Black indicates a control, Red indicates PD-CT; Green indicates PC-CT groups

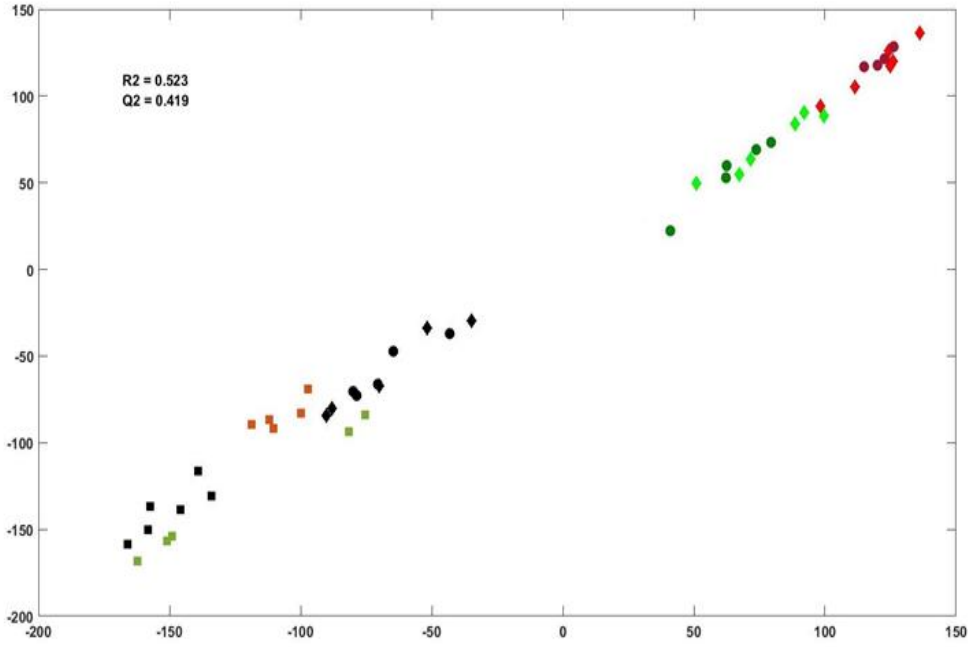

Figure 4. The score histogram of orthogonal partial least squares (OPLS) for APEC metabolites with treatment and control using two sub-MIC CT levels of 0.6 mg/mL, either PD-CT or PC-CT, at various time intervals. R^2 refers to estimates of the goodness of fit; Q^2 refers to the estimation of the goodness of samples. Black indicates a control, Red indicates PD-CT; Green indicates PC-CT groups

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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