Does lipidomic serum analysis support the assessment of digestive efficiency in chickens?

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ABSTRACT The increasing cost of conventional feedstuffs used in poultry diets has bolstered interest in genetic selection for digestive efficiency (DE) to improve the adaptation of the birds to various alternative feedstuffs. However, DE measurement through AMEn is time-consuming and constraining. To simplify selection for DE, the potential of serum composition to predict AMEn was evaluated based on 40 birds from two broiler lines (D+ and D−) divergently selected on the fecal AMEn of a difficult-to-digest wheat-based diet. Differences in serum coloration were suspected between the two lines, and thus a spectrophotometric analysis was carried out, revealing a significant difference in absorption between 430 nm and 516 nm, corresponding to the signature of orange–red lipophilic pigments such as xanthophylls. To go further, the liposoluble fraction of the serum was explored for its lipidome by mass spectrometry. Discriminant analysis revealed that a pattern of 10 metabolites, including zeaxanthin/lutein, can explain 82% of the lipidomic differences between the two lines. Colorimetry combined with lipidomics studies confirmed the relationship between digestive efficiency and serum composition, which opens up new possibilities for using it as a quick and easy proxy of digestive efficiency.

Key words: digestive efficiency, lipidomics, serum, biomarker, carotenoid

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INTRODUCTION

The intense selection performed during the past decades for chicken production-related traits has led to huge genetic progress when considering feed conversion ratio and growth rate, with broiler chickens being slaughtered 19% earlier, and requiring 24% less feed to reach the same weight in 2010 than in 1970 (Riffard et al., 2011). This is a success if considering solely the purpose of increasing animal protein production at a lower cost, which has been the goal of genetic selection in livestock until now. Nevertheless, this advance relies on the use of optimal feedstuffs in poultry diets, which are becoming more and more expensive and are increasingly in competition with human food. Therefore, digestive efficiency (**DE**), a component of feed conversion ratio, must also be improved in order to include less digestible feedstuffs in the diets. DE of energy (measured by AMEn), proteins, lipids, and starch have a genetic origin and have been successfully selected in a divergent selection experiment in chickens (Mignon-Grasteau et al., 2004). After eight generations of selection, the high DE (D+) and the low DE (D–) lines showed a difference of 40% in AMEn with no impact on the body weight (de Verdal et al., 2011). However, the only available way to assess DE is through balance trials, which are time-consuming and detrimental to animal welfare as animals are kept in individual cages, which consequently limits its integration in genetic selection schemes. Using indirect, non-invasive indicators of DE such as metabolomic biomarkers would thus be useful. However, currently none are available and no extensive metabolomics profiling of DE has been performed.

In a prior study, we showed that the metabolomics profile of the hydrosoluble fraction of serum was linked to DE (Beauclercq et al., 2018). In this study, we observed that the color of serum seemed to differ

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between efficient and non-efficient birds, thus suggesting that pigments also differed between the two lines. As pigments are located in the lipophilic fraction of serum, we proposed to screen the liposoluble fraction of D+ and D− broiler serum to search for potential biomarkers of DE within this fraction. Liquid chromatography-high-resolution mass spectrometry (**LC-HRMS**) was used to characterize the lipidomic composition of the lipophilic part of the serum.

MATERIALS AND METHODS

All animal care and experimental procedures needed for this study were approved by the Ethics Committee for Animal Experimentation of Val de Loire (Authorization No. 01047.02). This ethics committee is registered by the National Committee under the number C2EA 19.

All chemicals were bought from Sigma Aldrich (Saint-Quentin Fallavier, France) unless otherwise specified.

Birds and Sampling

This study was conducted on birds originating from the 20th generation of two genetic lines divergently selected for low (D−) or high (D+) DE through faecal AMEn of a difficult to digest wheat-based diet at 3 wk (Mignon-Grasteau et al., 2004; Carré et al., 2008). The animals were reared at UE1206 EASM (Unité Expérimentale Elevage Alternatif et Santé des Monogastriques, INRA, Le Magneraud, France), on the floor during the first 2 wk and then in individual cages to measure AMEn. During the whole experiment, the animals were fed ad libitum with the same diet as that used during the initial selection experiment, i.e., a diet adapted to nutritional requirements (2,943 kcal.kg^{-1} dry matter, 21% crude protein; 6% lipids), but of low digestibility due to its high content (55%) of Rialto wheat, a variety with high viscosity (Carré et al., 2002).

Birds were weighed at 17, 23, 25, and 26 d and their feed intake was recorded individually between 17 and 23 d, 23 and 25 d, and 25 and 26 d. Their feed conversion ratio between 17 and 26 d was calculated as the ratio of feed intake to growth during this period.

The balance trial was done between 23 and 25 d to get fecal AMEn and coefficients of digestive use of starch (**CDU$_S$**), nitrogen (**CDU$_N$**) and lipids (**CDU$_L$**) from 20 birds of each line (males and females) using a method based on total excreta collection, as described by Bourdillon et al. (1990). Gross energy, lipid, starch, and nitrogen content of individual freeze-dried excreta were measured for all birds using the Near Infrared Spectroscopy procedure (Foss, Hilleroed, Denmark) described by Bastianelli et al. (2010).

After the end of the balance trial, blood was drawn at the occipital sinus for serum composition analyses. Serum was prepared by keeping the blood at room temperature for 15 min until coagulation and centrifugation (3,000 g for 10 min). Sera were aliquoted and stored at −20°C until further analysis.

The equality of medians between the D− and D+ (n = 20) samples was tested for the DE traits with the non-parametric Mann–Whitney's test.

Serum Color

Samples of 200 μL from the serum of the 40 birds were transferred to a transparent 96-well plate (Greiner Bio-One, Kremsmünster, Austria) and their absorption spectra were acquired between 400 and 600 nm (2 nm steps) using an Infinite M200 spectrophotometer (Tecan, Männedorf, Switzerland). The equality of means between the D− (n = 20) and D+ (n = 20) serum absorption was tested for each 2 nm step at the 95% confidence level with Welch's t-test.

LC-HRMS Analysis

The liposoluble fraction of the serum of each bird was extracted based on the Bligh and Dyer method (Bligh and Dyer, 1959). Briefly, 375 μL of chloroform/methanol (1:2) were added to 100 μL of serum, the mix was then thoroughly vortexed before addition of 125 μL chloroform. A volume of 125 μL of water was subsequently added followed by mixing and centrifugation (15,000 g, 10 min, 4°C). The lower phase (200 μL) under the protein disk corresponding to the nonpolar fraction was recovered and put in glass tubes for further solvent evaporation in a SpeedVac (Thermo Fisher Scientific, Waltham, MA) at room temperature. The residue was then reconstituted with 100 μL of a 6:3:1 mix of acetonitrile (**ACN**)/water/isopropanol followed by centrifugation (15,000 g, 10 min, 4°C) before mass spectrometry analysis.

LC-HRMS analysis was performed on a UHPLC Ultimate 3000 system (Dionex, Sunnyvale, CA), coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific) and operated in positive ionization mode (**ESI+**). Chromatography was carried out with a 1.7 μm XB—C18 (150 mm × 2.10 mm, 100 Å) UHPLC column (Kinetex, Phenomenex, Torrance, CA) heated at 55°C. The solvent system comprised mobile phase A [isopropanol/ACN (9:1) + 0.1% (vol/vol) formic acid + 10 mM ammonium formate], and mobile phase B [ACN/water (6:1) + 0.1% (vol/vol) formic acid + 10 mM ammonium formate]; the gradient operated at a flow rate of 0.26 mL/min over a run time of 24 min. The multisteps gradient was programmed as follows: 0 to 1.5 min, 32% to 45% A; 1.5 to 5 min, 45% to 52% A; 5 to 8 min, 52% to 58% A; 8 to 11 min, 58% to 66% A; 11 to 14 min, 66% to 70% A; 14 to 18 min, 70% to 75% A; 18 to 21 min, 75% to 97% A; 21 to 24 min, 97% A. The autosampler temperature (Ultimate WPS-3000 UHPLC system, Dionex) was set at 4°C, and the injection volume for each sample was 5 μL. The heated

ESI source parameters were a spray voltage of 3.5 kV, capillary temperature of 350°C, heater temperature of 250°C, sheath gas flow of 35 arbitrary units (**AU**), auxiliary gas flow of 10 AU, spare gas flow of 1 AU, and tube lens voltage of 60 V for C18. During the full-scan acquisition, which ranged from 250 to 1600 m/z, the instrument operated at 70,000 resolution, with an automatic gain control target of 1×10^6 charges and a maximum injection time of 250 ms.

The instrumental stability was evaluated by multiple injections (n = 5) of a quality control (**QC**) sample obtained from a pool of 10 μL of all samples analyzed. This QC sample was injected once at the beginning of the analysis, between every 10 sample injections, and at the end of the run.

LC-HRMS Data Processing

XCMS online (https://xcmsonline.scripps.edu) was used to process the raw data for feature detection, retention time correction, and alignment using the default parameter for UHPLC Q-Extractive (Tautenhahn et al., 2012). Features with variance intensities greater than 30% in QC samples or with QC variance superior to sample variance were removed as well as those identified as background noise or poorly integrated after visual inspection (Want et al., 2012). The peak intensities were normalized by the median area of the spectra for each sample, transformed into a logarithm using the automatic transformation tool of SIMCA 13 (Umetrics, Umeå, Sweden), and centered to maximize the separation between the D+ and D− lines. The putative assignment of the peaks was based on a METLIN search of the accurate mass (Smith et al., 2005). Lipids were annotated according the LIPID MAPS Lipid Classification System (Fahy et al., 2005, 2009).

Chemometric Analyses

Principal Component Analysis (**PCA**) was performed on the data set as exploratory unsupervised analysis. Individuals outside of the 95% confidence interval of the Hotelling's T-square were considered to be outliers and excluded from the subsequent analyses.

Partial least squares discriminant analysis (**PLS-DA**), a method of supervised classification, was performed to predict the chicken group (i.e., D+ or D–) by explanatory quantitative variables (defined here by the peaks), using the SIMCA 13 Software (Barker and Rayens, 2003). The selection of metabolomics variables was carried out by iteratively excluding the variables with low regression coefficients and wide confidence intervals derived from jackknifing combined with low variable importance in the projection (**VIP**) until maximal improvement of the quality of the models. The model quality was evaluated after 7-fold cross validation by R^2_Y (goodness of fit), Q^2 (goodness of prediction), and CV-ANOVA (cross validation-analysis of variance). CV-ANOVA is a diagnostic tool for assessing the reliability of PLS-DA models; the returned P value is indicative of the statistical significance of the fitted model (Eriksson et al., 2008). The contribution of each predictor in the model was evaluated through the variable contribution plot.

RESULTS AND DISCUSSION

Bird Digestive Efficiency Traits

The composition of the liposoluble fraction of serum was investigated in 20 individuals from the D+ line and 20 from the D- line. Their average growth, feed conversion ratio, and DE values are described in Table 1. The individuals from the two lines have a similar body weight at 17 d. However, the feed conversion ratio of D− was 16% higher due to a lower DE (–16% for the CDU_L, –7% for CDU_N, and –12% in AMEn).

Serum Pigmentation and Digestive Efficiency

The coloring of the sera was noticeably different between the D+ and D− lines, which was confirmed by spectrophotometry. Indeed, the absorption spectra between 430 and 516 nm, corresponding to a yellow–orange color, were different ($P < 0.05$) between the two lines. The difference was maximal at 492 nm with a serum absorption 31% lower in D− than in D+ birds. The significance of differences between the lines for the absorption profile of the pigment molecules are presented in Figure 1a. This profile was characterized by two peaks at around 466 nm and 492 nm, which are close to the xanthophyll and carotenoid absorption spectra (Bernstein et al., 2010). It is worth noticing that the absorption spectra of those molecules are influenced by the solvent (Zang et al., 1997), as well as by the proportion of the different xanthophylls and carotenoids in the mix, which could explain the slight difference between our spectrum and the Bernstein's et al. spectra acquired in olive oil.

These differences in pigmentation may be an ideal diagnostic tool to discriminate between chickens with high or low DE because of its ease of implementation. It has previously been used in chickens to determine their status regarding coccidiosis infection, a parasitic disease of the intestine (Hamzic et al., 2015). In cases of infection, the intestinal absorption of pigments is disturbed and pigments are partially lost by leakage through the damaged wall of the cecum, leading to a reduced level of xanthophylls and carotenoids in the plasma from infected broilers (Ruff et al., 1974; Ruff and Fuller, 1975). The mechanism leading to the difference in serum pigmentation between D+ and D– could be similar as absorption capacity is probably lower in D− than in D+, including for pigments. Indeed, de Verdal et al. (2010) showed that the structure of the intestine was more

Table 1. Growth, feed conversion ratio, and DE traits of the D+ and D− line samples.

Traits[1]	D+ (n = 20)[2]	D− (n = 20)	*P* value[3]
Body Weight at 17d (g)	329 ± 30	350 ± 31	0.06
FCR 17/26 d ($g.g^{-1}$)	1.85 ± 0.12	2.14 ± 0.21	7.57×10^{-6}
CDU_S (%)	98.32 ± 0.65	89.30 ± 9.41	2.76×10^{-10}
CDU_L (%)	90.48 ± 3.71	74.17 ± 15.38	8.34×10^{-8}
CDU_N (%)	81.53 ± 2.54	74.75 ± 4.28	1.69×10^{-7}
AMEn ($kcal.kg^{-1}$ DM)	3477.58 ± 92.27	3053.65 ± 345.76	4.85×10^{-7}

[1]FCR 17/26: feed conversion ratio between 17 and 26 d; $CDU_{S, L, N}$: coefficients of digestive use of starch, lipids and nitrogen; AMEn: metabolizable energy corrected to zero nitrogen balance.
[2]Values are expressed as mean ± SD.
[3]Two-tailed Mann–Whitney's median value equality t-test.

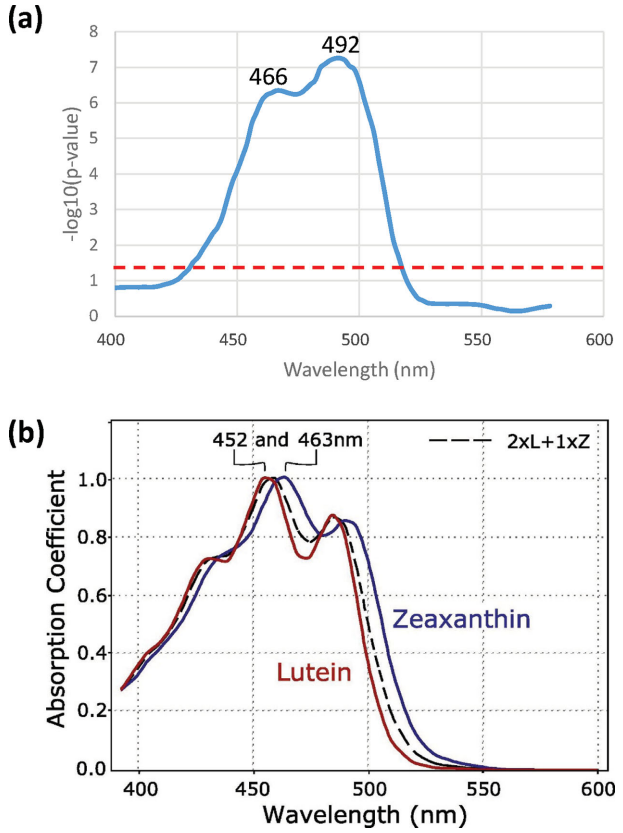

Figure 1. Significance of the difference between the two lines for the absorption profiles of lipohilic part of serum (1a) and reference profile of the xanthophylls reprinted from Bernstein et al. (Copyright 2010, with permission from Elsevier; 1b).

fragile in D−, which could favor losses by leakage in this line. However, it was unlikely that these differences were the result of a difference in sensitivity to coccidiosis since the diet included 0.05% of coccidiostats.

Spectrophotometry cannot identify with certainty the molecules responsible for these differences between the two lines without further biochemical characterization. For example, bilirubin, a product of heme catabolism present in serum, also absorbs at 460 nm and cannot be distinguished from xanthophylls and carotenoids by this method. However, xanthophyll and carotenoid, but not bilirubin, transfer to nonpolar solvents such as hexane (Brodersen, 1979). To obtain a better understanding, the LC-HRMS spectra of the lipophilic fraction of the sera from the same animals were thus acquired.

Serum Lipidome and Digestive Efficiency

The LC-HRMS (ESI+) spectra were processed using XCMS and 13,420 spectral features were identified (not including isotopic peaks). A total of 2,260 peaks were conserved after removal of those with poor qualities. The PCA analysis, when using all the data samples, showed that the five QC samples were clustered, which validates the stability of the analysis quality during the LC-HRMS acquisition. The PCA plots revealed that three samples could be considered to be outliers (2 D– and 1 D+) and thus were removed from the subsequent PLS-DA analysis (data not shown).

The PLS-DA model resulted in two predictive components with a cross-validated predictive ability (Q^2) of 0.80 and an overall proportion of the variation in DE explained by the model (R^2_Y) of 0.82. The reliability of this model assessed by CV-ANOVA was good, with a *P* value of 9.31×10^{-11}. The scatter plot resulting from the PLS-DA is shown in Figure 2a. The PLS-DA showed a clear separation between the D+ and the D– birds with high quality of prediction (Q^2), suggesting lipidome differences between the two lines. The model included 10 features each characterized by an exact mass and a retention time, which were listed in Table 2 as well as their importance in the model (VIP) and their loadings on the two components. The annotation of those peaks was performed based on the exact mass. That might have affected the precision of the annotation and could not distinguish some isomers or isobars. However, the difference between the exact mass and theoretical mass of the metabolite (expressed in ppm in Table 2) was low, which bring more confidence into this annotation of the metabolites. The putative molecules included in the model were lipids [ceramide (Cer), diglyceride (DG), phosphocholine (PC), phosphoethanolamide (PE), 3-O-(2-O-(2E-decenoyl)-alpha-L-rhamnopyranosyl- (1-2)-alpha-L-rhamnopyranosyl)-3-hydroxydecanoic acid, kamlolenic acid] as well as a terpenoid (24-noroleana-3,12-diene), a steroid (22:5 cholesteryl ester), and xanthophylls (lutein/zeaxanthin), the latter being the most

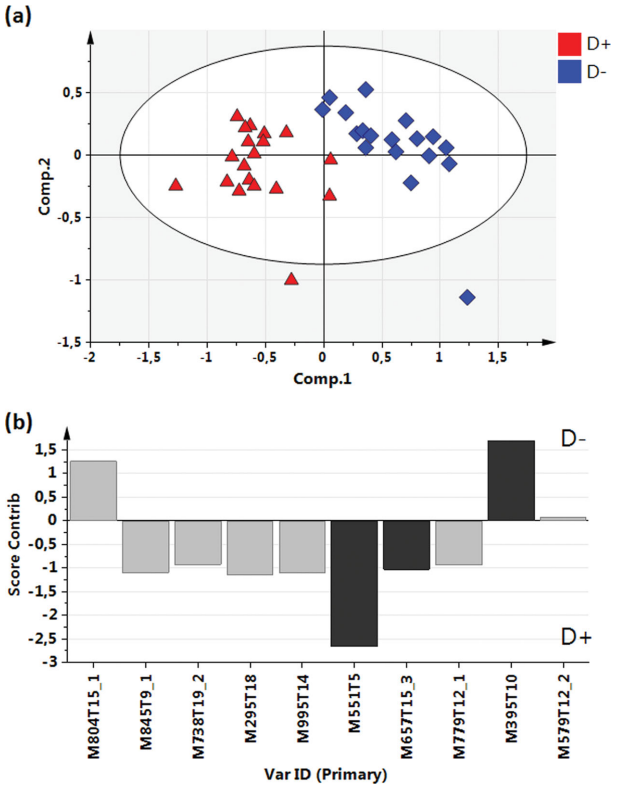

Figure 2. Metabolome score plot (a) and contribution plot indicating the contribution of the metabolites identified in PLS-DA (b). The individuals from the D− and D+ lines are represented by blue diamonds and red triangles, respectively, in the score plot. The features outside of 3 standard deviations in the raw data distribution were coloured in black in the contribution plot.

differential between lines with a fold change of 4.63. The contribution plots generated by SIMCA software (Figure 2b) were used to identify the metabolites allowing chicken-line discrimination. The M395T10 feature (24-Noroleana-3,12-diene) was the only one to highly and positively contribute (outside of 3 standard deviations in the raw data distribution) to the D– line, while M551T5 (lutein/zeaxanthin) was the highest contributor to the D+ line.

Biomarkers of Digestive Efficiency

The spectrophotometric approach as well as the PLS-DA model fitted on a selection of LC-HRMS features were two sources of biomarkers related to DE with their advantages and inconveniences. Spectrophotometric measurement of the absorption of the sera at 492 nm is easy to implement, but it is not as robust as the measurement of the metabolites included in the PLS-DA, which on the other hand requires a quite expensive analytical procedure. However, the number of features screened could be reduced according to the precision of the diagnosis needed or technical constraints for practical use. For instance, reducing the set of markers to the three features with the highest VIP (M551T5, M395T10, M295T18) did not hugely impact the explicative ability of the model ($R^2_Y = 0.73$) or the predictive ability ($Q^2 = 0.63$). Interestingly, M551T5, the variable with the highest VIP in the PLS-DA, was assigned to lutein/zeaxanthin, which has absorption spectra similar to the differential absorption profile between D+ and D-. Lutein and zeaxanthin, two isomers of $C_{40}H_{56}O_2$, represent 61% to 73% of yellow–orange pigment in corn kernels (O'Hare et al., 2015). They are also present at a lower concentration in wheat and soy kernels (Pardio et al., 2005; Roose et al., 2009).

The LC-HRMS approach applied to lipidic fractions led to a PLS-DA model with similar explicative abilities R^2_Y (0.82 vs 0.77) and higher predictive abilities Q^2 (0.80 vs 0.48) than the previous PLS model fitted to metabolites from serum polar extracts identified by NMR to predict the quantitative value of AMEn (Beauclercq et al., 2018). This could be explained by a higher sensitivity of LC-HRMS (nM to pM) in comparison to NMR (μM to mM) (Goldansaz et al.,

Table 2. Serum lipophilic fraction metabolites identified by PLS-DA discriminating between the D+ and D– lines.

ID	Retention time (min)	Exact mass (m/z)	VIP[1]	Loading comp.1	Loading comp.2	Putative metabolite[2]
M551T5	5.31	551.4250	1.95	−0.6269	0.3988	Lutein (1 ppm) Zeaxanthin (1 ppm)
M395T10	9.85	395.3669	1.20	0.3843	0.3529	24-Noroleana-3,12-diene (1 ppm)
M295T18	17.91	295.2265	0.87	−0.2848	−0.1813	Kamlolenic acid (1 ppm)
M804T15	14.99	803.5135	0.87	0.2836	0.1625	3-O-(2-O-(2E-decenoyl)-alpha-L-rhamnopyranosyl-(1–2)-alpha-L-rhamnopyranosyl)-3-hydroxydecanoic acid (2 ppm)
M995T14	13.57	994.6450	0.81	−0.2673	−0.0139	Unknown
M657T15–3	14.75	656.6186	0.80	−0.2646	−0.0462	Ceramide (18:0/22:0) + isomers (0 ppm) Diglyceride (17:0/20:0) + isomers (0 ppm)
M738T19–2	18.63	737.5611	0.74	−0.2387	−0.2213	22:5 Cholesteryl ester (3 ppm)
M845T9–1	9.35	844.5229	0.70	−0.2286	−0.1024	Phosphocholine (16:0/22:6) + isomers (3 ppm)
M779T12–1	12.43	778.5738	0.68	−0.2152	−0.2445	Phosphoethanolamide(22:4/18:1) + isomers (1 ppm)
M579T12–2	12.00	578.5141	0.67	0.0273	0.7467	Ceramide (14:2/22:1) + isomers (0 ppm)

[1]Variable importance in the projection.
[2]Putative annotation of the peaks based on their exact masses. The differences between the mass determined by spectrometry and the theoretical mass of the metabolites are indicated in ppm.

2017) or a lipophilic fraction more related to DE variations.

The colorimetric and lipidomic approaches opens up new possibilities to obtain a quick and easy proxy of DE that could be generalized to other chicken populations as metabolome and metabolic responses could be less dependent on the genetics than single nucleotide polymorphism or even the transcriptome in a given physiological state. Indeed, metabolomics and lipidomics are powerful means to non-invasively predict the quality of the production (Straadt et al., 2014; Beauclercq et al., 2016), feed efficiency and residual feed intake, ascertain disease propensity, evaluate dietary responses to different feeds, fertility, and other important economic or breeding traits associated with livestock (Goldansaz et al., 2017). This preliminary study is a first step in the discovery of DE biomarkers in chickens. The set of marker (exact mass, retention time, or visible wavelength) presented in this study might be used as a proxy for DE. However, their chemical natures should be confirmed at the molecular level to get a better understanding of the metabolic pathways influenced by DE; moreover, the biomarker set needs to be validated on a larger and independent population and diets with other compositions. This metabolic pattern will certainly assist in the development of more sustainable poultry production schemes by allowing a better combined nutritional and genetic approach to production within the context of a more variable and complex composition of broiler diets.

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