CAN METABOLOMICS FINGERPRINTING DETECT THE ANTIBIOTIC ADMINISTRATION IN PIGS?

Maria P. Fabrile[1], Augusta Caligiani[1], Sergio Ghidini[1], Giovanni L. Alborali[2], Silvio De Luca[1], Federico Scali[2], Veronica Lolli[1], Mauro Conter[3], Maria O. Varrà[1], Adriana Ianieri[1], Emanuela Zanardi[1*]

[1]Department of Food and Drug, University of Parma, Italy

[2]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna "Bruno Ubertini", Italy

[3]Department of Veterinary Science, University of Parma, Italy

*Corresponding author email: emanuela.zanardi@unipr.it

I. INTRODUCTION

Antimicrobial resistance embodies a relevant threat to public health leading to both the adoption of global action plans to optimize the use of antimicrobial agents and changing purchasing trends of consumers towards meat products deriving from animals raised without antibiotics (RWA) [1]. This green transition widely involves the meat sector leading to the spread of RWA claims on meat and meat-based products. Currently, the targeted analysis is the workhorse to quantify the residues of antibiotic administration; however, this approach may not be effective [2], leaving the question "Has this pig ever been treated with antibiotics?" unsolved. In this study, the untargeted Nuclear Magnetic Resonance (NMR)-based metabolomics approach was applied to compare antibiotic treated *vs.* untreated pigs.

II. MATERIALS AND METHODS

Heavy pigs (170 kg live weight) reared in four farms in Northern Italy were divided into two groups according to the antibiotic exposure during their entire life calculated with the Defined Daily Doses Animal for Italy per Biomass ($DDDAit_{biomass}$): control (CTRL, RWA) and treatment group (TX, $DDDAit_{biomass}$= 16.3-37.4 day/animal/$year_{2020}$), respectively. Liver (*n*=41; 22_{CTRL} *vs* 19_{TX}), kidney (*n*= 48; 27_{CTRL} vs 21_{TX}), and muscle (*n*=47; 31_{CTRL} vs 16_{TX}) were sampled on the same day at a commercial abattoir by a veterinarian operating on the offal-managing line to simultaneously collect the targeted food matrices, thus choosing the diaphragm as representative of muscle tissue and convenient sample. After collection, the samples were immediately frozen. Each sample was extracted following the biphasic extraction procedure Bligh and Dyer [3] slightly modified. After drying, polar and non-polar extracts, were resuspended in appropriate deuterated solvents to perform ^{1}H NMR acquisition by a ECZ600R NMR spectrometer operating at 600.17 MHz (JEOL). After shimming, spectra were recorded at 298 K, 32k (polar extracts) and 65k (non-polar extracts) datapoints over a spectral width of 24 ppm. 128 and 32 scans were acquired for polar and non-polar extract, respectively. Raw spectra were processed with MestreNova software and referenced to TSP (δ=0 ppm); correction for phase and baseline was manually performed. The peaks were visually inspected along the region δ 0-9 ppm and their area were expressed as relative percentage of TSP signal. Data matrices were exported to SIMCA 17 software for both unsupervised Principal Component Analysis (PCA) and supervised Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) separately on polar and non-polar extracts. Auto or Pareto scaling were performed. No missing data were detected, and Hotelling's T^2 test ($p \leq 0.05$) was used to identify outlier samples. The Variable Importance in Projection (VIP) analysis for OPLS-DA components was applied to identify the most relevant spectra signals for the discrimination of antibiotic treatment (VIP score ≥ 1).

III. RESULTS AND DISCUSSION

Liver displayed a clear discrimination between TX and CTRL groups in both polar and non-polar extracts [4]. Unlike the liver, no relevant findings emerged from the non-polar extracts of both kidney and muscle tissue, probably due to the different biological role. For PCA of polar extracts, satisfying values of goodness-of-fit (R^2) and the predictive ability (Q^2) were observed, as follows: liver (R^2= 0.751; Q^2=0.529), kidney (R^2= 0.808; Q^2=0.614), and muscle tissue (R^2= 0.765; Q^2=0.502), respectively. For OPLS-DA of polar extracts, a good intergroups variability was observed along the t[1] of the score plots of the three matrices, where the negative scores corresponded to TX samples and the positive scores to CTRL samples. No strict clusterisation along the t[2] was observed, thus indicating a high intragroup variability (Figure 1). Among all integrated bins, 17, 26 and 32 buckets were selected for liver, kidney, and muscle tissue, respectively, and then identified. To date, the assignment of ^{1}H NMR is completed for liver while still in progress for kidney and muscle. Overall, relevant signals belonging to the carbohydrates, mainly glucose, amino acids and organic acids were annotated in the ^{1}H NMR spectra.

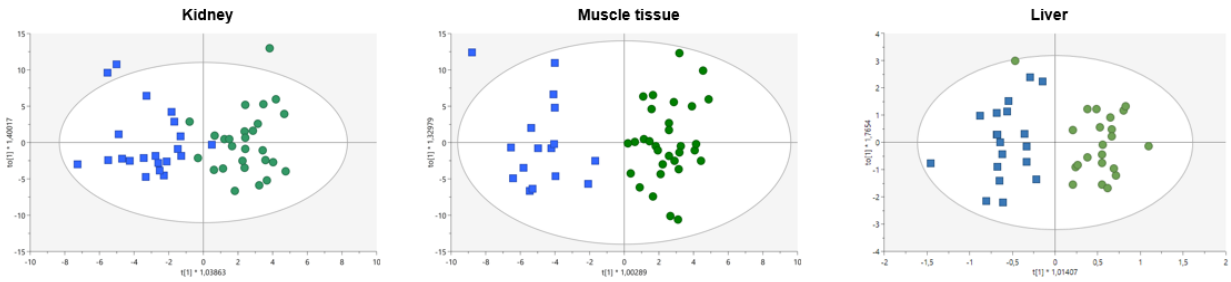

Figure 1. OPLS-DA of polar extract of kidney, muscle tissue, and liver. TX (blue square) and CTRL (green dot) groups are color-coded accordingly. Summary of statistical parameters: kidney [R^2X= 0.395, R^2Y= 0.756, Q^2=0.448], muscle [R^2X= 0.653, R^2Y=0.859, Q^2= 0.726]; liver [R^2X= 0.892, R^2Y= 0.774, Q^2=0.613].

IV. CONCLUSION

Results suggest that polar extracts of liver, kidney, and muscle tissue are worth of investigating for the research of biomarkers proof of antibiotics treatment. However, in the opinion of the authors the discrepancy found in non-polar fraction among different organs and tissue should be further studied to better understand the phenotypic outcome at molecular level.

ACKNOWLEDGEMENTS

This study was supported by the Italian Ministry of Health (Project “Classyfarm”), the University of Parma (Bando di Ateneo Ricerca 2022, Project abFREEPIG), and National Recovery and Resilience Plan, Mission 4 Component 2 Investment 1.3 – Call for tender No. 341 of 15/03/2022 of Italian Ministry of University and Research funded by the EU – NextGenerationEU (Project “ONFoods”).

REFERENCES

1. Bradford, H., McKernan, C., Elliott, C. & Dean, M. (2022). Consumers' perceptions and willingness to purchase pork labelled 'raised without antibiotics'. Appetite 171:105900.
2. Whelan, R., Tönges, S., Böhl, F. & Lyko, F. (2023). Epigenetic biomarkers for animal welfare monitoring. Frontiers in Veterinary Science 9:1107843.
3. Bligh, E.G. & Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37:911-917.
4. Fabrile, M.P., Caligiani, A., Ghidini, S., Alborali, G.L., De Luca, S., Scali, F., Lolli, V., Varrà, M.O., Ianieri, A. & Zanardi, E. (2022). Exploiting NMR-based untargeted metabolomics approach to unravel the administration of antibiotics in pig liver. In Proceedings and Abstracts of the 68th International of Meat Science and Technology (p. 226), 22-25 August, Kobe, Japan.