Exploiting NMR-based untargeted metabolomics approach to unravel the administration of antibiotics in pig liver

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Objectives: The use of label claims such as “antibiotic-free” or “raised without antibiotics” in the meat market leads to new challenges concerning tools for authentication and traceability. The objectives of this study were to evaluate metabolic changes and investigate putative metabolites related to antibiotic treatments, to identify potential biomarkers of antibiotic-free reared pigs. To this aim, pig liver, kidney, and muscle were investigated by a metabolomics approach. Preliminary results only related to liver are presented here.

Materials and Methods: A total of 41 heavy pigs (about 170kg live weight) belonging to 4 different farms of Northern Italy were selected. The pigs were divided into 2 groups (treatment group, n=19; control group, n=22), according to the daily dosage of the active substance (mg) used to treat 1 kg of pig related to the biomass of the animals present on the farm, known as Defined Daily Dose Animal for Italy ($DDDAit_{biom}$)[1]. This value indicates the days of treatment with antibiotics to which each animal raised on the farm may have been potentially exposed during the period under consideration (year 2020). In particular, $DDDAit_{biom}$ were 16.3 and 37.4 day/animal/year for pigs belonging to farms 1 and 2 (treatment group), and 0.38 and 0.14 day/animal/year for those belonging to farms 3 and 4 (control group). The Bligh and Dyer[2] method was employed for the recovery of polar metabolites and lipids as fol- lows: frozen liver tissue (100 mg) was manually grinded, added with a solution of ice-cold methanol:chloroform (2:1, v/v), and vortexed. After 30 min sonication in ice-cold water bath, ice-cold water and chloroform were added, and the sample newly vor- texed and centrifugated. Polar and non-polar phases were separately transferred to a new tube and dried under nitrogen flow. Both extracts were reconstituted prior to ^{1}H NMR analysis by sodium phosphate buffer in D_2O and sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeuteriopropionate (0.25M; pH 7.0) as internal standard for polar phase, and $CDCl_3$:CD_3OD (3:1, v/v) solution for non-polar phase. ^{1}H NMR spectra of both fractions, recorded on JEOL ECZR 600 spectrometer (600.17 MHz) using 65k data points (non-po- lar phase) and 32k data points (polar phase), were processed for signals integration after phase and baseline correction (MestReNo- va software, Escondido, USA). Raw data for polar and non-polar fractions were subjected to principal component analysis (PCA) and orthogonal partial last square-discriminant analysis (OPLS-DA) by SIMCA-P software (Umetrics, Sweden). The global quality of the models was evaluated considering the goodness-of-fit (R2X) and the predictive ability (Q2) as useful performance indica- tors. From OPLS-DA, the Variable Importance in Projection (VIP) scores were designed to find the strongest influence exerted by single NMR signal over samples grouping; as a rule, VIP values ≥ 1 were used to identify the most relevant metabolites.

Result and Discussion: Two data matrices N x R (N= samples; R= ppm range of integration pattern in spectral region 0-9 ppm) were built for a total of 3 444 and 3 116 values for polar and non-polar fractions, respectively. Concerning PCA, the first 2 PCs explained 39.0 and 50.9% of cumulative variance for non-polar and polar phase, respectively. The PCA model using projection onto two dimensions PC1 and PC2 showed a good clustering for non-polar (R2X= 0.725; Q2=0.419) and polar (R2X =0.751; Q2= 0.529) ex- tracts, indicating a tendency in differences in metabolites abundance between the two sample groups [treatment *vs* control]. Concerning OPLS-DA, the condition under investigation was also proved by the two groups of samples perfectly separated in the bi- dimensional space since control samples distributed along the positive t[1] component and the treated samples along the negative one. A mild degree of heterogeneity was observed within samples of the same group which were not closely clustered along the horizontal direction. A better performance was observed for non-polar extracts (R2X= 0.939; R2Y=0.948; Q2=0.869) compared to polar extracts (R2X= 0.892; R2Y= 0.774; Q2= 0.613). Seventeen and eleven NMR signals of polar and non-polar fractions, respec- tively, were found to be characterised by VIP values ≥ 1 and selected. Additional OPLS-DA was carried out to detect possible trends driven by the criterion based on different farms in which pigs were reared. The presence of a perfect separation among farms was observed in both fractions, confirmed by the good performance indicators for non-polar (R2X=0.967; R2Y=0.851, Q2=0.665) and polar extract (R2X=0.904; R2Y=0.519; Q2= 0.26). The assignment of ^{1}H NMR signals, the study of metabolic pathways, and the biological interpretation are in progress.

Conclusions: To the best of our knowledge, this is the first study considering metabolomic approach as investigation tool concerning the antibiotic administration in pigs. Metabolic differences between pigs subjected to antibiotic treatment from those untreated have been observed in liver; these preliminary results provide high potential with regard to the identification of treatment biomark- ers useful for meat authentication and traceability.

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