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Effects of Tetracycline Administration on the Proteomic Profile of Pig Muscle Samples (*L. dorsi*)

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Effect of tetracycline (TC) administration on the proteomic profile of pig muscle was evaluated by 2D electrophoresis and MALDI-TOF mass spectrometry. The TC content at slaughter was determined in *L. dorsi* samples by HPLC-DAD. Mean residual concentration of TC in the muscle of treated animals, calculated as the sum of TC and *epi*-TC was 126.3 μg/kg, indicating a rapid elimination of TC in this tissue. Several differential spots ($n = 54$, $p < 0.05$) were observed in protein profiles from control and treated animals. MALDI-TOF identification gave a positive match for 5 differential spots, that is, glycerol-3-phosphate dehydrogenase 1 (G3PD1), phosphoglycerate kinase 1, novelprotein (0610037L13Rik), leucine aminopeptidase 3 (LAP), and hypothetical protein isoform 2. Results show that proteomics could be a useful tool to reveal pharmacological treatments with TC, even if the possible uses of differential spots as biomarkers to detect illegal administration of TC require further studies. Different spot patterns as a consequence of TC treatments seem to be another interesting issue for the consequences on tissue metabolism and meat quality.

KEYWORDS: Tetracycline; proteomics; residue determination; glycerol 3-phosphate dehydrogenase; pig

INTRODUCTION

Tetracyclines (TCs) are broad spectrum bacteriostatic compounds, generally used as antimicrobials in human and veterinary medicine that interfere with protein synthesis in bacteria at the ribosomal level (*1*). Low-dose/long-time TC administration has been used in the past as growth promoter treatment in livestock production, but this use is banned today in the EU (*2*).

Furthermore, systematic antibiotic use promotes the development of resistant populations of bacteria and represents a considerable source of environmental contamination (*3*).

To preserve consumers' health, maximum residue limits (LMRs) have been established in the EU for veterinary drugs in different foods of animal origin (*4*). For tetracycline (TC), the LMRs comprise between 100 and 600 μg/kg, as the sum of TC and epi-tetracycline (*epi*-TC), depending on the edible part (*5*−*8*).

The official monitoring plans, implemented to guarantee the observance of the LMRs for TCs in foods, rely on microbiological, immunological, and chromatographic techniques. Nonetheless, confirmatory analyses must provide spectral or structural information about the target compounds, and are generally carried out with HPLC coupled to fluorescence detectors (FLD), mass spectrometers (MS), or diode array detectors (DAD) (*9*).

These techniques are able to reveal the presence of veterinary drug residues in edible parts but could be scarcely effective to detect illegal or incorrect uses of veterinary drugs if their administration is conveniently suspended before slaughter. As a general rule, the rapid metabolism of the veterinary drugs reduces their window of detection to a few days in edible tissues (*10*).

The ability of *-omics* technologies (genomics, proteomics, transcriptomics) to clarify the molecular mechanisms of meat quality is well established today (*11*), while the use of biomarkers of exposure and response to toxicity or susceptibility was recently proposed as an alternative to detect illegal uses of veterinary drugs ((*12*); http://www.biocop.org/theproject_objectives.html).

In a study performed on calves, Gardini et al. found that the administration of several anabolic compounds caused the differential expression of two proteins, adenosin kinase (AK) and reticulocalbin, in cytosolic and microsomal fractions of the liver (*13*).

Changes in protein patterns have been observed in strains of *Bacillus subtilis*, *Pseudomonas putida*, *Pseudomonas multocida*, and *Haemophilus influenzae* grown in the presence of TCs (*14*−*17*). The observation that the TCs are also inhibitors of matrix metalloproteinases is relatively recent (*18, 19*). Sadowski

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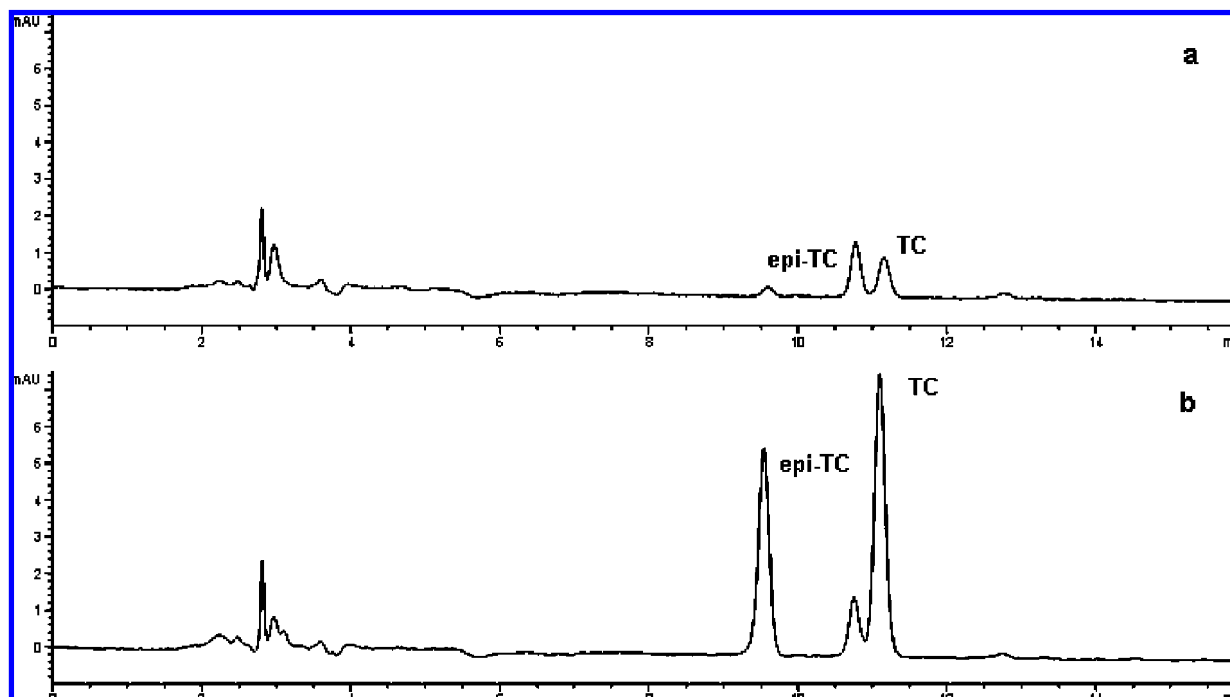

Figure 1. Cromatograms corresponding to purified *L. dorsi* extracts from a treated animal (**a**) and a control animal spiked at 1 mg/kg (**b**); 90 μL of each extract injected. Detection was performed at $\lambda = 365$ nm.

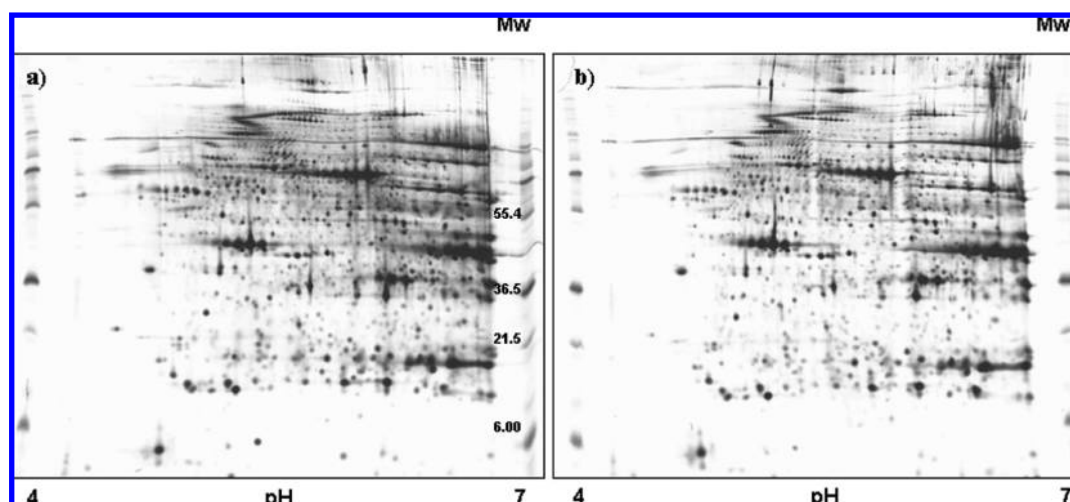

Figure 2. Silver stained gels of *L. dorsi* extracts from treated (**a**) and control (**b**) animals.

and Steinmeyer observed that TCs inhibited the synthesis of several metalloproteinases in chondrocytes cultures, through a genetic down-regulation (*18*).

A differential expression of 36 proteins, most of them related to carbohydrate and fatty acid metabolism, were found in mouse hepatocites after TC administration (*20*).

The use of proteomic techniques, based on 2D electrophoresis (2DE) and mass spectrometry (MS), could provide complementary information to demonstrate illegal pharmacological practices in livestock production or to develop alternative methods for screening analysis of veterinary drugs in meat (*12, 21*). However, to our knowledge no studies based on proteomic techniques are available for food producing animals treated with TCs.

The aim of this work was to compare the proteome profiles of meat samples (*L. dorsi*) obtained from pigs treated with TC with those of untreated (control) animals to put in evidence the differences between spot abundances as a consequence of the pharmacological treatment. The residual concentration of TC in muscle samples was also assessed by HPLC-DAD to evaluate the window of detection of TC administration or possible uses of the differential spots as biomarkers for TC treatment.

MATERIALS AND METHODS

Chemicals. Urea, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS), DL-dithiothreitol (DTT), IPG strips (pH 4−7, 18 cm), Pharmalyte 4-6.5 and 5-8, and dry strip cover fluid were purchased from Amersham Biosciences (Uppsala, Sweden).

Iodacetamide, formaldehyde, Tris(hydroxymethyl)aminomethane hydrochloride, bovine serum albumin (BSA), $MgSO_4$, citric acid, agarose, thiourea, ethylenediamine tetraacetic acid (EDTA), NaH_2PO_4, oxalic acid, and silver nitrate were from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, methanol, glutaraldehyde, sulfosalicylic acid, ammonium hydroxide, and sodium acetate trihydrate were from J.B. Baker (Mallinckrodt Baker Inc., Deventer, Netherlands). Glycerol (87%), sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), acetic acid, sodium hydroxide, and glycine were obtained from Merck KGaA (Darmstadt, Germany).

Ammonium persulfate (APS), *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and acrylamide/bis 40% were obtained from Bio-Rad

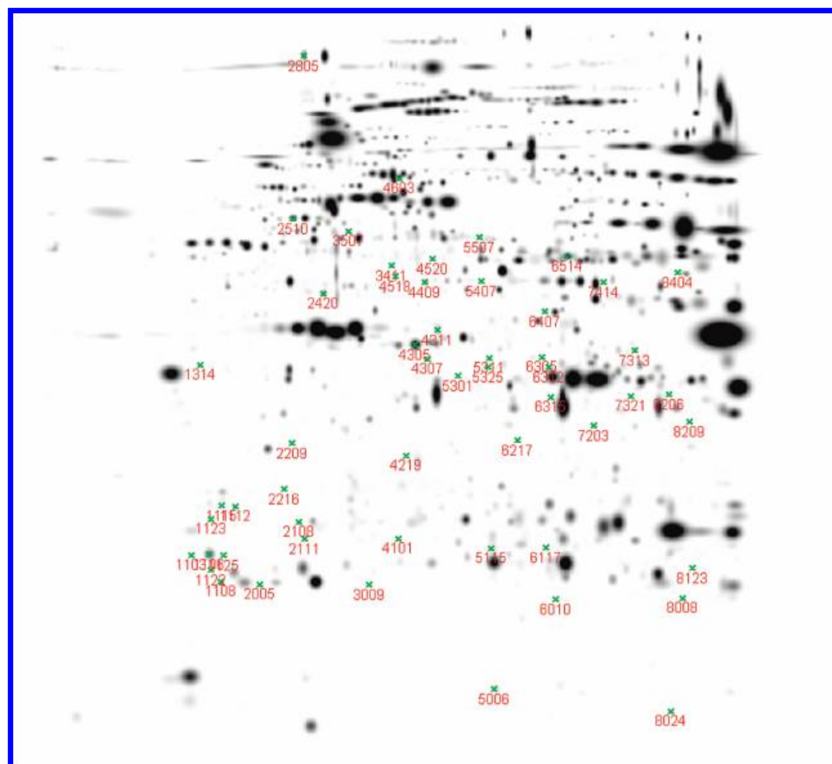

Figure 3. Master gel of sarcoplasmatic *L. dorsi* extract. Green cross indicates the localization of 54 differential spots ($p < 0.05$).

laboratories (Hercules, CA, USA). Protein molecular weight markers mark 12 were supplied by Invitrogen Corporation (Carlsbad, CA, USA), while Complete Protease Inhibitors were from Roche Diagnostics (Mannheim, Germany).

Sep-Pak plus C_{18} cartridges were purchased from Waters Corporation (Milford, MA, USA); bicinchoninic acid (BCA) protein assay from Pierce (Rockford, IL USA); tetracycline hydrochloride and epi-tetracycline hydrochloride from ACROS organics (Geel, Belgium); and TETRA 100 from Super's Diana S.L (Parets del Vallès, Spain).

Samples. Six male Landrace pigs were reared under controlled conditions up to 100 kg of weight. Three animals did not receive any medication (control), and the others three animals were treated orally with a daily dose of 7.5 g of TETRA 100 (Tetracycline Hydrochloride 20 g/100 g) for five days (treated), simulating a low-dose therapeutic treatment. The administration was performed via drinking water. One day after the administration of the last dose of TC, all animals were slaughtered, and samples of *L. dorsi* were collected for each animal, placed in dry ice, and deep frozen before two hours at −80 °C until analysis.

HPLC Analysis. TC residual content was determined in muscle samples following the method proposed by McNeil et al. with slight modifications (*22*). Five grams of frozen muscular tissue were extracted with 20 mL of Mcllvaine-EDTA buffer (*22*) and centrifuged at 4500 rpm for 5 min with Megafuge 1.0 (Heraeus Sepatech, Osterode Harz-Germany). The extraction was repeated twice with 10 mL of Mcllvaine-EDTA. The supernatants were mixed, centrifuged at 4500 rpm for 10 min, filtered through a paper filter, and made up to 50 mL with Mcllvaine-EDTA buffer. Thirty milliliters of the filtered extract were loaded on a Sep-pak plus-C_{18} cartridge previously conditionated with methanol and water (10 mL each). TCs were eluted with methanol containing 0.01 M oxalic acid, evaporated to dryness under nitrogen, and redissolved in 1 mL of the mobile phase A (0.01M oxalic acid/MeOH/ACN (80:10:10) (v/v/v)). LC analysis was carried out using an Agilent Technologies HP1100 series system equipped with a quaternary gradient pump, a DAD, and Chemstation Data Analysis System (Agilent, Palo Alto CA, USA). The chromatographic separation was performed at ambient temperature with a Luna Phenyl-Hexyl 5 μm (250 mm × 4.6 mm i.d) analytical column (Phenomenex, Torrance, USA). Elution of TC and *epi*-TC was realized with a gradient between solvent A (0.01M oxalic acid/MeOH/ACN (80:10:10) (v/v/v)) and solvent B (ACN). The composition of the mobile phase varied linearly from 90% A and 10% B (initial conditions) to 70% A and 30% B at 15.10 min. The identification of the peaks of TC and *epi*-TC was made comparing both retention time and spectra (collected between 220 and 500 nm) with those of the corresponding pure standard solutions. TC and *epi*-TC were quantified at $\lambda = 365$ nm.

Table 1. Mean ± Standard Deviation (SD) Intensities for 13 Differential Spots ($p < 0.01$) between Control and Treated animals ($n = 3$ Animals/Group)

spot number	group of animals	
	control (mean ± SD)	treated with TC (mean ± SD)
1112	61.7 ± 37.5	190.3 ± 29.3
1115	159.3 ± 60.1	398.4 ± 55.4
1122	210.3 ± 59.1	490.7 ± 41.9
1314	35.4 ± 4.5	54.2 ± 2.2
3009	75.9 ± 16.0	13.8 ± 6.5
4219	149.5 ± 19.3	252.2 ± 4.8
4305	4352.1 ± 46.7	3783.1 ± 198.1
5325	97.9 ± 16.8	46.7 ± 9.3
6010	114.2 ± 26.8	38.7 ± 8.1
6217	35.1 ± 5.7	76.1 ± 10.1
6305	nd[a]	219.0 ± 43.8
6312	nd	555.7 ± 158.6
8123	nd	78.8 ± 48.9

[a] nd = not detectable.

Quantification was made using an external calibration curve created by injecting a standard solution of TC and *epi*-TC in the range between 2.5 and 90 ng. Recoveries calculated with spiked control samples (50, 100, and 1000 ng/g) and the diluting factor were also taken into account.

Two-Dimensional (2D) Electrophoretic analysis. Five grams of frozen muscular tissue were homogenized with an Ultraturrax Mixer T25 (Janke & Kunkel, Staufen, Germany) in 30 mL of homogenization buffer (50 mM Tris, pH 7.4, containing 10 mM $MgSO_4$ and protease inhibitors). The homogenate was then centrifuged 30 min at 15400*g* at 4 °C with a Sigma centrifuge model 3K15 (Osterode am Harz, Germany) and the supernatant filtered through a paper filter. Total protein content in each extract was determined using a bicinchoninic

Table 2. Spot Identification by MALDI-TOF-TOF and Mascot Search Program

spot number	Identified protein by MALDI-TOF	MW (KDa)	database NCBInr	treated vs. control
6312[a]	glycerol-3-phosphate dehydrogenase 1 (soluble)	38284	gil60810758	over-expressed
5325[a]	phosphoglycerate Kinase 1 [sus scrofa]	44929	gil53792027	under-expressed
3009[a]	novel protein [0610037L13Rik]	7560	gil123244560	under-expressed
6514[b]	leucine aminopeptidase 3	56530	gil41393561	under-expressed
2111[b]	PREDICTED:hypothetical protein isoform 2	13262	gil55593263	over-expressed

[a] $p < 0.01$ between groups of animals (control and treated). [b] $p < 0.05$ between groups of animals (control and treated).

acid (BCA) protein assay kit considering BSA as a reference standard. A total of seven extracts were prepared to perform 2D-EF analysis: three extracts from control animals, three extracts from treated animals, and one extract obtained by mixing all the six samples.

The IPG strips (pH 4−7) were rehydrated directly with sample (100 μg of protein) solubilized in 350 μL of sample buffer containing 8 M urea, 2 M thiourea, 1.5% CHAPS, 50 mM DTT, and 1% Pharmalyte 4-6.5 and 5-8. Isoelectrofocusing (IEF) was performed using a Multiphor II Electrophoresis system (Pharmacia LKB, Uppsala, Sweden) at 20 °C, while the second dimension (SDS−PAGE) was performed in a Vertical apparatus Hoefer Dalt 2D system from Amersham Pharmacia Biotech (Uppsala, Sweden) at 12 °C, using 11% acrylamide gels (24 cm × 24 cm × 1 mm).

Proteins were fixed and silver stained as described in ref *23*. Once stained, gels were washed twice with deionized water, dried, and scanned using a 420 OE (PDI, BioRad Hercules, CA) flatbed scanner with a pixel size of 84.7 × 84.7 pixels. Digital images were treated using the PDQuest 7.1.1 program from BioRad (Hercules, CA), and quantitative density values were expressed as parts per million (ppm). A paired *t*-test for the intensity of all spots identified in the gels (∼800) was performed, considering the three gels from treated and nontreated animals ($n = 3$ each).

Additional 2D-EF gels ($n = 3$) were generated from a mixture of control and treated samples and stained with silver nitrate but without glutaraldehyde to be compatible with MALDI-TOF-TOF analysis for the protein identification.

Protein Identification by MALDI-TOF-TOF. Spots that presented significant variations between the two groups of samples were chosen to perform the protein identification. Selected spots were cut out of the gels and digested with trypsin as described by Lametsch et al. (*24*). The resulting peptides from the protein digestion were analyzed with the use of Ultraflex II, from Bruker Daltonics (Bremen, Germany). Both MS and MS/MS analysis were performed for protein identification. The database search was performed with the Mascot database search program (www.matrixscience.com), using a peptide mass tolerance of ± 70 ppm and fragment mass tolerance of ± 0.5 kDa compatible with oxidation as variable modification and carabamidomethylation as fixed modification.

RESULTS AND DISCUSSION

The residual content of TC (sum of TC + *epi*-TC) in muscle samples of treated animals ($n = 3$) (**Figure 1**) was 126.3 ± 57.9 μg/kg (standard deviation), while in control samples, no TC residues were detected (LOD= 20 μg/kg). These values are around the LMR, which is 100 μg/kg (TC + *epi*-TC) in muscular tissue (*7, 8*), and confirm the intense elimination of the drug after administration with negligible accumulation in muscular tissues (*5*).

Figure 2 shows representative 2D gels obtained for the two treatments (treated and control), while **Figure 3** shows the master gel obtained after digital image processing. Numbers indicate all of the spots ($n = 54$) that showed significant differences ($p < 0.05$) between the two groups of samples (treated and control).

Differential spot intensities (only spots with $p < 0.01$ between the two groups; **Table 1**) were either increased or decreased in treated animals compared to those of the control group.

Three spots clearly expressed in treated samples were not detected in the control group. As can be seen comparing **Table 1** with **Figures 2** and **3**, nearly all of the spots of interest at $p < 0.01$ presented molecular weights lower than or around 40 kDa.

The identification by MALDI-TOF-TOF MS (**Table 2**) gave a positive matching for 5 differential spots [i.e., glycerol-3-phosphate dehydrogenase 1 (G3PD1), phosphoglycerate kinase 1, novelprotein (0610037L13Rik), leucine aminopeptidase 3 (LAP), and hypothetical protein isoform 2].

Other differentially expressed spots were not successfully identified, probably because most of them were low abundant proteins that fall under the limit of detection for the MALDI-TOF-TOF analysis. More studies should be carried out to identify all of the differential spots revealed in 2D gels by introducing a prefractionation of the protein extracts or by modifying the conditions for 2D separation (IPG strips, loading conditions) to improve MS analysis. G3PD1 and hypothetical protein isoform 2 were more abundant in treated animals compared to control animals, whereas phosphoglycerate kinase 1, novel protein [0610037L13Rik], and leucine aminopeptidase 3 (**Table 2**) showed lower spot intensity.

G3PD1 (EC 1.1.1.8) is an oxidoreductase that catalyzes the interconversion of dihydroxyacetone phosphate to glycerol 3-phosphate. Dihydroxyacetone phosphate (DHAP) is the precursor for triacylglycerol synthesis in adipocytes and in much lesser extent than glycerol in other tissues (Kegg Database, (www.genome.jp/Kegg/Kegg2a.html)). This result is in concordance with the increase observed in the expression of genes involved in triglyceride synthesis and lipid metabolism after TC administration in rats (*25*). The gene controlling the synthesis of G3PD1 was also found to be over-expressed in the livers of TC-treated animals. A significant effect of TC on the proteins involved in fatty acid metabolism and lipid and carbohydrate metabolisms was also observed in rat livers (*20*). G3PD1 was not among the spots identified by Yamamoto et al., but it should be emphasized that they did not identify all of the differential spots.

G3PD1 expression has also been related with some meat quality parameters (color and cooking loss) and oxidative metabolism (*26, 27*) in pig muscle.

Phosphoglycerol kinase (EC 2.7.2.3) is a transferase enzyme that transfers a phosphate group from 1,3-biphosphoglycerate to ADP in the seventh step of glycolysis, while LAP (cytosol) is an exopeptidase hydrolyzing the peptide bond adjacent to a free amino group (Kegg Database, (http://www.genome.jp/kegg/kegg2a.html, consulted March 2008)). Both enzymes were less abundant in the muscular tissues of treated animals (**Table 2**). This is the first time that effects of TC on these two enzymes has been observed, even if LAP has been previously studied in *L. dorsi* pig muscle as a possible predictor of meat quality (*28*).

The other two identified differential spots, novel protein [0610037L13Rik] and hypothetical protein isoform 2, were previously described in muscular tissues (Mascot database search

program (http://www.matrixscience.com, consulted March 2008)), but no data about their function nor their possible mode of action are available in the literature.

Our results, even if preliminary, show that proteomic analysis can evidence a recent TC administration in pig. Further investigations should be conducted to evaluate the effects on a larger population, the window of detection of the proteomic approach, and finally the relevance of differential protein spots as biomarkers of exposure to implement screening methods of detection. The observed differences in spot intensities of specific proteins induced by antibiotic administration could have further interesting implications related to tissue metabolism and meat quality.

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