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ANDROGENS AND ESTROGENS BIOMARKERS IDENTIFICATION IN BOVINE URINE APPLYING

A METABOLOMICS APPROACH

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Experimental

Introduction

Despite steroids have been banned in the EU for growth promoting purposes, their illegal use in livestock is regularly suspected in Europe and in edible tissues imported from third countries. To tackle associated practices (natural hormones, designer drugs, low dose cocktails), innovative screening strategies based on biomarkers monitoring have already proven their efficiency, e.g. in highlighting a range of β -agonists practices [1]. Further, such approach has proved its applicability for control purposes under ISO17025 accreditation [2].

To extend the work to other anabolic practices, markers of effects related to steroids application in livestock have been investigated. Specific urinary patterns associated with three steroid treatments (17 β -nandrolone, 17 β -estradiol, 17 β -nandrolone+17 β -estradiol) in bovine have been investigated through RPLC-ESI(+/-)-HRMS.

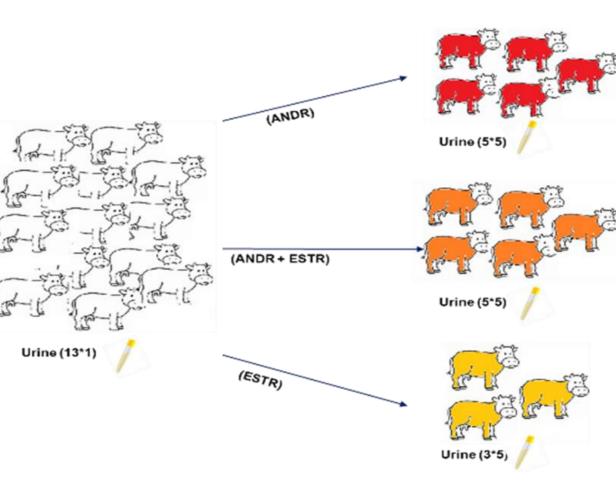
Results & Discusion

Steroid effect on urinary metabolome: Control versus treated animals

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Experimental Design

13 Friesian male veal calves aged 15-35 days were randomly divided in 3 groups and housed in box under the same controlled conditions for 6 months.



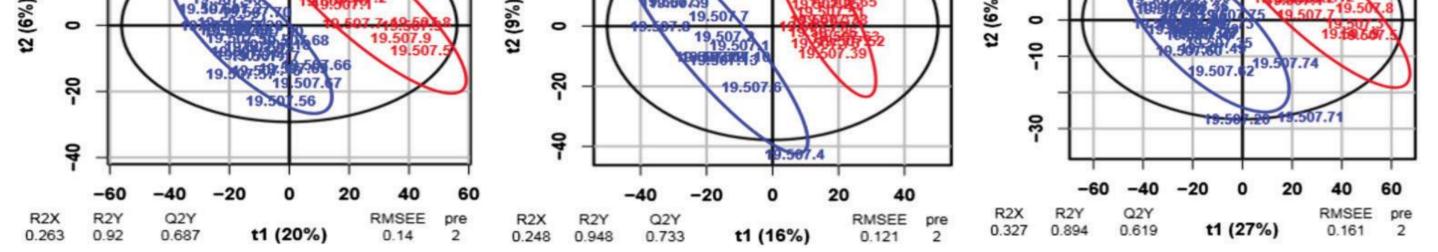
Animals were treated by intramuscular injection (IM) in the neck with either 17β -nandrolone decanoate ester (DECADURABOLIN®) or/and 17β -estradiol cypionate ester (ESTRADIOL DEPO®). Animals have been treated with steroids on 4 time points (D0, D7, D14, and D21). For each injection time point, five of them received 50-mg 17β -NT decanoate (ANDR), the other five received 3-mg 17β -E2 cypionate (ESTR), and the remaining three were treated with the mixture of 50-mg 17β -NT decanoate + 3 mg 17β -E2 cypionate (ANDR + ESTR). Control urine samples (CONTR, n = 13, collected at D0, before the first administration). In total, 78 urine samples were collected for about a month at regular time points, before and after the injections.

Sample preparation & metabolomics fingerprinting

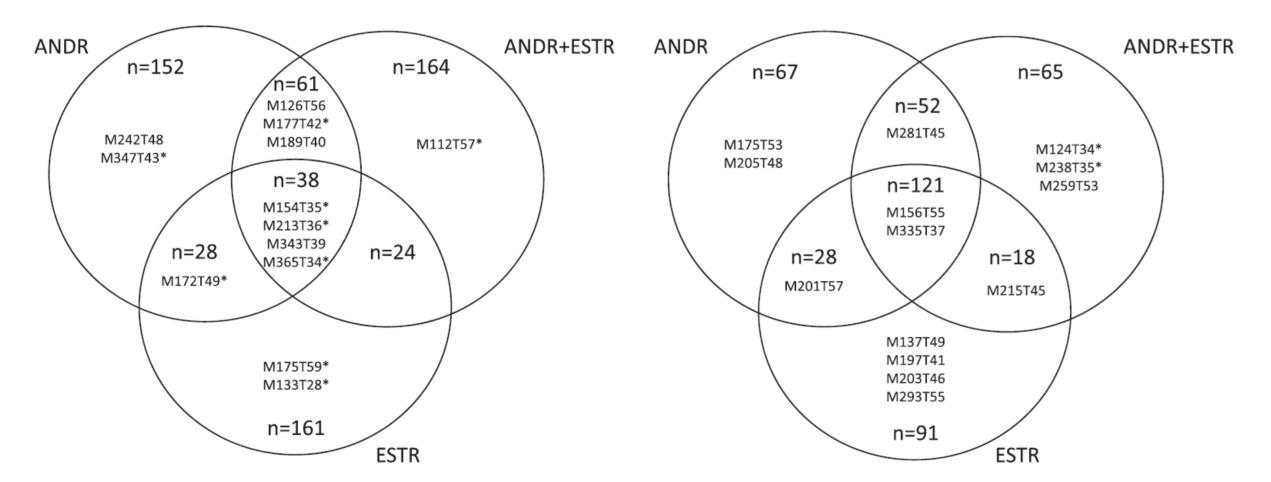
500 µL urine was normalized according to their specific gravity, filtered (10Kda) and centrifuged (14,000 g,30 min, 6°C). 30 µL were deposited in insert vials containing 10 µL at 2 ng/µL of evaporated mix metabolomics (L-leucine-5,5,5-d₃, L-tryptophan-2,3,3-d₃, indole-2,4,5,6,7-d₅–3-acetic acid, and 1,14-tetradecanedioic-d₂₄ acid) used as internal standards.

Chromatographic separation: Hypersil Gold C18 (2.1 x 100 mm, 1.9 μm). Elution solvents: 0.1% acetic acid in water (A), 0.1% acetic acid in acetonitrile (B). Elution gradient (A:B, v/v): 95:5 from 0 to 2.40 min; 75:25 at 4.50 min; 30:70 at 11 min; 0:100 at 14–16.5 min; 95:5 at 19–25 min. Flow rate: 0.4 ml/min, Injection volume: 5 μl, column's T° 35C.

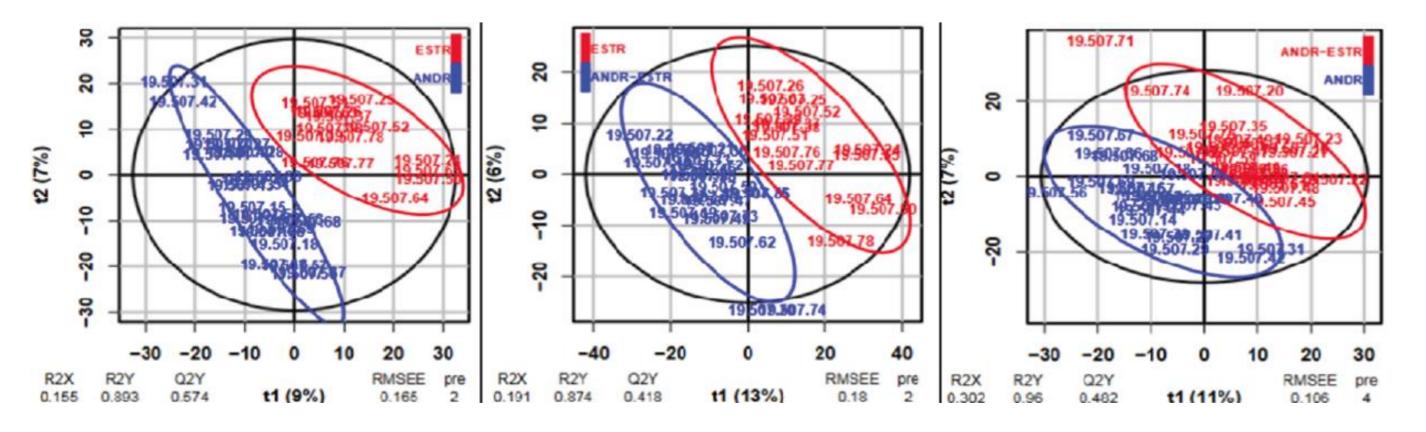
Acquisition on an Exactive-Orbitrap system (Thermo Fisher Scientific) in (ESI+/-), spray voltage 3.0 kV, capillary temperature 350°C, sheath gas flow rate 55 AU, gas flow rate 10 AU, and heater temperature 50°C. Full scan mass spectra acquired from 65 to 1,000 m/z, R 25,000 FWHM (at m/z 200) in centroid mode and a maximum time injection of 200 ms, Xcalibur (version 2.2) software.



Partial least squares discriminant analysis (PLS-DA) on ESI- datasets showed altered profiles between the groups studied. Similarities of effects between the three steroid treatments were also observed as shown on the Venn diagram below presenting the number of discriminant ions by conditions investigated or in common between treatments as well as the metabolites annotated in both ESI+/- datasets. (MxxTyy: M exact mass and T retention time in sec, * lower urinary concentration in considered group). Some ionic signals appeared to be impacted in the same way by the three treatments (Figure 3, n = 121 ions), allowing to conclude as to probable similar mechanisms of action. ESI +



Comparison of the different steroid treatment's effects



Data processing & Analysis

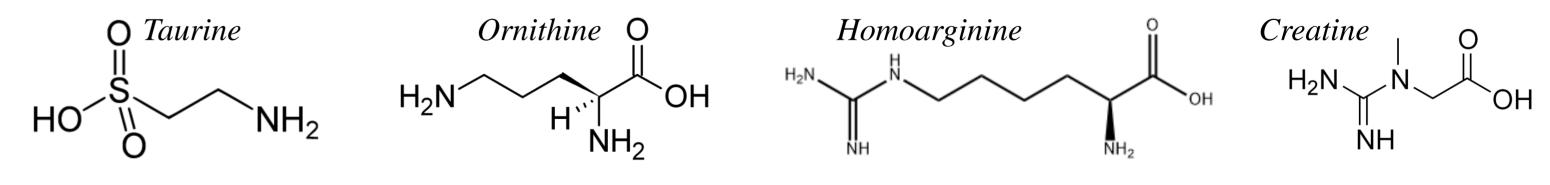
A log10 transformation and a "Pareto" scaling were applied on the data. Statistical analysis was carried out using both Workflow4Metabolomics platform (version 3.3) and SIMCA-P+[®] (Version 13.0.2, Umetrics AB, Sweden). Partial least squares discriminant analysis (PLS-DA) supervised analyzes were performed. Model's validity and robustness were evaluated by R2 (Y) and Q2 (Y), as well as by permutation tests (n = 1,000). The p-value threshold considered was < 0.05. Only discriminant ions with VIP > 1.5 were selected.

Structural identification

MS2 was performed on a selection of metabolites. Acquisition on a hybrid quadrupole-orbitrap (Q-Exactive[™]) mass spectrometer (ThermoFisher Scientific), heated electrospray (H-ESI II) source. Two inclusion lists were used for subsequent fragmentation in the positive or negative targeted-MS2 mode using higher energy collisional dissociation (HCD), resolving power 17,500 FWHM (at 200 m/z); AGC Target, 2 x10⁵; maximum IT, 100 ms; isolation window 1.0 m/z, and normalized collision energy (NCE) at 35%.

Biomarkers investigation

Twenty-eight candidate biomarkers could be further investigated using targeted MS² strategy. In particular, M189T40 was assigned to [M+H]+ of homoarginine, which was observed with higher concentration levels in androgen treated animals. The ion M133T28 was assigned to the $[M+H]^+$ of ornithine, presenting lower concentration in E2-treated animals. Finally, the only level-1 ion identified in ESI+ is M154T35 and was assigned to [M+Na]+ of creatine, showing lower concentration level in the urine of cattle treated with any of the steroids considered. In the ESI- fingerprints, the M124T34 could be identified (level-1) as [M-H]- of taurine. Taurine exhibited lower concentration levels in the urine of animals treated with 17 β -estradiol.



Partial least squares-discriminant analysis (PLS-DA) score plots on ESI- datasets demonstrated specific effects associated to each of the 3 experimental groups and a number of ions of interest could be specifically associated in bovine urine with nandrolone, estradiol, or a mix of nandrolone and estradiol treatment.

Biological pathways affected

The metabolites identified show that not only common but also specific metabolic pathways are activated by the various treatments. In particular, the pathways involving ornithine appear to be solicited only when estradiol alone is used, whereas creatine responds to treatment with estradiol whether used alone or as a cocktail. Taurine was selectively associated to the use of nandrolone combined with estradiol. Creatine is a naturally occurring compound which primary metabolic role is to generate phosphocreatine, subsequently used to regenerate adenosine triphosphate (ATP), it is therefore essential for energy and muscle metabolism. Ornithine and homoarginine are metabolites involved in creatine biosynthesis, while taurine is essential for a range of functions among them the development and function of skeletal muscle, which may explain in the present context its role as steroid biomarkers.

Conclusion

While specific fingerprints with strong differences could be highlighted between urinary metabolite profiles within urine samples collected on control and steroid treated animals, it appeared further that significant discriminations could also be observed between steroid treatments, evidencing thus specific patterns and candidate biomarkers associated to each treatment. An MS-2 structural elucidation step enabled identification of biomarkers mainly involved in energy pathways, in relation to skeletal muscle functioning [3]

[1] Dervilly-Pinel Metabolomics 2015[2] Dervilly-Pinel Food Additives Contaminants 2018[3] Ouzia Drug Test Anal. 2021

Poster presented at Euroresidue IX, 22-25 May 2022, The Netherlands