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Plasma metabolomic profiling based detection of drug specific responses to different bovine growth promoting regimes

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A B S T R A C T

The use of anabolic substances for growth promoting purposes in food producing animals is prohibited within the EU, yet ongoing applications of hormones such as oestradiol prove both difficult to detect and to distinguish from endogenous presence. Additionally, the misuse of glucocorticoid compounds (dexamethasone and prednisolone), which are permitted for therapeutic applications but can also promote improved animal health through long-term dosing, is reported to be increasing posing potential health concerns for consumers. Twenty-four male beef cattle were randomly assigned to four groups ($n = 6$) for experimental treatment over 40 days consisting of a control untreated group, and three treatment groups administered oestradiol, dexamethasone or prednisolone at levels known to reflect growth promoting practices. Untargeted metabolomic profiling of plasma collected from each animal midway through the study treatment period, was performed following reverse phase separation employing an UHPLC-QTof-MS system operating in positive electrospray ionization mode. Metabolomics analysis revealed metabolite perturbations in plasma common to all treated animals, with additional metabolites found to be specifically associated to the various differing growth promoting regimes. OPLS-DA modelling was used to discriminate plasma profiles of oestradiol, dexamethasone, or prednisolone from control untreated cohorts with 56, 48 and 58 ions found to be altered by the respective administered treatments. This culminated in 99 shared ions which could differentiate between plasma samples from untreated or variously growth promoter treated animals. Further assessment of these metabolites identified 24 ions to be significantly altered in comparison to control animals, of which 3, 11 and 8 ions were pertinent to oestradiol, dexamethasone or prednisolone administrations respectively and 2 relevant to more than one treatment. Incorporation of such markers, principally associated with lipid and fat metabolism responses to exogenous administrations, which are specific to growth promoting treatment types could be used in screening approaches to facilitate more effective confirmatory analysis.

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1. Introduction

The implementation of screening based testing for detection of drug residues in food producing animals is a required action within the European Union as stipulated in EC Regulation 2002/178/EC (2002). Testing is assigned through National Residue Control Plans (NRCP) coordinated by European Residue Laboratories (EURLs) and results are reported to the European Food Safety Authority (EFSA) for annual review. EURLs are required to test 0.4% of slaughtered cattle numbers to meet minimum legislative requirements (Directive, 1996), and while routine regulatory monitoring finds sufficient compliance (DAFM, 2015), additional random on-farm sampling indicates continued illicit use of chemical agents within beef producing animals (Leporati et al., 2015; Imbimbo et al., 2012; Chiesa et al., 2016). The financial gains arising from illegal

ABBREVIATIONS: EDTA, Ethylenediaminetetraacetic acid; MW, molecular weight; m/z, mass to charge ratio; AMRTP, accurate mass and retention time pair; QC, quality control; FC, Fold Change; RSD, relative standard deviation; ANOVA, analysis of variance; UHPLC, Ultra High Performance Liquid Chromatography; MS, Mass Spectrometry; PCA, Principal Component Analysis; OPLS-DA, Orthogonal Partial Least Squares Discriminant Analysis; VIP, variable importance of projection.

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growth promoting administration encourages their use and exposes consumers to toxicological risk from contaminated food materials due to a combination of irregular drug use and ineffective testing (Ronquillo & Hernandez, 2017).

Current test methods are dependent on direct detection analysis of known compounds with confirmatory analysis typically reliant on gas (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) methods. Despite improved sensitivity through progress in these advanced technologies, analytical challenges to the detection of growth promoter use persist (Stolker & Brinkman, 2005; Ginkel and Sterk, 2016). These challenges include the detection of emerging unknown compounds, identification of drug use at low doses, and effective discrimination between endogenous forms of hormones and exogenous administrations either as therapeutics or for illicit purposes (Courtheyn et al., 2002; Mooney, Elliott, & Le Bizec, 2009; Pinel et al., 2010). The latter includes glucocorticoid and oestradiol derivatives which are increasingly abused (EFSA, 2013; Sterk, Blokland, De Rijke, & Van Ginkel, 2014) due to their natural presence which is indistinguishable from external application. In this way confirmatory methods have incorporated isotope ratio (IRMS) techniques to discriminate exogenous metabolites based on the ratio of $^{13}C/^{12}C$ (Janssens et al., 2013). However, such analyses are only available through confirmatory test methods and robust screening tools are needed. As such more research in this field is directed towards assessment of an animal's biological response to drug administration as a feasible alternative approach to discriminate biomarkers significant to xenobiotic exposure (Nebbia et al., 2011). In this way, Marin et al. (2008) were able to discern dexamethasone administration in finishing bulls by monitoring blood parameters, whilst Mooney et al. (2008) and Doué et al. (2015) have demonstrated biochemical screening of sex-hormone and bone markers as indicative of steroid misuse.

The range of sample matrices available for anabolic screening tests varies and is dependent on the regulatory body requirements on whether the drug to be tested is acquired from live or slaughtered animals (Directive, 1996). External biological material such as urine, hair and blood can be sampled on farm, whilst consumable parts are only available after slaughter. Some metabolomic studies have been conducted with urine to distinguish treatment of oestradiol, β-agonist and prohormones (Courant et al., 2009; Dervilly-Pinel et al., 2011; Jacob, Dervilly-Pinel, Biancotto, Monteau, & Le Bizec, 2015; Rijk et al., 2009), however there are concerns of false positive results due to faecal contamination (Arioli, Fidani, Casati, Fracchiolla, & Pompa, 2010) and also endogenous prednisolone levels caused by stress (Pompa et al., 2011). Similarly, hair analysis may be subject to environmental contamination and obscured by the method of drug delivery, whilst drug residues are known to diffuse rapidly (Vanhaecke, Antignac, Courtheyn, Le Bizec, & De Brabander, 2011). For the purpose of screening, blood can be collected on farm and Noppe, Le Bizec, Verheyden, and De Brabanderd (2008) previously reported a higher occurrence of steroid hormones within the blood due to the circulating action from anabolic tissues whilst metabolomic profiling has revealed detectable biomarkers within the plasma collected (Graham et al., 2012).

Metabolomic fingerprinting has been promoted as a non-targeted approach whereby the entire metabolite profile is compared to unveil markers which differ between animal cohorts (Dettmer, Aronov, & Hammock, 2007; Fiehn, 2002). The acquisition of such a vast amount of data requires both bioinformatic tools to generate models which can distinguish the disrupted homeostatic state due to exogenous drug administration, and predictive techniques that assign acquired data to an assumed response (Antignac et al., 2011; Courant, Antignac, Dervilly-Pinel, & Le Bizec, 2014). Recent research incorporating the whole profile of blood metabolites to discriminate cattle exposed to growth promoting agents has been described (Dervilly-Pinel et al., 2012; Graham et al., 2012; Nzoughet et al., 2015a; Regal et al., 2011), yet progress towards applicable screening approaches is as yet unrealised. Metabolites contributing to differentiating profiles have been investigated (Dervilly-Pinel et al., 2012; Pinel et al., 2010; Riedmaier, Becker, Pfaffl, & Meyer, 2009) but their reliability is often obscured by biological and environmental conditions and the specific relevance of metabolite profiles to individual growth promoter treatments is not clear.

The focus of the current study has centred on the detection of metabolomic markers significant to the misuse of glucocorticoid (dexamethasone and prednisolone) and oestradiol compounds in bovine animals for meat enhancement purposes. Glucocorticoid agents are readily available for therapeutic veterinary applications but may be misused through long-term low-dose regimes which sustain animal health whilst encouraging lean meat production (Antignac, Le Bizec, Monteau, Poulain, & Andre, 2001; Cannizzo et al., 2011). The use of oestradiol for growth promoting purposes is currently prohibited by the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) (Directive, 2003), and whilst effective monitoring procedures have been established, it's availability outside the EU is thought to contribute to a black market of illicit use (Courtheyn et al., 2002; Regal, Alberto, & Fente, 2012) with administrations difficult to distinguish from variable endogenous levels in cattle (Regal et al., 2011). We for the first time unveil the bovine plasma metabolome changes, detected by ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS), significant to the administration of oestradiol, dexamethasone or prednisolone. The results illustrate the use of advanced statistical models incorporating ions altered by various treatment types to predict growth promoter exposure, whilst putative identifications highlight the possible underlying metabolite functions specific to administered compounds.

2. Materials and methods

2.1. Chemicals and reagents

LC-MS grade methanol (MeOH) and formic acid (HCOOH) were purchased from Sigma Aldrich (UK). Leucine enkephalin (Leu-Enk) was sourced from Waters (UK) and ultra-pure water (18.2 MΩ cm^{-1}) was generated in-house using a Millipore system (Millipore, USA).

2.2. Experimental design and plasma sample collection

Samples were obtained from an experimental treatment study using growth promoting regimes reflective of suspected on-farm practices conveyed in the literature (Cannizzo et al., 2008; Courtheyn et al., 2002; De Maria et al., 2009). Authorized by the Italian Ministry of Health and bioethics committee of the University of Turin, the study cohort consisted of twenty four male *Charolais* cattle aged 17–22 months old randomly assigned to four treatment groups: Group O ($n = 6$) received 0.01 mg/kg intramuscular injection of 17β-oestradiol-3-benzoate (Sigma-Aldrich, Milan, Italy) weekly on day 12, 19, 26, 33 and 40; Group D ($n = 6$) were administered an oral dose of 0.7 mg/day dexamethasone-21-sodium phosphate (Desashock Fort Dodge Animal Health, Bologna, Italy) for 40 days; Group P ($n = 6$) were given 15 mg/day prednisolone acetate orally (Novosterol, Ceva Vetem SpA, Milan, Italy) for 30 days; Group C ($n = 6$) were control untreated animals. All animals were kept in separate housing and fed a diet of silage,

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