Multi-block integration of four LC-HRMS metabolomic datasets to improve screening of growth promoter administration in production animals

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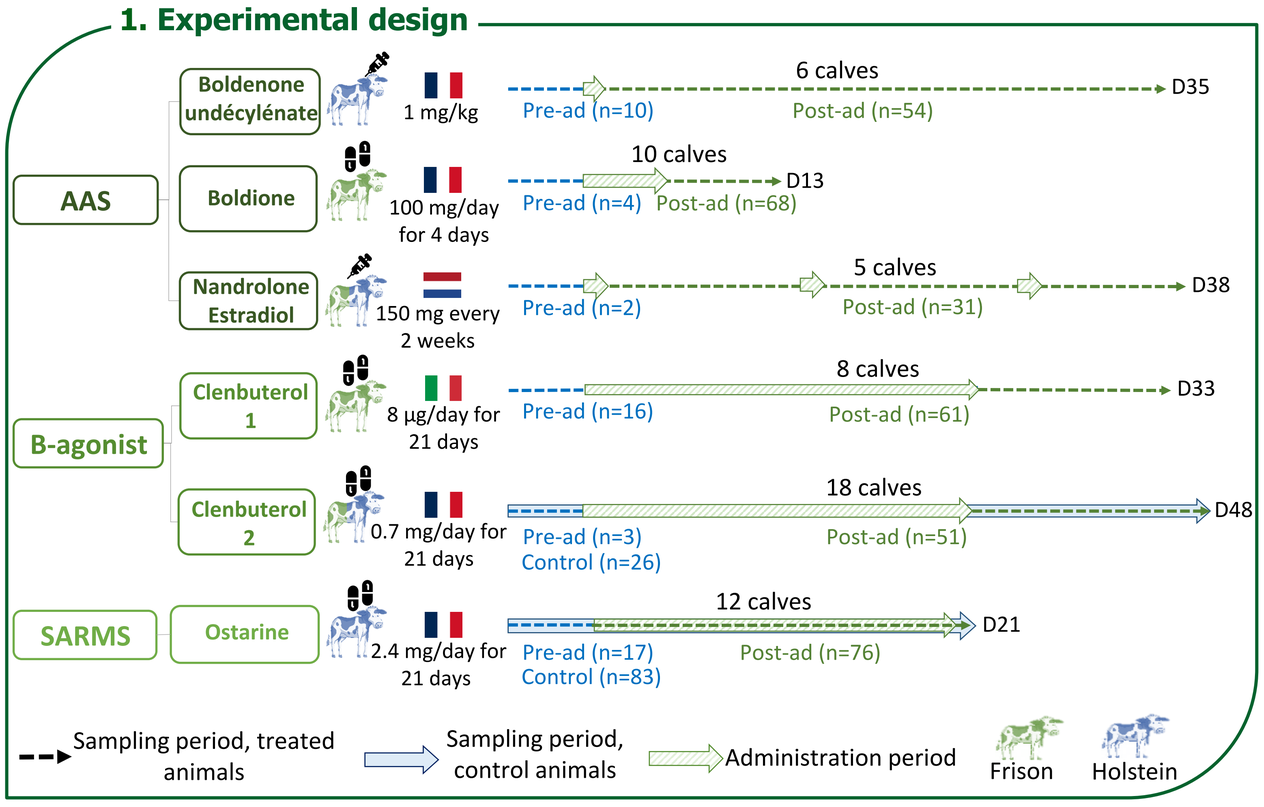
INTRODUCTION

To ensure the safety of food products of animal origin, screening for the administration of prohibited growth promoters in production animals is a priority for public authorities. In this regard, metabolomics is a preferred strategy for the discovery of new effect biomarkers. Numerous studies have demonstrated the potential of these approaches as proof of concept, but they only addressed a single class of compounds and had limited metabolome coverage. Thus, the present study aimed to establish a comprehensive classification model for urine samples from cattle treated with five different growth promoters, based on a more exhaustive evaluation of the metabolome.

EXPERIMENTAL

1. Experimental design



2. Sample preparation

Density based normalisation
→ 10 kDa filtration
→ Centrifugation (13000 g 10 min 4 °C)
→ Supernatant collection

3. Metabolomics analysis

RPLC analysis
Hypersil Gold
Flow Rate : 0,4 mL/min
A : H2O + 0.1% AcetAc
B : ACN + 0.1% AcetAc

HILIC analysis
BEH amide
Flow Rate : 0,4 mL/min
A : H2O + 20 mM ammonium formate and 0.1% formic acid in water.
B : ACN + 0.1% AcetAc

HRMS analysis - **Q-Exactive**

MS acquisition
- Full scan @ 70k with polarity switch

MS/MS acquistion
- Iterative DDA Top5 @ 35k for annotation

4. Data processing

Peak picking
- NA < 20 %
- Fold Change > 2 (sample/blank)
- IQR (InterQuartile Range) filtering

Signal normalization
- LOESS
- Log 10
- Pareto

Sample normalization
- PQN (Probabilistic Quotient Normalization) based on QC samples
- Time 0 centered

(Narduzzi *et al.* 2020)

5. Statistical analysis

Univariate analysis
- Wilcoxon test with FDR (False Discovery Rate) correction
- Fold change

Multivariate analysis
- PCA, C-OPLS
- Multiblock integration
- UVE (Uninformative Variation Elimination)
- Model validation: cross validation and permutation test

(Boccard *et al.* 2013)

6. Annotation

Level 5: m/z
Level 4: Molecular formula (adduct, isotope)
Level 3: Candidate based on in silico analysis (SIRIUS)
Level 2: MS and MS/MS match with spectral database
Level 1: Standard comparison

(Schymansky *et al.* 2014)

References

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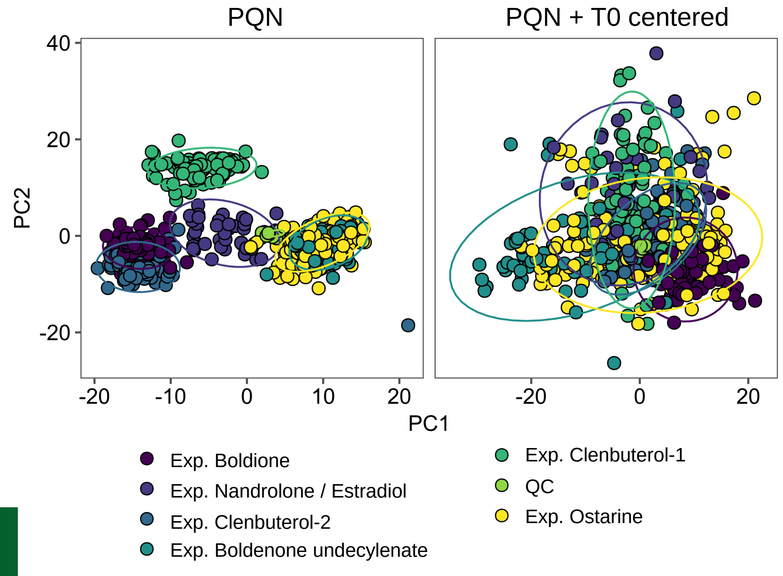
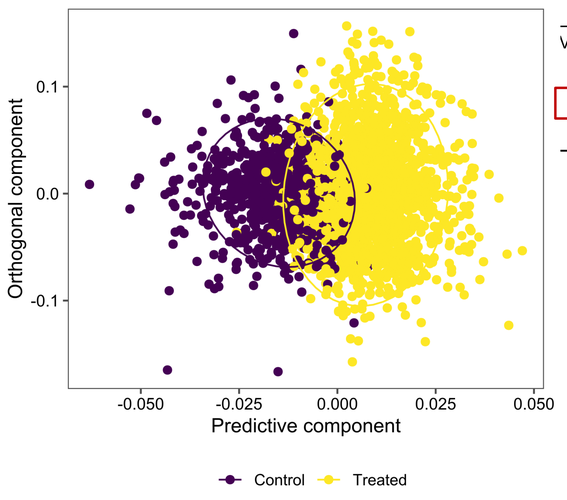
RESULTS AND DISCUSSION



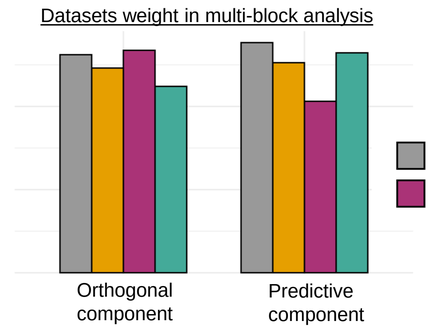
1. Data processing

The quality of the data was verified using PCA. Although no instrumental variability was observed (as indicated by grouped QC samples), specific phenotypes for each experiment were highlighted despite PQN normalization and could masking the effects of growth promoter administration. To address the significant inter-individual variability and standardize the data obtained from each experiment, T0 normalization was performed using samples collected before administration. After normalization, the data appeared homogeneous across different individuals.

2. Classification model and multiblock integration

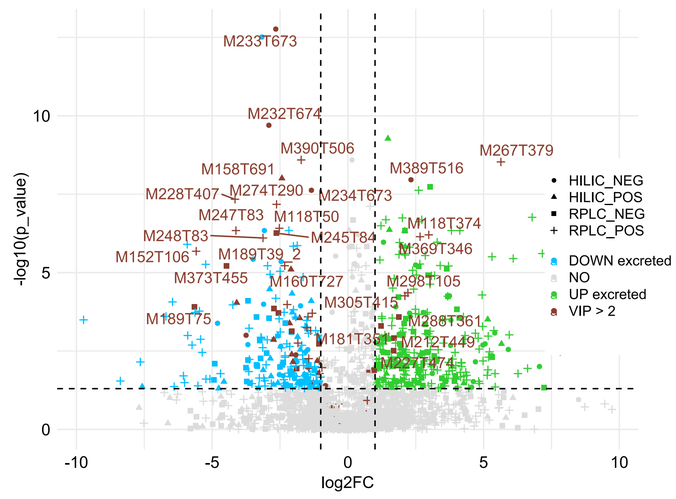


Block	HILIC_NEG	HILIC_POS	RPLC_NEG	RPLC_POS	Multiblock
Variable number	252	260	447	1134	2093
R2Y	0.507	0.535	0.565	0.682	0.678
Q2Y	0.448	0.429	0.565	0.619	0.662
R2X	0	0	0	0	0

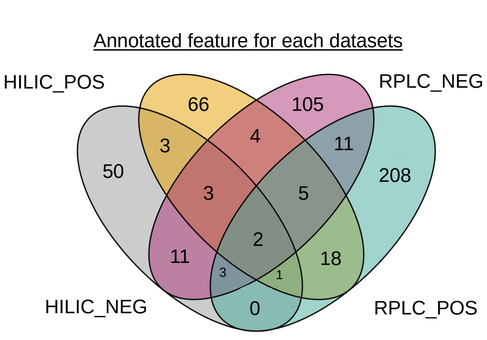


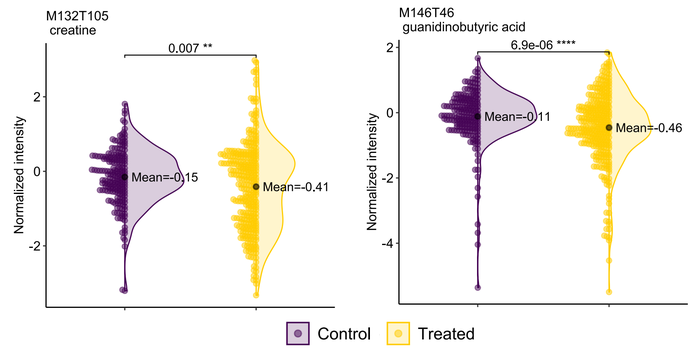
Following metabolomics analyses, four datasets were obtained: RPLC_POS, RPLC_NEG, HILIC_POS, HILIC_NEG. Multivariate analyses highlighted differences in the metabolome between control samples and those collected after administration of anabolic agents regardless of the compound. Furthermore, considering the model performance (Q2Y), multi-block integration improved the classification and prediction of samples compared to individual models. In this regard, multi-block analyses also demonstrated that all four datasets were important in predicting treated samples.

3. Biomarkers discovery



Combining both univariate and multivariate analysis with compound annotation allowed potential biomarkers discovery. Interestingly, known compounds discovered in previous metabolomics studies on common experiments also appeared significantly different when including a wide variety of anabolic agents. This is notably the case for creatine (Dervilly *et al.* 2018) and guanidinobutyric acid, a metabolite derived from the arginine and proline metabolic pathway (Stojilkovic *et al.*, 2019). Metabolite annotation also demonstrated the complementarity of the four datasets, with only two metabolites in common.





CONCLUSION

In conclusion, the combination of (1) six different experiments and (2) four LC-HRMS analyses, never reported previously to our knowledge, illustrates the complementarity of LC-HRMS analyses in terms of metabolic coverage. Their multi-block integration enabled the implementation of a comprehensive classification model capable of predicting the administration of growth promoters.

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