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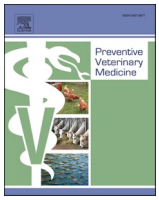

Metabolic profile of Charolais young bulls transported over long-distance

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ABSTRACT

Long-distance transport can cause stress to beef cattle impairing health and growth performances. This study aimed to deepen the knowledge of the effects of long-distance transport on young bulls' metabolic profile to identify reliable blood parameters for monitoring their health and welfare. Eighty Charolais young bulls, transported for 12 hours to the final fattening unit, were weighed and blood sampled at three time intervals: before leaving the commingling centre (**day −1**), upon arrival at the fattening unit (**day 0**), and 7 days post-arrival (**day 7**). These bulls were part of a larger study aimed at testing whether the animals have some benefit from the supplementation of live yeast and selenium through slow-release rumen boluses that were administered to half of them before departure from France ('Yeast' vs 'Control' group). The effect of the supplementation on the parameters considered in this study was included in the statistical analysis to account for the structure of the experimental design. Transport affected the initial body weight of the animals, which dropped on day 0 and it was not fully recovered on day 7. Most plasma traits of protein, energy, hepato-muscle, and mineral profiles were affected by transport. Cortisol was also assessed and peaked at day 0. The footprint of the long-distance transport on bulls' metabolic profile indicated a combination of stress, energy deficit, and muscle damage, with a certain degree of dehydration and liver impairment. Plasma traits measured on day −1 highlighted that stressful conditions and physiological responses of the bulls to recover homeostasis already started during the commingling phase before departure. No effect of supplementation was detected, except for higher selenium plasma level in Yeast bulls at day 7. Among blood parameters, non-esterified fatty acids, total protein, cortisol, glucose, and iron were those responsible for most of the variation in metabolic profile of bulls undergoing long journey. Therefore, these traits might be used as major biomarkers to assess stress in transported beef cattle, helping to identify critical situations for which proper mitigating actions should be taken. The outcomes of this study suggested that preventive measures against transport stress in beef cattle should start at the commingling of the animals in the collection centers, thus before departure.

1. Introduction

About 1.4 billion bovines, swine, poultry, ovine, caprine, and equines traveled across Europe in 2019, and long-distance journeys were 125000 in 2015 (Massot et al., 2021). Long-distance transport can cause severe stress to animals (Ashenafi et al., 2018) and public concern about livestock transfer has been increasing in recent years. The European Council Regulation (1/2005) on animal protection during transport defined as 'long' a journey that exceeds 8 hours, starting from when the first animal of the consignment is moved. The European Food Safety

Abbreviations: ADG, average daily gain; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APP, acute phase protein; AST, aspartate aminotransferase; AUC, area under the curve; BW, body weight; CHOL, cholesterol; CK, creatine kinase; Control, control group of bulls; CREA, creatinine; CV, coefficient of variation; Day 0, the day of arrival to Italy; Day −1, one day before departure from France; Day 7, after one week of arrival to Italy; GGT, gamma-glutamyl transferase; GLOB, globulins; GLU, glucose; GPX, glutathione peroxidase; LDH, lactate dehydrogenase; MDG, mean decrease Gini; NEFA, non-esterified fatty acids; RF, Random Forest; ROC, receiver operating characteristic curve; TG, triglycerides; TP, total proteins; UREA, total blood urea; Yeast, group of bulls that received a supplementation.

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Authority (**EFSA**) is fully committed to improving the current situation of the livestock transported across Europe by refining the regulations in force or finding feasible alternatives (EFSA, 2022). In this scenario, the Italian situation regarding beef cattle is of particular interest. Every year, around 900000 young bulls and beef heifers are imported mainly from France to complete their fattening in Italian beef farms (ISMEA, 2022). Coming from pasture-based suckler herds, the animals are commingled in dedicated collection centres before being transferred to the Italian fattening units (Herve et al., 2020). In the collection centers, animals from different areas and farms are stratified according to sex and body weight (**BW**), and the resulting batches are then uploaded to the trucks (Santinello et al., 2022). Upon arrival at the Italian fattening unit, beef cattle face an adaptation period to the new housing and management system. Over the first 20–30 days from arrival, their diet is gradually shifted from forage-based to high-energy-based, with concentrates representing more than 60 % of the total matter at the end of this transition period (Cozzi, 2007). Commingling, transport, and change in climate, housing, and feeding are stressful factors that predispose the incoming animals to disease outbreaks (Taylor et al., 2010; Santinello et al., 2022). As a result, the mortality rate peaks during the first month of the fattening cycle (Rumor et al., 2015).

Previous studies demonstrated the role of transport in inducing changes in the physiological profile of young cattle (Buckham Sporer et al., 2008; Earley et al., 2017). Indeed, some blood parameters, such as cortisol and creatine kinase (**CK**), have already been used as indicators of stress in transported beef cattle (Ashenafi et al., 2018; EFSA, 2022). Therefore, the metabolic profile appears a promising tool to monitor the physiological status of the transported cattle.

This study aimed to deepen the knowledge about the effects of long-distance transport on the metabolic profile of Charolais young bulls by analyzing a complete panel of blood protein, energy, hepato-muscle, and mineral parameters. Blood parameters and BW were monitored from the day before departure to the end of the first week after the arrival at the fattening unit to investigate variations in metabolic profile and identify potential biomarkers that can be useful to monitor stress in transported bulls and thus tailor more specific mitigation strategies.

2. Material and methods

All experimental procedures were approved by the Animal Care and Use Committee of the University of Padova (Ethical Approval Code: n. 400074 5/2021).

2.1. Animals and fattening unit management

Data used in this study referred to two batches of 40 Charolais young bulls each that were transported from 2 French collection centres (Auvergne region) to an Italian commercial fattening unit (Veneto region) in October 2021. Bulls arrived at the French collection centers 1 or 2 days before departing to Italy at an average age of 286 ± 24 days (±SD). At the French collection centres, bulls were housed in straw-bedded pens of 8 mates and were fed hay and water *ad libitum*. This study was the first part of a research project aimed at testing some practical solutions to mitigate the adaptation stress of bulls to the change in feeding regime during the receiving period of 30 days at the final fattening unit. With this aim, the young bulls of each batch were allocated to 2 supplementation groups of 20 animals each, balanced according to animals' BW, named 'Yeast' and 'Control'. The day before departure, Yeast bulls received 2 slow-release boluses (Levucell® SC 10 TITAN, Lallemand SAS, Blagnac, France) that contained the live yeast strain *Saccharomyces cerevisiae* CNCM I-1077 and a selenium-enriched yeast (ALKOSEL®, Lallemand SAS, Blagnac, France) produced from *S. cerevisiae* NCYC R397. Boluses were designed for a slow release of 1.5 g/d of live yeast (i.e., 1.5×10^{10} CFU/d) and 1.5 mg/d of selenium (**Se**; i.e., 0.95 ppm selenomethionine for a target dry matter intake of 8 kg) over one week of bolus administration. Sample size calculation was performed using the POWER procedure of SAS software 9.4 (SAS Institute Inc., Cary, NC). Due to the lack of sufficient literature on the trend of blood traits during transport specific to the genotype and origin of the cattle considered in this study, the average daily gain (**ADG**) was the variable selected to determine the minimum number of experimental units, with an expected mean and SD of 1.40 ± 0.23 kg/d for the whole fattening cycle (Gallo et al., 2014). A two-sample t-test was used, setting the type I error (α) to 0.05 and the test power (1-β) to 0.80. Assuming a difference (δ) in growth between Yeast and Control groups of 0.15 kg/d, 38 individuals per group were necessary. Assuming a 5 % prudential estimate of potential loss of animals per group, the group size was set to 40 bulls each.

According to the individual inspection performed by a veterinarian, all bulls were clinically healthy before transport. The journey from France to Italy lasted 12 hours covering a distance of 950 km. The transport was carried out in compliance with the current European regulations for cattle transport (European Council Regulation, 1/2005/EC; EC, 2018). The European Council Regulation (1/2005) on animal protection during transport is the main law regulating the transport of live animals between European Union (**EU**) Member States and providing for checks on animals entering or leaving the EU. It lays down detailed rules that aim at safeguarding animal welfare and preventing injury or unnecessary suffering to the transported animals. Besides authorizations and official controls issues, the regulation details general requirements for the transport of animals, such as fitness to travel, prior arrangements before transport, means of transport, loading and unloading facilities and transport practices, space allowances, duties of transporters, feed and water provision, rest periods, handlers skills, treatment of sick animals, specific additional requirements by animal species and type of transport (by road, sea or air), and requirements for emergencies or failure to apply the welfare rules. For long journeys (more than 8 hours) between EU countries and destinations outside the EU, the regulation sets up specific requirements regarding trucks' roof, floor and bedding, feed and water provision, partitions, ventilation systems and temperature monitoring.

In this study, therefore, each truck driver checked the physical status of the animals, provided the bedding straw, and checked the functionality of water troughs in the truck before starting the loading procedure. The two trucks leaving from the collection centres in France carried 40 young bulls each, providing an average space allowance of 2 m^2/head. At the arrival to the Italian fattening unit, young bulls of each truck were unloaded in 4 pens of 10 mates each without mixing bulls coming from different collection centres. The space allowance within the pen was of 7.5 m^2/bull and the manger space was of 0.5 m/bull. All pens had a deep litter straw bedding and were equipped with two waterers to allow *ad libitum* drinking. In the first 7 days at the Italian fattening unit, bulls were fed *ad libitum* hay along with an increasing amount of a total mixed ration based on corn silage (25 % as fed basis), wet sugar beep pulps (13 %), corn meal (12 %), wheat bran (11 %), dehydrated alfalfa (10 %), soybean meal (9 %), and mineral premix (2 %). The mineral premix contained 19.1 % calcium, 0.33 % phosphorous, 0.17 % magnesium, and 0.06 % sodium. The total mixed ration had an average dry matter content of 55.4 % and mean contents of ash, crude protein, aNDF, and starch of 5.7 %, 14.0 %, 39.2 %, and 20.0 %, respectively.

2.2. Body weight and blood measurements

Individual BW measurements and blood samplings were performed: (i) at the French collection centres before traveling (day −1); (ii) upon arrival at the Italian fattening unit (day 0); and (iii) after one week (day 7). Animal handling and moving were performed by trained personnel to reduce stressful conditions and the procedures were conducted only after the veterinarian checked bulls' health. An electronic livestock scale with a precision of 0.5 kg (Ghislandi&Ghislandi, Covo, Bergamo, Italy) was used to individually weigh the bulls.

Blood samples were collected by the veterinarian of the collection

centres in France and by the veterinarian of the fattening unit in Italy. All samples were taken from the jugular vein of each bull using 9 mL Vacuette® LH - lithium heparin blood collection tubes (Greiner Bio-One GmbH, Kremsmünster, Austria) and placed into a refrigerated box filled with dry ice until the end of the sampling session. Blood tubes were centrifuged at 1500 × *g* for 15 minutes at 4°C within 2 hours after the collection and plasma was aliquoted and stored into 2 mL cryovials (S.I. A.L. Cryovials, Rome, Italy) at –80°C until the analysis. Blood analyses were carried out at the laboratory of the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) and blood parameters included the protein profile (albumin, **ALB**; creatinine, **CREA**; globulins, **GLOB**; total proteins, **TP**; total blood urea, **UREA**), the energy profile (cholesterol, **CHOL**; glucose, **GLU**; triglycerides, **TG**), the hepato-muscle profile (alkaline phosphatase, **ALP**; alanine aminotransferase, **ALT**; aspartate aminotransferase, **AST**; **CK**; lactate dehydrogenase, **LDH**; gamma-glutamyl transferase, **GGT**; total and direct bilirubin), and the mineral profile (calcium, **Ca**; chlorine, **Cl**; iron, **Fe**; potassium, **K**; magnesium, **Mg**; sodium, **Na**; phosphorous, **P**; Se). Levels of cortisol and non-esterified fatty acids (**NEFA**) were assessed as well. All plasma components were analysed by a biochemical analyser (COBAS C501, Roche Diagnostics GmbH, Mannheim, Germany) using commercial diagnostic kits from Roche Diagnostics. The NEFA determination was performed with enzymatic colorimetric methods (Randox Laboratories Ltd., Ardmore, United Kingdom) using COBAS C501 instrumentation.

2.3. Statistical analysis

Statistical analysis was performed using the SAS software 9.4 (SAS Institute Inc., Cary, NC) to assess the effect of long-distance transport on young beef bulls' blood metabolic profile. Yeast supplementation by means of slow-release boluses was addressed to mitigate the potential negative effects at the rumen level due to the change in feeding regime carried out during the receiving period (first 30 days) at the final fattening unit. The assessment of the effects of yeast supplementation on the rumen environment was not within the scope of the present study, and no significant effects of this treatment were expected on bulls' blood parameters during the study period. However, the supplementation factor was included in the statistical model used for data processing to account for the structure of the experimental design and avoid potential bias due to it. The BW loss due to transport was calculated for each bull as the difference between BW at day −1 and BW at day 0, whereas the ADG from day 0 to day 7 was calculated as (BW, day 7 – BW, day 0) / 7.

To assess the trend of BW over time (from day −1 to day 7), the following repeated mixed model (MIXED procedure of SAS) was used (Eq. 1):

$$y_{ijklm} = \mu + Time_i + Supplementation_j + Pen_k(Supplementation_j) + (Time \times Supplementation)_{ij} + Age_l + animal_m + e_{ijklm}$$

where y_{ijklm} is the dependent variable; μ is the overall intercept of the model; $Time_i$ is the fixed effect of the *i*th day of measurement ($i = -1, 0, 7$); $Supplementation_j$ is the fixed effect of the jth supplementation group (j = Yeast, Control); $Pen_k(Supplementation_j)$ is the fixed effect of the *k*th pen (k = 1–8) nested within the *j*th supplementation group; $(Time \times Supplementation)_{ij}$ is the fixed interaction effect between day of measurement and supplementation group; Age_l is the fixed effect of the *l*th age of the bulls at arrival modeled as covariate; $animal_m$ is the random effect of the *m*th bull (m = 1–80) ~N(0, σ^2_{bull}), where σ^2_{bull} is the bull variance; and e_{ijklm} is the random residual ~N(0, σ^2_e), where σ^2_e is the error variance. Post-hoc multiple comparisons of least squares means were performed using Bonferroni's correction.

Regarding blood parameters, values outside the mean ±3 SD were considered outliers and discarded from the dataset (Cozzi et al., 2011). All blood variables were then checked for normal distribution using the UNIVARIATE procedure and Shapiro-Wilk goodness-of-fit test in SAS. Variables with Shapiro-Wilk (**W**) values ≥0.95 indicated normal distribution (Quiroz-Rocha et al., 2009). Only CK was not normally distributed: it was log-transformed before the analysis and results were then back-transformed. The trend of blood parameters over time (except for total and direct bilirubin) was analysed by the same model used for BW through the MIXED procedure of SAS (Eq. 1). Total and direct bilirubin were returned by the laboratory as binary variables and were considered physiological when <2.5 μmol/L and <1.5 μmol/L, respectively. Therefore, two categories for total and direct bilirubin were created and the GLIMMIX procedure of SAS was used with the same effects of Eq. 1, specifying that the distribution of the response variable was binary. Results for these variables were then provided as odds ratio. Statistical significance was declared at $P < 0.05$.

2.3.1. Blood biomarkers of transport stress

A further analysis was performed aiming at identifying the most relevant blood variables to describe the variation of the metabolic profile due to the long-distance transport. To this aim, a random forest (**RF**) model was built using 'randomForest' and 'caret' packages in the RStudio software (R version 2023.03.0; RStudio PBC, Boston, MA; Liaw and Wiener, 2002; Kuhn, 2014). As accurately described by Machado et al. (2015) and Hastie et al. (2009), RF is a machine learning method for classification and regression analysis based on an ensemble of randomized decision trees to define its output. During the process of model training, this method provides estimates of variable importance measured by the Gini index, which evaluates the average decrease in the nodes' impurity. The mean decrease Gini (**MDG**) defines the importance of a given variable representing the Gini index reduction for the variable summed over all nodes for each tree in the forest, normalized by the number of trees (Xi and Ishwaran, 2012). Thus, the higher the MDG, the more crucial the variable is for maintaining the predictive power of the RF model (Machado et al., 2015).

The original dataset was randomly and uniformly split into a training set (70 %) and an independent testing set (30 %), as it is a common strategy when external validation data is unavailable (Machado et al., 2015). The training set was used for cross-validation, and the testing set was used to evaluate the final model performance. The predictor variables consisted of all the blood traits collected on day −1 and day 0.

The RF model was trained using the training set (70 % of the original data) and the complete set of variables with the 'randomForest' package in RStudio. The number of trees was set to 500 (Machado et al., 2015), and the number of variables sampled for node splitting was optimized using the 'caret' package. To better estimate performance and generalization power, and to prevent overfitting, a repeated 10-fold cross-validation was adopted during model training.

To determine the most important variables that influenced the variation in the entire metabolic profile of each animal that underwent transport, the cross-validation was iterated 2000 times to ensure stable values of model performances. In each iteration of the cross-validation, the model's performance metrics were calculated, including accuracy, sensitivity, specificity, receiver operating characteristic (**ROC**) curve, area under the curve (**AUC**) through confusion matrices (Machado et al., 2013). For each blood trait, MDG values were calculated and averaged across all iterations to obtain an overall estimate of the model's performance. Finally, the MDG values for each blood trait were averaged over all iterations to determine the most important predictors.

3. Results

3.1. Body weight

Average bulls' BW decreased from 444 kg before leaving France (day −1; SEM = 1.86) to 417 kg upon arrival in Italy (day 0; SEM = 1.85; $P <$ 0.05; Fig. 1). The average shrinkage was 6.1 % of the mean bulls' BW measured at day −1 (Δ = –27 kg; CV = 38 %). Bulls' BW at day 7 was 432 kg (SEM = 1.89; $P < 0.05$; Fig. 1), with an average ADG of 2.33 kg/d during the first week in the Italian fattening unit (CV = 59 %). No

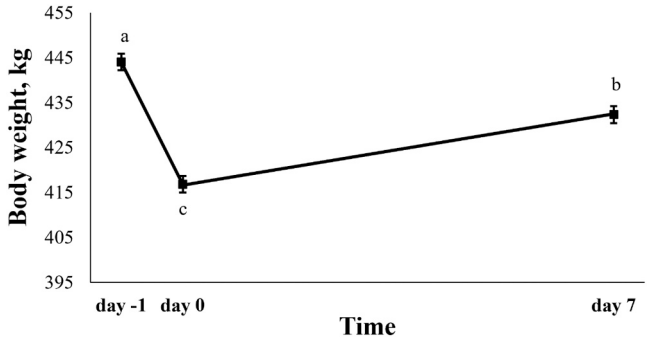

Fig. 1. Least squares means and standard errors of body weight over time (day −1 = 1 d before arrival to Italy; day 0 = the day of arrival to Italy; day 7 = after one week) for Charolais young bulls. Different letters identify significant differences ($P < 0.05$).

differences due to the yeast supplementation were detected over time in terms of BW variations (BW loss and ADG; $P > 0.05$).

3.2. Metabolic profile

Descriptive statistics of the plasma traits analysed in this study are presented in Table 1. Time was responsible for the great variation observed in plasma traits, except for P (Table 2). The supplementation did not affect any blood parameter during the period considered in this study, with the sole exception of Se (Table 2). The effect of time on plasma traits is detailed in Table 3 and described as follows. Albumin concentration peaked at day 0 and strongly decreased at day 7, whereas CREA showed a significant drop at day 0. Globulins had a linear increase from day −1 to day 7, and TP were higher at days 0 and 7 than at day −1. Urea showed higher levels on days −1 and 0 compared with day 7. Higher levels of CHOL, GLU, TG, and NEFA were observed on day −1, with a peak of GLU, TG, and NEFA at day 0. Plasma levels of the hepato-muscle profile enzymes were generally higher on day −1 and day 0 compared with day 7 (ALP, CK, GGT) or peaked at day 0 (ALT, AST, LDH). Total and direct bilirubin showed similar trends, with the highest probability of observing physiological values (i.e., <2.5 μmol/L and <1.5 μmol/L, respectively) at day 7. Calcium and Na plasma levels had similar trend over time, peaking at day 0 and having intermediate values at day 7. Plasma Cl concentration was higher on days −1 and 0 than on day 7. Iron showed a negative drop on day 0 and the highest value on day 7, whereas K peaked on day 7. Magnesium plasma concentration dropped on day 0 and was still low on day 7. Phosphorus was the only plasma trait that did not vary over time in this study ($P > 0.05$). Plasma Se concentration increased linearly with time (Table 3) and a significant Time × Supplementation interaction was observed (Fig. 2). Yeast and Control groups had similar Se plasma levels on day −1 (43.9 and 42.0 μg/L, respectively) and on day 0 (66.1 and 60.4 μg/L, respectively), but at day 7 Yeast group had 110.3 μg/L of Se and Control had 70.1 μg/L

Table 1
Descriptive statistics of plasma traits of Charolais young bulls.

Trait	Mean	CV[a], %	Minimum	Maximum	W[b]
Protein profile					
Albumin, g/L	33.3	8.0	27	40	0.99
Creatinine, μmol/L	151.3	16.2	99	221	0.99
Globulins, g/L	41.0	14.0	30	58	0.98
Total protein, g/L	74.3	6.4	61	88	0.99
Urea, mmol/L	3.5	29.3	1.3	6.9	0.98
Energy profile					
Cholesterol, mmol/L	2.31	24.8	1.00	3.82	0.97
Glucose, mmol/L	5.0	11.0	3.5	6.6	0.99
Triglycerides, mmol/L	0.21	22.9	0.10	0.33	0.99
Non-esterified fatty acids, mEq/L	0.81	38.9	0.10	1.75	0.98
Hepato-muscle profile					
Alkaline phosphatase, U/L	112.6	38.1	33	244	0.99
Alanine aminotransferase, U/L	25.3	18.3	12	39	0.95
Aspartate aminotransferase, U/L	99.0	24.6	55	234	0.95
Creatine kinase, U/L	387.9	92.9	95	2882	0.96[c]
Lactate dehydrogenase, U/L	1284	13.9	695	1788	0.96
Gamma-glutamyl transferase, U/L	14.7	29.7	4	28	0.98
Mineral profile					
Calcium, mmol/L	2.40	4.6	2.07	2.69	0.99
Chlorine, mmol/L	99.2	2.5	91	105	0.97
Iron, μg/dL	80.7	39.2	29	164	0.99
Potassium, mmol/L	4.79	9.4	3.71	5.90	0.98
Magnesium, mmol/L	0.84	10.1	0.61	1.37	0.97
Sodium, mmol/L	139.4	1.7	125	145	0.95
Phosphorous, mmol/L	2.32	10.9	1.69	2.97	0.99
Selenium, μg/L	65.0	41.9	18	148	0.99
Stress profile					
Cortisol, nmol/L	48.4	60.2	4	142	0.99

[a] CV = coefficient of variation.
[b] W = coefficient of the Shapiro-Wilk normality test.
[c] Value obtained after logarithmic transformation of the variable.

Table 2
Analysis of variance of plasma traits of Charolais young bulls: F- and *P*-values of the effects of Time (day −1 = 1 d before arrival to Italy; day 0 = the day of arrival to Italy; day 7 = after one week), Supplementation (received or not live yeast and selenium ruminal boluses before departure), and their interaction.

	Time		Supplementation		Time × Supplementation	
Trait	F	*P*	F	*P*	F	*P*
Protein profile						
Albumin	58.14	<0.001	0.25	0.618	0.11	0.893
Creatinine	13.22	<0.001	0.03	0.856	0.21	0.810
Globulins	36.97	<0.001	0.22	0.643	1.48	0.231
Total protein	40.55	<0.001	0.09	0.764	1.55	0.215
Urea	9.00	<0.001	2.11	0.148	1.17	0.312
Energy profile						
Cholesterol	26.17	<0.001	1.63	0.203	1.11	0.331
Glucose	30.09	<0.001	3.38	0.068	4.47	0.220
Triglycerides	54.91	<0.001	0.29	0.591	0.31	0.733
Non-esterified fatty acids	25.09	<0.001	0.04	0.841	1.23	0.296
Hepato-muscle profile						
Alkaline phosphatase	173.90	<0.001	0.77	0.381	0.40	0.669
Alanine aminotransferase	68.98	<0.001	0.01	0.928	0.08	0.924
Aspartate aminotransferase	59.90	<0.001	0.01	0.950	3.01	0.080
Creatine kinase	32.63	<0.001	3.16	0.078	0.05	0.955
Lactate dehydrogenase	154.35	<0.001	1.16	0.284	1.19	0.306
Gamma-glutamyl transferase	58.41	<0.001	0.14	0.710	0.62	0.539
Total bilirubin[a]	17.80	<0.001	0.05	0.826	1.08	0.343
Direct bilirubin[a]	12.24	<0.001	0.15	0.704	0.89	0.413
Mineral profile						
Calcium	6.91	0.001	0.01	0.926	0.98	0.376
Chlorine	82.30	<0.001	0.08	0.774	2.41	0.093
Iron	127.23	<0.001	0.95	0.330	0.12	0.890
Potassium	24.29	<0.001	0.07	0.788	0.62	0.537
Magnesium	6.98	0.001	0.03	0.861	0.04	0.961
Sodium	4.71	0.010	0.53	0.469	0.51	0.602
Phosphorous	0.27	0.763	0.01	0.952	0.86	0.425
Selenium	300.12	<0.001	21.42	<0.001	67.45	<0.001
Stress profile						
Cortisol	17.84	<0.001	1.99	0.161	0.63	0.536

[a] Binary variable, analysed using the GLIMMIX procedure of SAS software 9.4.

Table 3
Effect of time on the plasma traits of Charolais young bulls that underwent long-distance transportation.

Trait	Time[a]			SEM	P-*value*
	day −1	day 0	day 7		
Protein profile					
Albumin, g/L	33.6[b]	34.6[a]	31.7[c]	0.3	<0.001
Creatinine, μmol/L	151.3[b]	145.0[c]	157.4[a]	2.8	<0.001
Globulins, g/L	38.5[c]	41.4[b]	43.2[a]	0.6	<0.001
Total protein, g/L	72.0[b]	76.0[a]	74.9[a]	0.5	<0.001
Urea, mmol/L	3.6[a]	3.7[a]	3.2[b]	0.1	<0.001
Energy profile					
Cholesterol, mmol/L	2.42[a]	2.40[a]	2.11[b]	0.06	<0.001
Glucose, mmol/L	4.8[b]	5.3[a]	4.9[b]	0.1	<0.001
Triglycerides, mmol/L	0.22[b]	0.23[a]	0.18[c]	0.01	<0.001
Non-esterified fatty acids, mEq/L	0.71[b]	0.99[a]	0.72[b]	0.03	<0.001
Hepato-muscle profile					
Alkaline phosphatase, U/L	134.0[a]	130.5[a]	76.7[b]	3.8	<0.001
Alanine aminotransferase, U/L	25.7[b]	27.7[a]	22.6[c]	0.5	<0.001
Aspartate aminotransferase, U/L	102.3[b]	111.2[a]	83.4[c]	2.5	<0.001
Creatine kinase, U/L	345.6[a]	393.6[a]	216.8[b]	1.1	<0.001
Lactate dehydrogenase, U/L	1321[b]	1393[a]	1141[c]	16.4	<0.001
Gamma-glutamyl transferase, U/L	17.0[a]	15.2[b]	12.2[c]	0.4	<0.001
Total bilirubin (OR: <2.5 μmol/L)[b]	0.37[b]	0.29[b]	0.80[a]	0.06	<0.001
Direct bilirubin (OR: <1.5 μmol/L)[b]	0.56[b]	0.72[b]	0.98[a]	0.42	<0.001
Mineral profile					
Calcium, mmol/L	2.37[b]	2.42[a]	2.40[ab]	0.01	0.001
Chlorine, mmol/L	100.3[a]	100.2[a]	97.1[b]	0.2	<0.001
Iron, μg/dL	73.3[b]	59.4[c]	109.7[a]	2.5	<0.001
Potassium, mmol/L	4.64[b]	4.68[b]	5.04[a]	0.04	<0.001
Magnesium, mmol/L	0.86[a]	0.83[b]	0.83[b]	0.01	0.001
Sodium, mmol/L	138.8[b]	139.9[a]	139.6[ab]	0.3	0.010
Phosphorous, mmol/L	2.33	2.31	2.33	0.03	0.763
Selenium, μg/L	42.9[c]	63.2[b]	90.2[a]	2.0	<0.001
Stress profile					
Cortisol, nmol/L	36.6[c]	60.4[a]	48.9[b]	3.1	<0.001

[a,b,c]Means with different superscripts within the same blood parameter and effect differ significantly ($P < 0.05$).

[a] day −1 = 1 d before arrival to Italy; day 0 = the day of arrival to Italy; day 7 = after one week of arrival to Italy.

[b] Total and direct bilirubin were binary variable analysed using the GLIMMIX procedure of SAS software 9.4. Thus, results are reported as odds ratio (OR) that indicated the probability that total and direct bilirubin were within physiological limits (<2.5 and <1.5 μmol/L, respectively) at each time of sampling.

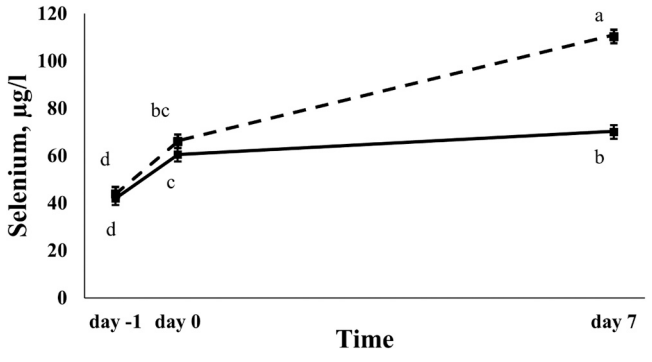

Fig. 2. Least squares means and standard errors of selenium concentration in plasma for the interaction effect between supplementation group (Yeast = dashed line; Control = solid line) and time (day −1 = 1 d before arrival to Italy; day 0 = the day of arrival to Italy; day 7 = after one week) for Charolais young bulls. Different letters identify significant differences for the interaction ($P < 0.05$).

(SEM = 2.86; P < 0.001; Fig. 2). Cortisol plasma concentration considerably increased at day 0, and lowered again at day 7.

3.2.1. *Blood biomarkers of transport stress*

The accuracy, specificity, and sensibility of the RF model over all iterations of 10-fold cross-validation averaged 0.78, 0.76, and 0.80, respectively. The average AUC of the ROC curves over all repetitions of cross-validation was 0.87 (Supplementary Figure S1). The MDG revealed that the most influential blood parameters for transport variance were (MDG = 6.92), TP (MDG = 4.02), cortisol (MDG = 3.83), Se (MDG = 3.69), GLU (MDG = 3.66) and Fe (MDG = 3.26), followed by GLOB (MDG = 2.61), K (MDG = 2.52), and LDH (MDG = 2.23) (Fig. 3). Stability of MDG values across iterations can be found in Supplementary Figure S2.

4. Discussion

Commingling and transport can negatively affect the health and welfare of young stock (Van Engen and Coetzee, 2018). This study aimed to deepen the knowledge on the effect of long-distance transport on blood metabolic profile of Charolais young bulls. The BW variation was also analysed. The supply chain of young beef cattle described in the present study is common for Southern Europe, and travel conditions are similar for several EU and non-EU Member States. Much different travel conditions (e.g., absence of adequate space allowance, ventilation system or water supply) could lead to different results on blood parameters.

4.1. *Body weight*

The BW shrinkage is one of the most common effects of long-distance transport in cattle due to factors like duration of the journey, driving quality, animal handling procedures, feed and water restrictions, and climate conditions (González et al., 2012; Van Engen and Coetzee, 2018). In the current study, BW loss was observed upon arrival in Italy (day 0), but the average shrinkage was quite limited compared to the values from 5 % to 12 % reported in the literature (Coffey et al., 2001; Chirase et al., 2004; Werner et al., 2013). Bulls started to recover their BW loss after the arrival at the Italian fattening unit, but the BW measured at the French collection centres (day −1) was not fully recovered after the first week of the receiving period (day 7; Fig. 1). As expected, the yeast supplementation did not affect bulls' BW loss and ADG over time. Other authors suggested several interventions addressed to limit the shrinkage in cattle stressed by the long journey, such as the adoption of dedicated feeding plans and the provision of drinking solutions enriched with electrolytes and energy before and during transport, as well as at the farm of destination (Coffey et al., 2001; Fike and Spire, 2006).

4.2. *Metabolic profile*

It is not easy to retrieve specific reference intervals referred to beef cattle for the numerous blood traits analysed in this study. Confidence intervals for some blood parameters were produced by Cônsolo et al. (2018) on young beef crossbred heifers (~290 d of age) and by Contiero et al. (2018) on clinically healthy Holstein Friesian bulls hosted in an artificial insemination station, whereas Mottaran et al. (2015) specifically studied the blood mineral profile of transported Charolais and Limousin young bulls. The mean values of most of the plasma traits analysed in this study fell within the reference intervals reported in the literature (Table 1). Only GLU, NEFA, AST, and CK were above the upper limits (Cônsolo et al., 2018; Contiero et al., 2018). Transportation affected young bulls' metabolic profile, as all the plasma traits significantly varied according to time, with the sole exception of P (Table 2). As expected, Se plasma level was affected by Yeast supplementation, but no other blood parameter showed variations according to treatment group during the study period (Table 2).

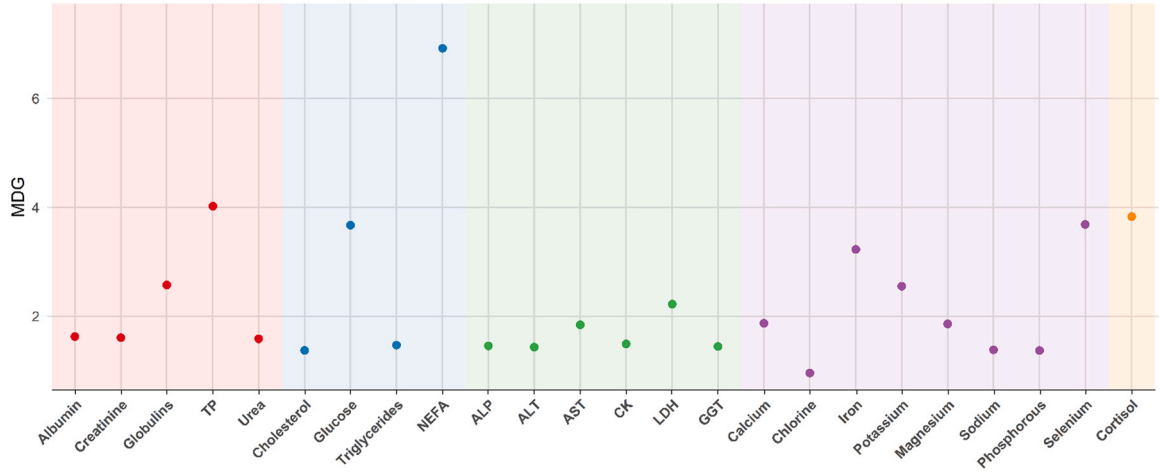

Fig. 3. Mean decrease Gini (MDG) describing variable importance as estimated by the random forest model run using blood variables of metabolic profile of Charolaise young bulls that underwent long-distance transport, calculated on each 10-fold cross-validation, iterated 2000 times. Protein profile (red background): albumin (g/L), creatinine (μmol/L), globulins (g/L), total protein (TP; g/L), urea (mmol/L). Energy profile (blue background): cholesterol (mmol/L), glucose (mmol/L), triglycerides (mmol/L), non-esterified fatty acids (NEFA; mEq/L). Hepato-muscle profile (green background): alkaline phosphatase (ALP; U/L), alanine aminotransferase (ALT; U/L), aspartate aminotransferase (AST; U/L), creatine kinase (CK; U/L), lactate dehydrogenase (LDH; U/L), gamma-glutamyl transferase (GGT; U/L). Mineral profile (violet background): calcium (mmol/L), chlorine (mmol/L), iron (μg/dL), potassium (mmol/L), magnesium (mmol/L), sodium (mmol/L), phosphorous (mmol/L), selenium (μg/L). Stress profile (orange background): cortisol (nmol/L).

4.2.1. *Protein profile*

Total protein plasma concentration results from the combination of ALB and GLOB fractions, whose ratio varies to keep the blood osmotic pressure constant (Piccione et al., 2011). In this study, the peak of both TP and ALB observed at day 0, along with the increase of GLOB, could indicate a degree of hemoconcentration due to dehydration (Parker et al., 2003), despite none of the three traits were above the physiological limits reported in other studies (Contiero et al., 2018) and hematocrit was not assessed. The highest value of GLOB observed on day 7, counterbalanced by the strong ALB decrease just below the inferior physiological limit reported by Contiero et al. (2018), could indicate the presence of an inflammatory status caused by both transport and adaptation to the new housing and feeding conditions. Indeed, ALB is the major negative acute phase protein (**APP**) in cattle and GLOB is composed of both immunoglobulins and positive APP (Tothova et al., 2014). Other studies reported that cattle take up to 5 days to overcome the stress due to long-distance transport (Swanson and Morrow-Tesch, 2001; Ashenafi et al., 2018).

The higher levels of UREA observed on days −1 and 0 compared with day 7 could be due to an increase in tissue protein catabolism as well as a reduction in tissue protein synthesis (Cole et al., 2016) that could arise from the lower feeding of bulls during the commingling and transport phases. The negative peak of CREA concentration observed at day 0 is consistent with this hypothesis, as CREA is a by-product of the breakdown of creatine and phosphocreatine in muscles and its reduction in plasma indicates a prolonged active tissue protein catabolism (Ndlovu et al., 2007), which reflects in a drop in muscular mass.

4.2.2. *Energy profile and cortisol*

The trends observed for CHOL, GLU, TG, and NEFA, most of them showing a significant increase on day 0, were signs of an important energy mobilisation by the body reserves of the bulls that identified a situation of energy demand starting from day −1 and peaking at day 0, with mitigation at day 7.

Cortisol is a biomarker of stress in transported animals (Ashenafi et al., 2018; Van Engen and Coetzee, 2018) and the significant increase in its concentration that was observed on day 0 fully agreed with the literature. Both the limited feed intake and the stress due to commingling and transport activate the hypothalamus pituitary adrenal axis releasing cortisol, which is involved in nutrient mobilisation during stressful and fasting conditions (Van Engen and Coetzee, 2018). Thus, GLU is mobilised from glycogen reserves in muscles and the liver, and gluconeogenesis occurs (Ashenafi et al., 2018). Cortisol also increases lipid mobilisation (Van Engen and Coetzee, 2018), and blood TG increases along with CHOL, which plays a major role in lipid transport and lipogenesis (Bourgon et al., 2017).

Consistent with our findings, an increase in NEFA linked to the transport of beef cattle has been reported also in previous studies as indicator of body fat mobilisation due to energy demand (Earley et al., 2012; Melendez et al., 2021). Mirroring the recorded pattern for BW shrinkage, in the present study the energy demand of the bulls was high across the whole observation period, as both GLU and NEFA remained above the upper physiological limits reported in the literature (Cònsolo et al., 2018; Contiero et al., 2018).

4.2.3. *Hepato-muscle profile*

The AST and ALT enzymes rapidly increase in blood when muscular or hepatic damages occur (Zaitsev et al., 2020). The same causes along with stress have been associated with the increase in LDH plasma concentration (Klein et al., 2020). The CK is a recognized marker of stress and muscular damage in transported cattle (EFSA, 2022), and its concentration in blood increases also after high muscular activity and fatigue (Otter, 2013). Other studies reported marked variations of biomarkers of muscular damage linked to beef cattle transport (Averós et al., 2008; Earley et al., 2012; Werner et al., 2013). The values of AST, ALT, LDH, and CK recorded in this study on day −1 identified signs of muscular stress and damage already before the transport of the bulls, likely due to the commingling at the collection centre. Mixing unfamiliar animals is a known stressor associated with increased agonistic interactions during the establishment of a new social hierarchy (Mench et al., 1990). Therefore, preventive actions to limit stress should start before transport, especially if cattle are commingled in collection centres.

Even the trends of ALP, GGT, and bilirubin denoted a degree of hepatic stress or damage that started before transport and peaked at day 0, with a recovery at day 7. Biomarkers of liver function in transported beef cattle have been scarcely investigated and results of this study showed that the liver can undergo subacute disorders under fasting and stressful conditions. Indeed, ALP and GGT usually increase in blood when cholestasis or subacute or chronic liver disease occur, whereas higher

bilirubin plasma concentration can be due to both increased indirect bilirubin in case of higher release of hemoglobin or myoglobin by the cells, or increased direct bilirubin in case of hepatic stress or damage (Otter, 2013). The variations in protein and energy profiles discussed above fully support the hypothesis of a framework of muscle damage and liver fatigue in transported bulls.

4.2.4. Mineral profile

Variations of P plasma concentration over time were not significant in this study, but all other minerals showed variations linked to transport. The trend recorded for Ca, with a peak on day 0, was in line with the results by Ashenafi et al. (2018) who observed higher Ca blood levels in transported animals due to the increased muscle activity (and damage) that led to higher Ca release in the extracellular fluids.

Magnesium is mainly involved in enzymatic reactions but also in muscle contraction and nerve impulse transmission (Suttle, 2010). In this study, Mg dropped on day 0 and did not recover by day 7, but never went out of its physiological range (Cônsolo et al., 2018). Fasting can cause a quick fall in Mg concentration (Suttle, 2010) and a decrease in Mg level has been documented in transported cattle (Ashenafi et al., 2018).

Both Na and Cl are strictly related to the hydration status of the animal, thus it is plausible that their trends with higher values on day 0 were due to a degree of dehydration, according to the patterns of ALB and TP that also peaked on the same sampling day (Swanson and Morrow-Tesch, 2001; Suttle, 2010).

Iron and K were the only metabolites that showed values slightly outside the available reference limits for beef cattle (Mottaran et al., 2015). Iron was below the limits at day 0 and K showed generally high values, with a peak at day 7. Mottaran et al. (2015) recorded 76 % of Charolais bulls imported from France with low Fe levels after transport. Iron is mainly linked to proteins in the body, most to hemoglobin and then to myoglobin and transport and reserve proteins (e.g., transferrin and ferritin; Herdt and Hoff, 2011). Despite dietary poor supply can be a cause of low Fe blood levels, inflammation should also be considered, as some studies reported that an inflammatory status can cause a rapid drop in blood Fe, considering also that transferrin acts as a negative APP (Herdt and Hoff, 2011). A suspect of inflammatory status has arisen also from the analysis of the plasma protein and hepato-muscle profiles previously discussed.

Because K can be rapidly moved between intracellular and extracellular compartments, variations in its plasma concentration can be difficult to interpret. Higher plasma K levels can be due to transient hyperkaliemia after meals (dietary origin), intense muscle exercise, and the attempt of the body to compensate for metabolic acidosis (Goff, 2006). Bulls of this study might have experienced all these situations.

Selenium in cattle is mostly present as glutathione peroxidase (**GPX**) within the erythrocytes with a primary antioxidant function and its concentration depends mainly on dietary supply (Herdt et al., 2000). Mottaran et al. (2015) reported that only 30 % of bulls transported from France to Italy have an adequate concentration of GPX. In the present study, the mean value of Se on day −1 (before the bolus administration) was slightly below the reference limits of 51–85 μg/L reported by Mehdi and Dufrasne (2016), confirming a deficiency status in bulls before transport. Despite the discussion of supplementation effect is not within the aim of this study, the trend observed for plasma Se suggested the effectiveness of the slow-release of this micromineral by the boluses, as plasma Se concentration increased with time and a significant Time × Supplementation interaction was observed (Fig. 2).

4.2.5. Blood biomarkers of transport stress

The main strength of this study was the possibility of analyzing a comprehensive panel of blood traits in transported beef bulls. The RF approach and MDG calculation enabled the identification of key biomarkers that influenced the variations in the metabolic profile of young bulls during transport. These biomarkers can be considered for promoting routine blood analyses to tailor targeted interventions. The RF model showed good performance metrics and identified NEFA as the main blood biomarker. This confirmed the evidence that bulls underwent energy deficit in the period comprising commingling and transport. Indeed, GLU had also a high MDG, ranking within the five major traits identified, and in turn increased after transport, denoting important energy mobilization by the body reserves, as previously discussed. The high MDG of cortisol and TP confirmed the activation of stress response mechanism and indicated a potential dehydration or inflammatory response in bulls. Iron was identified as another important indicator, supported by evidence of Fe deficiency in imported beef cattle from France (Mottaran et al., 2015). This evidence suggests that the ranking of mineral biomarkers may change according to cattle origin. The importance of Se was somewhat expected, given its supplementation in the study, and should be carefully considered as well.

In a restricted panel of analytes for assessing transport stress in beef cattle, the outcomes of RF would suggest to include also GLOB, K and LDH, which mirror and support the biological meanings of major biomarkers already discussed. Despite CK is commonly considered as one of the most important indicators of stress in transported beef cattle (Ashenafi et al., 2018; EFSA, 2022), it did not result as a major biomarker by the RF analysis. This might be explained by the evidence that signs of metabolic impairment were observed already in *pre*-transport blood samples, when CK levels were already high likely due to negative interactions among unfamiliar batch-mates and handling procedures during commingling.

4.2.6. Welfare implications

It is worth mentioning that the transport of the study bulls complied with the EU Regulation (2005/1/EC), and none of the bulls died during and after the transport. Variations in blood traits according to time were significant, thus indicating a metabolic response by the animals to new stressors (commingling, transport, and new fattening unit). The footprint of the long-distance transport on bulls' metabolic profile denoted a combination of energy deficit and muscular damage, with a certain degree of dehydration and liver impairment, that peaked consistently with the bulls' BW shrinkage. However, blood parameters rarely fell outside the physiological limits reported in the literature (Mottaran et al., 2015; Cônsolo et al., 2018; Contiero et al., 2018), meaning that severe alterations were limited to some of them. Therefore, it can be hypothesized that good management practices tailored by accurate definition of bulls' metabolic alterations could further help reduce animal stress.

5. Conclusions

The present study reported that plasma traits of the metabolic profile are sensitive indicators of the stress experienced by young bulls during long-distance transport, suggesting their potential as reliable biomarkers for stress assessment. The observed post-transport shrinkage in bulls' BW aligns with changes in the metabolic profile, reflecting a combination of stress, energy deficit, and muscular damage that might begin even before transport commences. Despite some degree of transport-related stress is unavoidable for cattle, proactive monitoring of metabolic profiles could aid in early detection of significant alterations, facilitating targeted interventions to mitigate stress and minimise BW loss. Key blood biomarkers identified in this study offer practical insights for promoting routine monitoring practices.

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CRediT authorship contribution statement

Isabella Lora: Writing – original draft, Validation, Methodology,

Conceptualization. **Matteo Santinello:** Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization. **Massimo De Marchi:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Federico Righi:** Methodology, Conceptualization. **Alberto Guerra:** Methodology, Conceptualization. **Bruno Martin:** Writing – review & editing, Validation, Methodology. **Eric Chevaux:** Writing – review & editing, Validation, Project administration, Methodology. **Mauro Penasa:** Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Giulio Cozzi:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Clothilde Villot:** Writing – review & editing, Validation, Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Matteo Santinello, Isabella Lora, Giulio Cozzi, Mauro Penasa, Alberto Guerra, Federico Righi, Massimo De Marchi have no conflicts of interest. Clothilde Villot, Eric Chevaux, and Bruno Martin are employed by Lallemand SAS

Data Availability

None of the data were deposited in an official repository. However, data and models are available upon reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2024.106296.

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