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ORIGINAL ARTICLE

# Metabolomic profiling of elite female soccer players: urinary biomarkers over a championship season

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## Abstract

**Introduction** In soccer, most studies evaluate metabolic profile changes in male athletes, often using data from a single match. Given the current landscape of women's soccer and the effects of biological sex on the physiological response and adaptation to exercise, more studies targeting female athletes and analyzing pre- and post-game moments throughout the season are necessary.

**Objectives** To describe the metabolomics profile of female soccer athletes from an elite team in Brazil. The study observed the separation of groups in three pre- and post-game moments and identified the discriminating metabolites.

**Methods** The study included 14 female soccer athletes. Urine samples were collected and analyzed using Nuclear Magnetic Resonance in pre-game and immediate post-game moments over three national championship games. The metabolomics data were then used to generate OPLS-DA and VIP plots.

**Results** Forty-three metabolites were identified in the samples. OPLS-DA analyses demonstrated a progressive separation between pre-post conditions, as supported by an increasing $Q^2$ value (0.534, 0.625, and 0.899 for games 1, 2 and 3, respectively) and the first component value (20.2% and 19.1% in games 1 and 2 vs. 29.9% in game 3). Eight out of the fifteen most discriminating metabolites appeared consistently across the three games: glycine, formate, citrate, 3-hydroxyvalerate, glycolic acid, trimethylamine, urea, and dimethylglycine.

**Conclusion** The main difference between the three games was the increasing separation between groups throughout the championship. Since the higher VIP-scores metabolites are linked to energy and protein metabolism, this separation may be attributed several factors, one being the accumulation of fatigue.

**Keywords** Soccer · Metabolomics · Nuclear magnetic resonance · Elite athletes

## 1 Introduction

Metabolomics is characterized as a science focused on the study small molecules from living organisms, called metabolites (Bongiovanni et al., 2022; Markley et al., 2017). This science is applied in various fields of study such as exercise physiology and metabolism, known as 'sportomics', and has been adopted in the study of sports such as cycling, soccer, basketball, and rugby (Bragazzi et al., 2020; Gowda & Raftery, 2021; Bongiovanni et al., 2022).

The application of metabolomics in soccer is quite recent. Its intermittent nature, with long duration and high intensity, causes changes in energy metabolism and the metabolic profile of athletes (Cao et al., 2020).

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Additionally, changes have been identified in the metabolites of purine pathways, glycolysis, Krebs cycle, amino acids, fatty acids, and lactate (Zhao et al., 2020). These alterations can also vary according to the type of sample used (blood, urine, saliva), the lifestyle of the athletes, sex, age, and duration of exercise (Alzharani et al., 2020). Thus, some studies have evaluated pre- and post-game moments, while others have also assessed soccer players in training with cycle ergometers or high-intensity interval training (HIIT), using data analysis techniques such as PCA, OPLS-DA, and pathway enrichment analysis to identify which metabolites or pathways are highlighted/most utilized at a given moment in the game, as well as the temporal effect of sampling on the athletes' metabolome behavior (Pitti et al., 2019; Cao et al., 2020; Zhao et al., 2020).

However, most of these studies still used samples from male athletes. Given the current scenario of women's soccer and considering the physiological differences between male and female athletes and how they influence response and adaptation to exercise (Ansdell et al., 2020), there is a need for more studies focused on the female audience, as studies involving metabolomic analysis and women's soccer are still scarce (Rodas et al., 2022).

A single study that evaluated both male and female soccer players through mass spectrometry using urine samples associated with external load revealed significant metabolic differences between biological sexes. For female athletes, there was a focus on the metabolism of tryptophan, β-alanine, GABA, sarcosine, and hypoxanthine, with β-alanine being associated with higher player loads for both sexes. The other mentioned metabolites are involved in mechanisms of skeletal muscle maintenance and exercise-induced muscle adaptation (Rodas et al., 2022). However, this study monitored the athletes during pre-season and in-season periods, aiming to characterize the fluctuations in metabolites detected by Metabolomics. Therefore, we highlight the need for a metabolomic description also in post-game or post-season moments, a goal not yet applied to women's soccer, considering the modifications that can be observed due to the metabolic stress caused by a soccer match.

Based on the above, especially considering the growing popularity of women' soccer and the limited number of studies evaluating female soccer players, the present cross-sectional study aimed to describe the metabolomic profile of female soccer players competing in the national championship, observing the group separation in pre- and post-game moments in OPLS-DA analyses over 3 consecutive championship games, and describing the discriminant metabolites from urine samples.

## 2 Methods

### 2.1 Population/sampling

The sample was selected non-probabilistically by convenience and comprised 14 elite female athletes (aged 19–32 years) from a top-tier Brazilian soccer team. The athletes' anthropometric characteristics are detailed in Table 1. They were monitored over three games of the National Championship held between June and July 2022 (Fig. 1). The athletes' training routine was daily, with sessions lasting between 60 and 145 min.

### 2.2 Data Collection

Initially, a visit to the data collection laboratory was conducted to familiarize with the athletes and their routines. Subsequently, a second visit was made to collect anthropometric data. Once the season began, urine samples were collected at their training field according to the championship game dates (Fig. 1).

For context, the three matches were played against different opponents, always with the home team advantage for the analyzed team and held at the same time (3 pm GMT-3). The first (0–1, goal in the first half) and second (0–2, goals in the second half) games were losses, while the third was a 1–0 victory (goal in the second half). Also, exercise intensity during the games and training sessions between games were controlled by session time, the session rating of perceived exertion (sRPE) and sRPE training load—the product

**Table 1** Anthropometric characterization data of the sample

| | Age | Body mass(kg) | Height(cm) | BMI(kg/m$^2$) | Body density(g/cm$^3$) | % Body fat |
|---|---|---|---|---|---|---|
| Mean | 22.6 | 56.0 | 162 | 21.4 | 1.06 | 18.8 |
| Standard deviation | 3.46 | 5.99 | 7.64 | 1.54 | 0.04 | 4.70 |
| IC95% | 21.09 to 24.71 | 52.9 to 59.1 | 158 to 166 | 20.59 to 22.21 | 1.039 to 1.081 | 16.34 to 21.26 |
| Minimum | 19.0 | 45.8 | 148 | 19.3 | 1.00 | 12.7 |
| Maximum | 32.0 | 69.0 | 175 | 23.8 | 1.10 | 26.5 |

Values are expressed as mean ± standard deviation.

*BMI* Body mass index

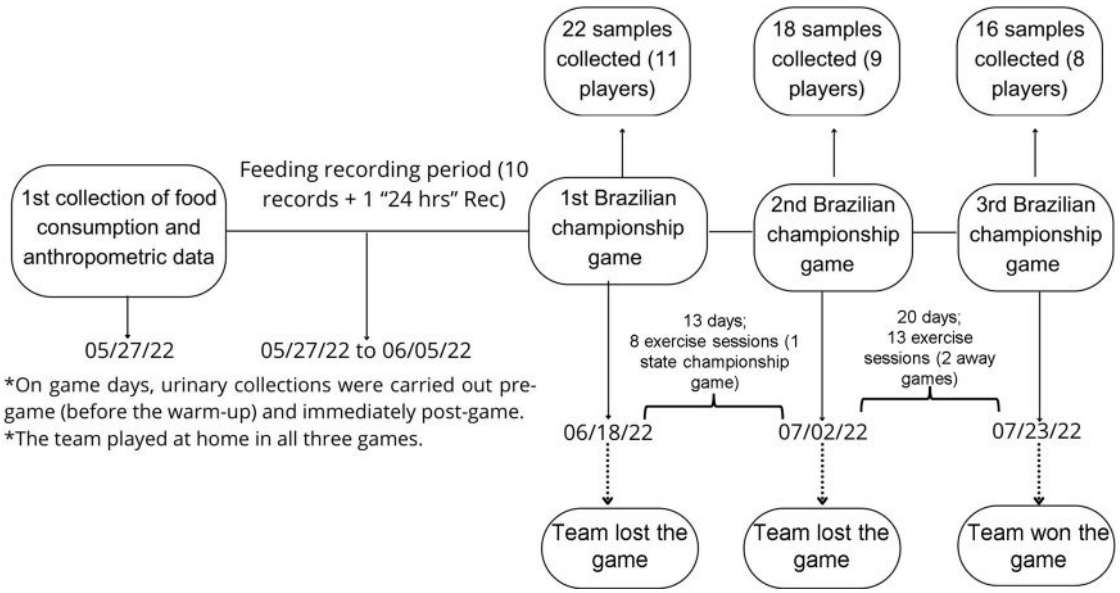


**Fig. 1** Experimental Design of the study

between exercise time and RPE (sRPE-TL) (McLaren et al., 2017). There were eight exercise sessions between games 1 and 2, with one of them being a state championship match, not included in the analysis. Between games 2 and 3 there were thirteen exercise sessions, being two of them away games where we didn't have access to the players for urine collection.

## 2.3 Urine Collection

Using 25 ml collection containers labeled with the athletes' names, date, and collection time (pre-or post-game), samples were collected on game days, before warm-up (to avoid physical activity interference with the metabolome) and immediately after the matches in the locker room as athletes returned from the field. The athletes were instructed to discard the initial urine stream and then use the collection container. The collected samples were transported in a thermal container with ice to a laboratory refrigerator for storage and subsequent Nuclear Magnetic Resonance (NMR) analysis. All athletes involved in the game provided urine samples, but only those who played as starters and substitutes (n = 14) who played at least 20 min after substitution in at least one game were included in the study. The athletes were free to refuse to collect urine samples for any reason (e.g. inability to urinate 30 min after the game, unwillingness to participate in the study, injury in subsequent matches or not listed by coach). So, out of the 14 athletes involved in this analysis, 11 were included in the first game, 9 in the second game, and 8 in the third game. Among the 14 athletes, seven played in all three games, and the rest played in one or two games.

## 2.4 Anthropometry

Weight and height were measured using an anthropometric scale with a stadiometer (Adult Mechanical Scale 180 kg, Welmy®, Santa Bárbara do Oeste, Brazil). Body fat percentage and skinfold thickness were measured using a skinfold caliper (Lange Skinfold Caliper, Cambridge Scientific Industries®, Cambridge, USA), following the three-skinfold protocol (suprailiac, thigh, and triceps) by Jackson, Pollock, and Ward (1980) to determine the athletes' body fat percentage using the following formulas: Body Density = 1.0994921—(0.0009929 × sum of skinfolds) + (0.0000023 × squared sum of skinfolds)—(0.0001392 × age) and Body Fat Percentage (%) = (495 / Body Density)—450. The athletes did not train or warm up before anthropometric data collection.

## 2.5 Data processing and analysis

### 2.5.1 Metabolomic analysis

Samples were analyzed using a global/unbiased metabolomic approach. Analysis was performed using a BRUKER 600 MHz, AVANCER III nuclear magnetic resonance (NMR) spectrometer (Bruker®, Karlsruhe, Germany) with a 5 mm probe at 300 K (PABBO) using the pulse sequence: noesygppr1d. Samples were prepared by taking 1.5 ml and transferring them to Eppendorf tubes for centrifugation at 14,000 rpm for 15 min (MIKRO 220R) and stored individually at -80 °C. For analysis, 300 µL of the supernatant from each sample was transferred to a 5 mm NMR tube with 300 µL of 1 mM phosphate buffer solution ($D_2O$, pH = 7.4, TSP = 1 mM) for 20 min in the BRUKER AVANCER

spectrometer, operating at 600 MHz for hydrogen analysis with a broadband 5 mm PABBO probe at 300 K. Water signal suppression was achieved with presaturation using the following parameters: NS: 128 (number of scans); D1: 4.00 s (delay time); TD: 64 K (spectrum points); SW: 20 ppm (spectral width); O1P: 4.69 ppm (water signal position); AQ: 5.11 s (acquisition time).

Spectra for each sample were obtained using TopSpin® 3.6.5 software (Bruker®, Karlsruhe, Germany) and identified with Chenomx profiler® 9.05 software (Chenomix®, Edmonton, Canada), confirmed against the Human Metabolome Database (HMDB) (www.hmdb.ca). Pre-processing, involving overlapping, alignment, and quantification of spectra, was done using R software (version 4.2.2) (Lucent Technologies®, Georgia, USA) with the PepsNMR package version 3.17. Data were then exported to an.xls table format, with samples identified in rows and the 43 metabolites found in columns.

### 2.5.2 Statistical analysis

Multivariate metabolomic analysis was conducted using the online platform Metaboanalyst 5.0 (University of Alberta, Alberta, Canada) for Orthogonal Projections to Latent Structures (OPLS) analysis and graph generation, using normalization by sum, logarithmic transformation, and Pareto scaling. $Q^2$ and $R^2$ values > 0.5 were considered to validate the model by permutation (n = 1000). The sample size (n) in the OPLS plots varied because some athletes were injured, unable to urinate, or not selected for certain matches, and in these cases, their urine samples were not included in the analysis. PCA analyses were performed but resulted in the non-formation of distinct groups. So, to offer additional insights into the overall data structure and group formation, these can be found in the supplementary material (Figures S1 and S2).

## 3 Results

To characterize the sample (n = 14), data on age, body mass, height, and BMI were collected and are presented in Table 1. After NMR analysis of the urine samples, 43 metabolites were identified. These identified metabolites are listed in Table 2 along with their respective minimum and maximum ppm signals.

In the first game, the groups differentiated but still tangentially touched. As the games progressed, the pre- and post-game groups became increasingly separate and distinct. Based on the OPLS-DA model VIP metrics plots were also generated, showing the top 15 most relevant metabolites influenced by exercise as depicted by OPLS-DA (an arbitrary cutoff value of VIP > 1 was considered) according to pre- and post-game moments for each match. The choice of showing the 15 most relevant metabolites had the intent to find and describe changes in metabolites

**Table 2** Identified metabolites and their minimum and maximum ppm signals

| Metabolites | Chemical Shift (δ) | Multiplicity |
|---|---|---|
| 2-Hydroxyisovalerate | 0.864 | Triplet |
| Leucine | 0.92 | Triplet |
| Isobutyrate | 1.064 | Doublet |
| 3-Aminoisobutyrate | 1.18 | Doublet |
| 3-Hydroxyisovalerate | 1.262 | Sigleto |
| Lactate | 1.305 | Doublet |
| Mevalonic Acid | 1.121 | Singlet |
| Alanine | 1.47 | Doublet |
| Pyruvate | 2.332 | Singlet |
| Succinate | 2.4 | Singlet |
| Citrate | 2.501 | Doublet of doublets |
| Methylamine | 2.589 | Singlet |
| Dimethylamine | 2.713 | Singlet |
| Methyllguanidine | 2.822 | Singlet |
| Trimethylamine | 2.9 | Singlet |
| Dimethylglycine | 2.921 | Doublet |
| Creatinine | 3.023 | Singlet |
| Malonic acid | 3.108 | Singlet |
| Dimethylsulfone | 3.134 | Singlet |
| TMAO | 3.264 | Singlet |
| Methyluric acid | 3.281 | Singlet |
| Methanol | 3.335 | Singlet |
| Alpha-Hydroxy-Isobutyrate | 3.351 | Singlet |
| Taurine | 3.405 | Triplet |
| Glycine | 3.559 | Singlet |
| Pi-methylhistidine | 3.671 | Singlet |
| Guanidinoacetate | 3.79 | Singlet |
| Phosphocreatine | 3.928 | Singlet |
| Glycolic acid | 3.943 | Singlet |
| Creatinine | 4.031 | Singlet |
| tartrate | 4.351 | Singlet |
| Trigonelline | 4.433 | Singlet |
| Glucose | 5.224 | Doublet |
| Urea | 5.712 | Singlet |
| Trans-aconitate | 6.587 | Singlet |
| Hydroxyphenylacetic | 6.849 | Doublet |
| Tyrosine | 6.889 | Doublet |
| Methylhistidine | 7.005 | Singlet |
| Phenylacetic acid | 7.342 | Triplet |
| Uracil | 7.493 | Doublet |
| Hippurate | 7.529 | Triplet |
| Formate | 8.452 | Singlet |
| 1-Methylnicotinamide | 9.282 | Singlet |

that were present and were more relevant for the pre-post comparison in all three games, but the model was constructed considering all metabolites for all cases. The side columns indicate the game moment (pre- or post-game) and the colors red or blue represent higher or lower significance of the metabolites at a given moment, as seen in Figs. 2, 3, and 4. It is worth noting that the team lost the first two games of the national championship (scores of $0\times1$ and $0\times2$) and won the third (score of $1\times0$). The $R^2$ and $Q^2$ values for games 1, 2, and 3 considering all

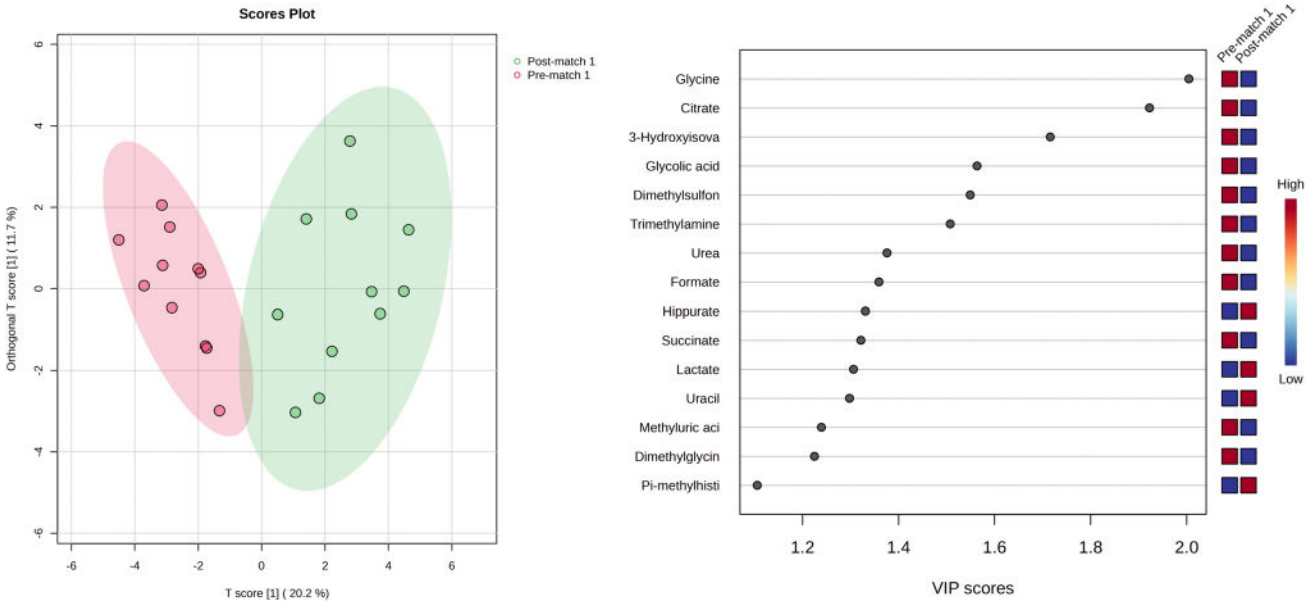


**Fig. 2** OPLS-DA and VIP-plot graphics from pre and post-match 1. The pink and green colors represent the pre- and post-match 1 moments, respectively, and each small dot represents an athlete. Next to the VIP are the 15 most relevant metabolites (VIP > 1) based on the OPLS-DA model and their representativeness at each moment of play. N = 11; $R^2 = 0.844$; $Q^2 = 0.534$. See Figure S3 for the model's validation process

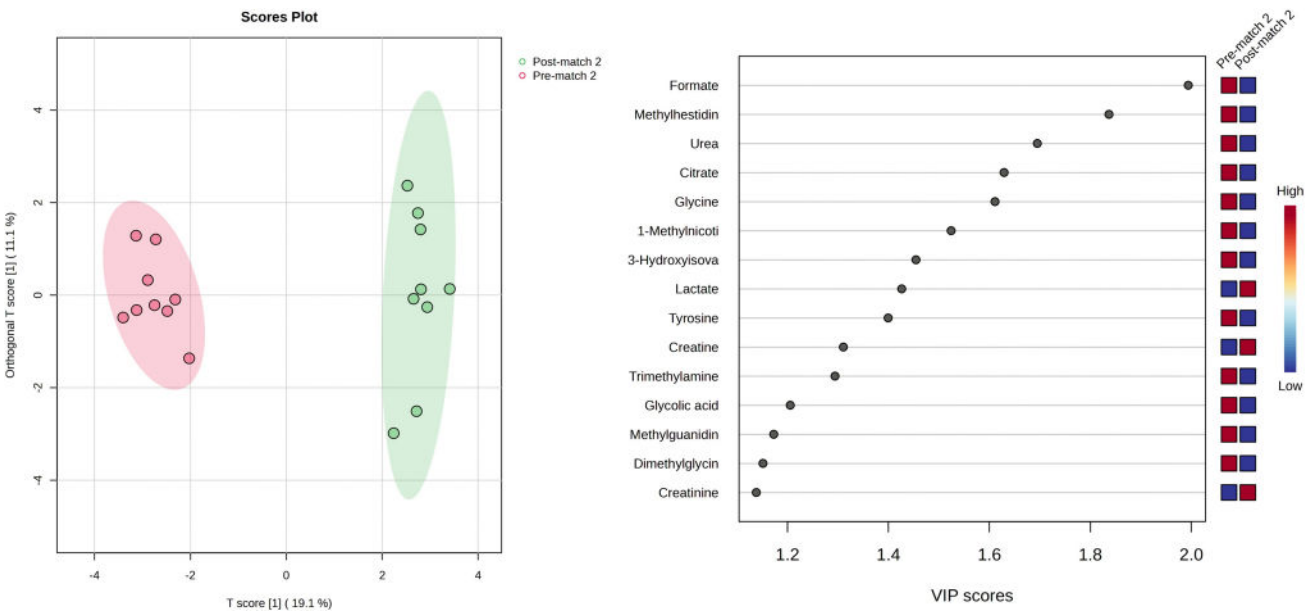


**Fig. 3** OPLS-DA and VIP-plot graphics from pre and post-match 2. The pink and green colors represent the pre- and post-match 2 moments, respectively, and each small dot represents an athlete. Next to the VIP are the 15 most relevant metabolites (VIP > 1) based on the OPLS-DA model and their representativeness at each moment of play. N = 9; $R^2 = 0.983$; $Q^2 = 0.625$. See figure S4 for the model's validation process

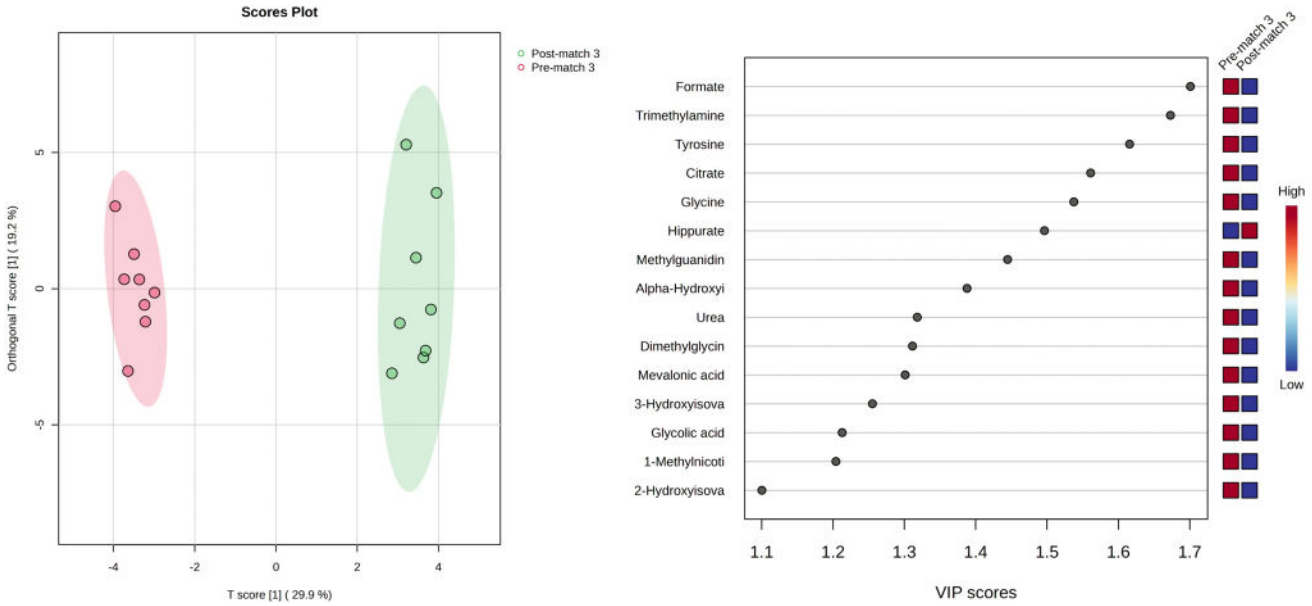


**Fig. 4** OPLS-DA and VIP-plot graphics from pre and post-match 3. The pink and green colors represent the pre- and post-match 3 moments, respectively, and each small dot represents an athlete. Next to the VIP are the 15 most relevant metabolites (VIP > 1) based on the OPLS-DA model and their representativeness at each moment of play. N = 8; $R^2 = 0.991$; $Q^2 = 0.899$. See Figure S5 for the model's validation process

metabolites were respectively: 0.844 and 0.534; 0.983 and 0.625; 0.991 and 0.899, confirming the internal and external validity of the model. Validation figures can be found in the supplementary material (Figures S3, S4 and S5). This $Q^2$ values, added to the component 1 value (X block in Figs. 1, 2 and 3) suggest that there is an increasing separation between groups throughout the championship.

The VIP plots show that although the top 15 most relevant metabolites to the model varied slightly with each game, eight appeared among the most relevant in all three games: glycine, formate, citrate, 3-hydroxyisovalerate, glycolic acid, trimethylamine, urea, and dimethylglycine. These metabolites are generally related to amino acid metabolism, energy metabolism, and the tricarboxylic acid cycle.

To add context about the effort exertion during the games and training sessions between the games, RPE, exercise time and session RPE are displayed in Fig. 5. Using the game days as reference for performance (depicted in black) it is possible to notice some training days matching or even higher than game days. It is important to notice there were off days not included in the picture, being five off days between games 1 and 2 and 9 off days between games 2 and 3. In off days, the athletes could either be completely dismissed or do very light sessions with the medical staff for recovery purposes. Also, it is worth noting that out of the eleven players included in game 1 analysis, seven of them answered an RPE of 7 or higher. For the second match, three out of nine players recorded an RPE of 7 or higher, and in the third match, it was five out of eight players.

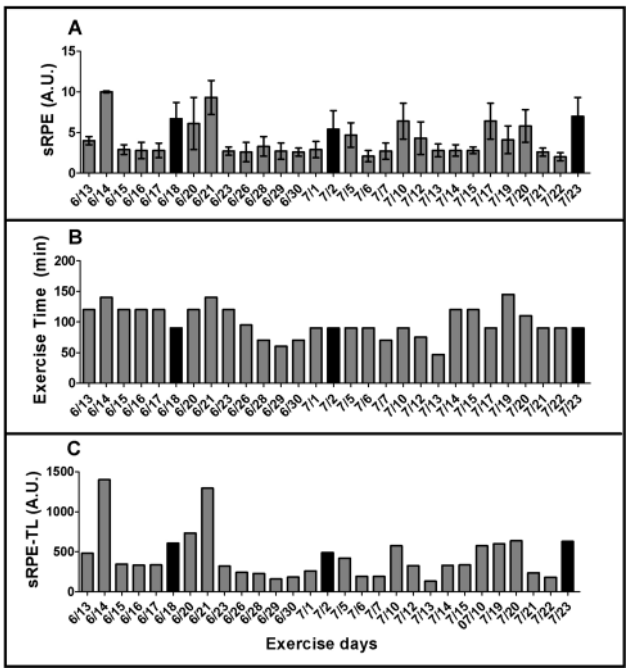


**Fig. 5** Exercise exertion control. Game days included in the study are highlighted in black, while gray columns refer to other exercise sessions. The Session rate of perceived exertion (sRPE) are depicted in panel A, followed by exercise time in panel B and session RPE training load (the product between time and RPE) can be seen in panel C

## 4 Discussion

Our study utilized the OPLS-DA technique to analyze urine sample results, revealing a clear separation of the groups at pre- and post-game moments. Also, a progressive separation between pre-post conditions was demonstrated, as supported by an increasing $Q^2$ value (0.534, 0.625, and 0.899 for games 1, 2 and 3, respectively) and the first component value (20.2% and 19.1% in games 1 and 2 vs. 29.9% in game 3). These findings suggest that the effort during the game and other aspects such as the accumulated fatigue along the season, the opposition level or even the match outcome could be the sources of this discrimination among samples, indicating the potential of using metabolomics in urine to investigate the physiological impact of team sports matches. Although further studies are necessary to confirm a cause-and-effect relationship between the external load of the game and its internal impact on athletes' metabolism, detailed descriptive studies like this one are essential for fingerprinting the physiological paradigms that will inform future robust clinical trials on this topic.

The literature, so far, have used metabolomics for discriminating pre-post efforts in some contexts. A study by Cao et al. (2020) investigating the effects of fatigue in 11 male adolescent soccer players also achieved group separations at pre- and post-exercise moments in their urine, via mass spectrometry. However, the authors examined these moments before and after a highly controlled intensity cycle ergometer test (three aerobic intensity sets at 55–60 rpm/min for six minutes and a load of 150w, followed by a 30-s Wingate test), not a team sport match with unpredictable effort. Another study evaluated the metabolomic profile of 26 male soccer players, analyzing plasma, urine, and saliva samples using mass spectrometry. The OPLS-DA analysis results also showed clear separations between the pre- and post-training groups, corroborating the findings of the present work (Alzharani et al., 2020). Additionally, the study by Pitti et al. (2019) evaluated saliva samples from 17 female soccer players using NMR and still observed a separation between pre- and post-game samples in terms of metabolite concentrations via PCA analysis.

Considering the above, metabolomic analyses appear capable of detecting the impacts of exercise in the urine of both male and female athletes. Despite the expected physiological differences between male and females, a meta-analysis was not able to detect hormonal changes mediated by biological sex in soccer players after a match (Slimani et al., 2017). However, in addition to physiological differences, it is important to notice that biological sex plays an important role in soccer players' fitness levels (Mujika et al., 2009). Senior males covered 30 to 100% more distance than their female counterparts in the Yo-YoIR1, a test measuring the ability to perform intermittent high-intensity exercise for prolonged periods of time (Bradley & Vescovi, 2015; Mujika et al., 2009). On the other hand, differences were around 10–20% for tests measuring parameters as sprint, vertical jump performance and agility with and without the ball (Mujika et al., 2009). These results suggest that despite present for top speed, the gap in fitness between sexes is more prominent for the aerobic metabolism.

Regarding the VIP-plot graphs in our study, it is observed that the same metabolite behaved differently in each match. It is plausible to speculate that contextual variables of the match (opponent, championship stage, season fatigue accumulation) might influence which metabolites are deemed most important by the VIP-plot. Some studies evaluating metabolomics in male and female soccer players report that metabolites such as lactate, pyruvate, and succinate can increase their concentrations post-exercise, acting as markers of the degree of tissue hypoxia reached during exercise (Pitti et al., 2019; Vike et al., 2022), possibly because they are metabolites derived from the anaerobic phase of glycolysis. This is especially expected in women's soccer, considering that high blood lactate concentrations were found in these athletes in short (12-min) games (Farhani et al., 2024). In the present study, this behavior was observed in lactate, which increased in post-game samples. However, pyruvate, while present in the samples, was not among the top 15 metabolites in the games. Nonetheless, being essential to energy metabolism, pyruvate generally shows increased production during exercise, especially strenuous ones (Sun et al., 2017). Succinate emerged as a discriminant only in the first game, but was elevated pre-game, unlike other studies such as Alzharani et al. (2020), where succinate was elevated post-training, justified by increased malate concentrations.

Citrate can be considered a marker of post-exercise fatigue and is generally elevated in this condition (Alzharani et al., 2020; Cao et al., 2020). However, in the present study, citrate was found to be decreased post-game compared to pre-game in all matches. This could be attributed to a higher participation of citrate in aerobic metabolism (citric acid cycle), and increased lactate levels serve as a marker of metabolic acidosis that might reduce aerobic metabolism activity (Chycki et al., 2018). Supporting this notion, a study evaluating male athletes in 80 m sprint sessions found that citrate also decreased post-exercise, even with a large enhancement of lactate levels (Pechlivanis et al., 2010). Similarly, glycolic acid, which was also discriminant in all three games, is an intermediate in the glyoxylate metabolism (related to the Krebs cycle) (Yamaguchi & Ogawa, 1997). This metabolite was elevated pre-game compared to post-game.

Formate, a metabolite involved in metabolic acidosis processes and energy production reduction through cytochrome oxidase inhibition, is considered a marker of exercise-induced fatigue (Sun et al., 2017). Sun et al. (2017) observed a reduction in formate levels in male athletes post an 800 m run, a behavior similarly observed in our study where formate was higher pre-game and decreased post-game for all three games. Pechlivanis et al. (2010) also found similar results with a reduction in post-run formate, associating this finding with the dehydrogenation processes methanol undergoes until converted to formate. These dehydrogenations require NAD +, which high-intensity exercises tend to deplete, consequently influencing formate level reduction.

McFadden et al., (2020a, 2020b) reported that female soccer athletes cover greater distances at moderate and endurance intensity compared to male soccer athletes, while the later spent more time in speeds 19 $km.h^{-1}$ and above. This might suggest that female athletes do not recruit anaerobic metabolism as much during matches, favoring more aerobic metabolism. However, McFadden et al., (2020a, 2020b) compared biological sexes using the same absolute intensities for intensity zone division, and did not identified differences in time spent on heart rate zones (relative intensity). Other studies suggest considering biological sex when dividing these intensity thresholds (Bradley & Vescovy, 2015; Vescovy & Favero, 2014), as male and female soccer players exhibit different metabolic behaviors at similar absolute intensities (Cardoso de Araujo et al., 2020; Farhani et al., 2024), highlighting the need for metabolic impact assessments based on relative intensities to the physiological processes of each biological sex. Thus, even when performed at lower absolute intensities, women's soccer matches tend to be relatively more anaerobic, potentially explaining our study's results where acidosis markers increased while oxidative metabolism-related metabolites decreased.

Glycine and tyrosine amino acids are involved in balancing protein synthesis and catabolism (Pitti et al., 2019). In the current study, glycine was elevated pre-game compared to post-game (for all matches), similarly to the studies by Pechlivanis et al. (2010) and Sun et al. (2017) in male athletes. Pechlivanis et al. (2010) suggest this glycine behavior as a possible indication of renal function alteration due to exercise-induced lactic acidosis. In contrast, Pitti et al. (2019), who analyzed saliva samples of female athletes, found glycine increased post-exercise, a divergent finding possibly linked to the type of biofluid used. Tyrosine also behaved similarly to glycine in our study, unlike Pitti et al. (2019), where it increased post-game (games 2 and 3). Dimethylglycine could be related to protein catabolism resulting from physical and muscular exertion (Marinho et al., 2022). In our analysis, it was higher pre-game than post-game in all three matches.

There are reports of urea concentration in urine, produced in the liver from ammonia due to protein catabolism, to be increased after a day of winter training season and decreased after ten days of recovery in male soccer players (Kim et al., 2022). In our study, it was more present pre-game and decreased post-game across all three matches, possibly due to pre-game training sessions. Another metabolite indicative of muscle damage or renal function decline, creatinine, increased post-marathon (blood samples) (Bester et al., 2021). This also occurred in our study, but it was only discriminant in the second game, possibly indicating more muscle damage in athletes during that match. Souglis et al. (2018) demonstrate how a women's soccer match can significantly affect muscle damage markers in soccer athletes of both biological sexes. Additionally, for male athletes, Selmi et al. (2022) report muscle damage and anabolic/catabolic balance alteration induced by training, linked to factors like players' perceived recovery state.

Like creatinine, trimethylamine can indicate renal function, potentially elevated in urine in cases of acute renal dysfunction/injury due to intense exercise (Hyang Yeon Kim et al., 2022). In our study, it was elevated pre-game and decreased post-game across all three matches. Sun et al. (2017) observed a reduction in 3-hydroxyisovalerate concentration post-run in male runners, like our study where it was higher pre-game and reduced post-game, possibly related to renal function (Sun et al., 2017). In the same study, 2-hydroxyisovalerate, associated with elevated oxidative stress, increased post-run, a behavior opposite to our third game where it decreased post-match. This suggests athletes might have started the third match with higher oxidative stress and accumulated fatigue from previous matches and/or training, suggested by the greater group separation over the games observed in OPLS graphs. However, some associations described are speculative and require further studies for empirical validation.

Alpha-hydroxyisobutyrate, or 2-hydroxyisobutyrate, a metabolite related to ketone body production (Marinho et al., 2022), remained high pre-game and reduced post-game, being discriminant only in the third match. This suggests athletes might have been using fat as an energy substrate in training sessions before the game, returning to glucose for energy during the match due to intensity demands. In our study, methylguanidine, considered a marker of oxidative stress, was discriminant in games two and three, behaving similarly to Pechlivanis et al. (2012), where it reduced post-run in one group of male athletes that performed 80 m runs with a short interval between them (10 s). Hipurate, associated with cell damage (Marinho et al., 2022), was discriminant in two games (first and third matches), and was elevated post-game. Aspects like accumulation of fatigue or a higher exertion of effort (the third was the only winning match out of the

three analyzed) can explain the markers of cell damage being more discriminant in the third match, suggesting the need to consider contextual match elements in metabolomic analysis. 1-Methylnicotinamide, involved in nicotinate and nicotinamide metabolism (Wang et al., 2021), also stood out in two games, remaining higher pre-game.

Metabolites such as dimethylsulfone, methylhistidine (considered a marker of muscle protein breakdown) (Pechlivanis et al., 2010), methyluric acid (a caffeine metabolite) (Zaworski et al., 2020), possibly related to consumption, supplementation, or use of caffeine-containing medications, and mevalonic acid were discriminant in only one game, elevated pre-game and reduced post-game. Uracil and pi-methylhistidine also stood out in one game but were low pre-game and elevated post-game.

Regarding the context of efforts performed during the matches and training sessions between match days, the training had a schedule of one-game weeks. The distribution of sRPE, exercise time and sRPE-TL in Fig. 5 shows a high demand of training when compared to match days. sRPE was not maximum for all the players post-match, as it is known the effort may vary for different playing positions (Datson et al., 2014). However, both sRPE and sRPE-TL exceeded the load of the matches included in this study, configuring a state of stress that can lead to fatigue (Costa et al., 2022). In male players, post-match high values of sRPE ($\geq 7$) were able to differentiate the metabolic profile from athletes with lower sRPE (Marinho et al., 2022). For the first match, 7 out of eleven players (64%) had an sRPE $\geq 7$, while in the second it was three out of nine (33%), and five out of eight (63%) in the third. Fatigue in soccer can be assessed by subjective scales or performance decrement in physical tests involving sprints, jumps of voluntary contractions (Costa et al., 2022), but this was not performed here. Hence, fatigue would not be treated as a dependent variable, but the load control for the players included in this study suggests that fatigue accumulation may had played a role in the increasing separation between pre/post-match comparisons.

Describing these metabolites reveals that some remained consistent across all three games, while others varied in relevance depending on the match. This could be attributed to limitations of the present study, such as differences between games in competition phases, different opponents, and tactics adopted in each game, potentially causing injuries in some athletes and lineup changes. Additionally, the small sample size ($n = 14$) is also a limitation. These limitations do not undermine the presented results, considering the difficulties to perform this kind of data collection in a real-life scenario with actual athletes during championship games, as it reflects the day-to-day routine of a soccer squad.

## 5 Conclusion

Our results showed that over the course of three games in a championship, the pre-post metabolomic profile difference of female soccer players was notably increased. This separation may be attributed to several factors, such as the opponent characteristics, fatigue accumulation over the competition, match outcome, etc. This alteration was observed through OPLS-DA graphs, and the more discriminant metabolites identified in the VIP plot were primarily related to energy and protein metabolism. There was a trend towards an increase in anaerobic glycolysis metabolism at the expense of aerobic oxidative metabolism, which may be a characteristic inherent to women's soccer, differing from results previously found in men's soccer.

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**Data availability** The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** Prior to the commencement of the study, the athletes were informed about all the procedures to be conducted during the research, as well as the possible risks and benefits. Subsequently, they signed the Informed Consent Form (ICF). This study is part of a larger project titled "Analysis and Improvement of Athlete Performance," approved by the Research Ethics Committee (CEP) of the Federal University of Alagoas (UFAL) under the protocol number CAAE: 29269020.8.0000.5013 and Opinion: 4297907, in accordance with the ethical principles outlined in the Declaration of Helsinki of 1964. The overarching project was supported by the National Council for Scientific and Technological Development (CNPq) under the code 408972/2021-1.

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