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A small sided game session affects salivary metabolite levels in young soccer players

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Abstract.
BACKGROUND: The use of saliva for monitoring metabolic variations in physical exercise and in different sports gained ground in recent years. Several studies showed that saliva reflects biochemical changes useful for analytical purposes in clinical investigations and in physiological research.
OBJECTIVE: The aim of this study was to explore the profile of salivary metabolite changes due to a session of small sided games (SSG) in elite soccer players, searching for a correlation between metabolic changes and athlete performance as GPS-measured distances covered in the match.
METHODS: Ten under-20 elite soccer players participated to the study. The game had an overall duration of 24 min and it consisted of 4 bouts of 6 min duration with 2 min passive recovery between exercise bouts. Saliva samples were collected before and after the game and physiological parameters evaluated, namely the distances covered by players and blood lactate. Samples were analyzed by Nuclear Magnetic Resonance spectroscopy. Orthogonal Projection of Latent-Structure (OPLS) was used to process the data.
RESULTS: Multivariate data analysis showed that the SSG session affected salivary metabolite levels in players. We observed no relationship between concentrations of hematic and salivary lactate, nor found any changes in the metabolic profiles that correlate with the blood lactate values. Among the identified metabolites, taurine was instead found to correlate with distances covered by players during the game.
CONCLUSIONS: Altogether these results point to a potential use of saliva to follow metabolic changes during an athletic competition, and opens the possibility of using this non-invasive biofluid for the study of athlete training state and performance.

Keywords: Small sided game, saliva, metabolome, salivary metabolites, soccer, NMR analysis

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1. Introduction

High-strength endurance sports such as soccer, are known to generate many metabolic changes in athletes. The majority of studies concerning the impact of physical exercise, investigated a limited number of analytes with the aim of discovering biomarkers able to correlate with the actual performance in competition and predict the progress in the improvement [4,29,41,54,55]. Although giving a deep insight into the metabolic energy switch, such studies are scarcely applicable as routine analyses to everyday sport team life. In fact they are based on invasive practices, such as muscle biopsies and blood analysis, which are scarcely tolerated by both team managements and athletes. Furthermore, they provide an important single measured parameter, although not exhaustive enough with respect to athlete metabolism.

Metabolomics is a new "omic" discipline, which is the farther step of genomics, transcriptomics and proteomics, and involves the determination of comprehensive metabolite profiles in biological matrices [26]. Such an approach allows a qualitative and quantitative measurement of metabolic changes due to physiological stimuli, as physical exercise or pathophysiological conditions, and it can provide global insight into physiological processes. Great achievements have been obtained in metabolomics research, especially in biomedical sciences [62]. Although the application of this approach is growing rapidly, there are many challenges that must be addressed before it can reach its full potential. Biological interpretation is a key step to successfully understand metabolomic data; as in other areas of clinical chemistry, it must be recognized that pre-analytical factors, i.e. factors that occur prior to the final analysis, may influence the metabolic profile of biological specimens [22]. Since studies have long been focused on blood metabolites and other physiological measurements to investigate the body's responses to exercise, it became evident that exercise induced changes also in other biofluids and these need to be measured in order to study athlete metabolism after a physical effort [6,15,28].

The use of saliva for monitoring metabolic variations in physical exercise and in different sports gained ground in recent years. Several studies showed that saliva reflects biochemical changes useful for analytical purposes in clinical investigations and in physiological research [15]. Moreover saliva monitoring has as a strong point in that it may be used routinely since sample collection is not invasive and very quick. In a recent work we showed how Nuclear Magnetic Resonance (NMR) spectroscopy can be used to investigate metabolic profiles of soccer players' saliva with the advantage of allowing a specific and simultaneous determination of a high number of metabolites. Our results demonstrated that variations in metabolite concentration correlated with the performance score obtained in the Yo–Yo level 1 intermittent recovery test, a physical test able to evaluate athletes' ability to repeatedly perform high intensity exercise [42]. In high performance sports it has been well documented that the maximum benefits are achieved when the training stimuli are similar to competitive demands [32]. The use of small sided games (SSG) has been introduced in the training programs in order to reproduce the physical, technical and tactical requirements of real match play [1,24]. Studies indicate that SSGs reproduce game conditions close to the competitive matches in terms of intensity, number of players and pitch size [32,40]. Due to this fact, game-based conditioning using SSG has become a popular method of developing specific aerobic fitness for soccer players [23,27]. The aim of this study was to explore the profile of salivary metabolite changes due to a session of SSGs in elite soccer players and to correlate metabolic changes with athlete performance evaluated as GPS-measured distances covered in the match. To this end a multivariate data analysis was performed. The preliminary analysis was the principal component analysis (PCA), an unsupervised method that does not separate classes but it is useful to identify outliers. Then, an Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) was run [58]. This method allows discrimination between two groups, filtering out variations that are not directly related to this separation.

The supervised methods were employed mainly to study the contribution of the variables in group separation and to find differentiating biomolecules. In the end, correlation of spectroscopic data to the GPS distance an OPLS analysis was calculated. With this method the variables (spectra) are correlated to a response (GPS).

2. Materials and methods

2.1. Exercise intervention

Ten elite soccer players under-20 SS Lazio participated to the study. The subjects avoided any intense physical activities the day before the measurements and had the same diet controlled by team management. The protocols were performed during the mid-season. The SSGs were conducted with two goalkeepers and a maximum of two ball touches in a 30 × 40 m pitch. The game had an overall duration of 24 min and it consisted of 4 bouts of 6 min duration with 2 min passive recovery between exercise bouts. Distances covered by players during each game were collected using portable GPS devices (SPI Elite; GPS Sports Systems Ltd., Canberra, Australia), sampling at 1 Hz. Soon after the entire training, capillary blood samples were drawn from the ear lobe of players, using a sterile lancet (Accu-Check Softclix, Roche – 5 m). Blood samples were analyzed for lactate concentration by means of a blood lactate analyzer (Lactate-AU5 Pro, Arkray, Japan). Informed consent and local ethics committee approval were provided.

2.2. Saliva sampling

Saliva samples, pre- and post-exercise, were collected for passive drooling. Participants were seated with their head tilted forward, allowing saliva to pool in front of the mouth. Saliva was allowed to be dribbled out of the mouth into polypropylene tubes until a sufficient amount (i.e. 2 ml) was obtained [12]. The saliva samples were stored at −80°C until use. At the time of analysis samples were deproteinized by ultrafiltration. Prior to filtration, 3 KDa cut-off centrifugal filter units were washed several times with 2 ml of H_2O and centrifuged at 4000*g* for 20 min to remove residual glycerol bound to the filter membranes. The washing procedure was repeated until control by NMR spectroscopy of the filtrate showed no residual presence of glycerol. A volume of 1 ml of each saliva sample was transferred in the filter device and centrifuged at 4000*g* for 1 h at 4°C. An amount of 400 ml of filtrate was diluted with 100 ml of buffer (250 mM phosphate buffer pH 7.4 containing 1 mM 3-(trimethylsilyl)propionic acid-d_4 sodium salt, TSP, 2% NaN_3, 10% D_2O). The final solution was mixed and transferred to a 5 mm NMR tube.

2.3. NMR spectroscopy

Spectra were acquired on a Bruker 600 MHz Avance spectrometer equipped with an inverse 5 mm BBI probe with z-gradients. Standard methods for the acquisition of saliva NMR spectra were used [7]. ^{1}H-NMR spectra were acquired at 25°C using the NOESYPR1D (1D Nuclear Overhauser effect spectroscopy with water pre-saturation) pulse sequence (RD-90°-t_1-90°-t_m-90°-acquire) with $t_1 = 4$ µs $t_m = 100$ ms, spectral width of 20 ppm, acquisition time of 4 s, relaxation delay of 1 s, 256 transients. Water saturation was performed using a continuous-wave field, both during relaxation delay and mixing time. All FIDs were zero-filled to 128k data point and subjected to line broadening of 0.5 Hz. All spectra

were calibrated, phased and baseline-corrected manually before being used for estimation of metabolite concentrations.

For the statistical analyses, FIDs were processed using the MestReNOVA software. Intelligent bucketing was used to integrate each spectrum with a bin size of 0.03 ppm. The water region between 4.6–5 ppm was excluded. Noise regions of the spectra were excluded by setting the corresponding bin to zero. The spectra were normalized according to the "Probabilistic Quotient Normalization" method [20].

2.4. Identification and quantification of metabolite

NMR spectra acquired were imported in Chenomx NMR suite software (version 8.0). The software allowed the identification and quantification of metabolites present in the Chenomx library. As internal standard, TSP with a final concentration of 0.2 mM was used.

2.5. Multivariate data analysis

Multivariate data analysis was carried out using SIMCA-P (version 14 Umetrics AB, Umea, Sweden). Both univariate and Pareto scaling modes were tested. Pareto scaling was chosen as it is more appropriate for spectroscopy data [31]. A principal component analysis (PCA) was run to obtain a general overview of the variance of the NMR sample. This method provides the possibility to detect and exclude outliers, defined as observation outside the 95% confidence region of the model.

Orthogonal projection of latent-structure discriminant analysis (OPLS-DA) models was employed to study the contribution of the variables in group separation (before and after exercise) eliminating variation in the data that were unrelated to the variation between classes. The robustness of the models is indicated by the following parameters: R^2Y, predicted percentage of the response; R^2X, variation of X explained by the model and Q^2, goodness of prediction. R^2 varies between 0 and 1, Q^2 varies between -1 and 1. When Q^2 value is higher than 0.5 the predicted model is good [52].

Spectral data were also analyzed in relation to lactate blood concentration and to distance covered (GPS data) by orthogonal projection of latent-structure analysis (OPLS). This method connects the information in two blocks of variables (X and Y) to each other. The Y vector was constituted by the lactate concentration value or the covered distance.

3. Results

3.1. Compound identification and quantification

Saliva samples from ten soccer players, after (A) and before (B) a session of SSGs were collected and analyzed using ^{1}H NMR. Representative spectra are shown in Fig. 1. A first PCA analysis was performed in order to detect the presence of possible outliers. The resulting plot clearly showed that the metabolite content of saliva belonging to player 9 before exercise (9B) was significantly different from any of the other samples (data not shown). A simple inspection of spectra in Fig. 1 already identifies a series of peaks in the region 3.2–3.8 ppm with higher intensities for sample 9B than in other spectra. For this reason, this sample was excluded from the analysis. On the other hand, the spectrum of the same player after the exercise (9A) shows no significant deviation from other samples. This fact suggests that the extra peaks observed in the spectra for 9B are due to external contamination, *although other sources cannot be ruled out*.

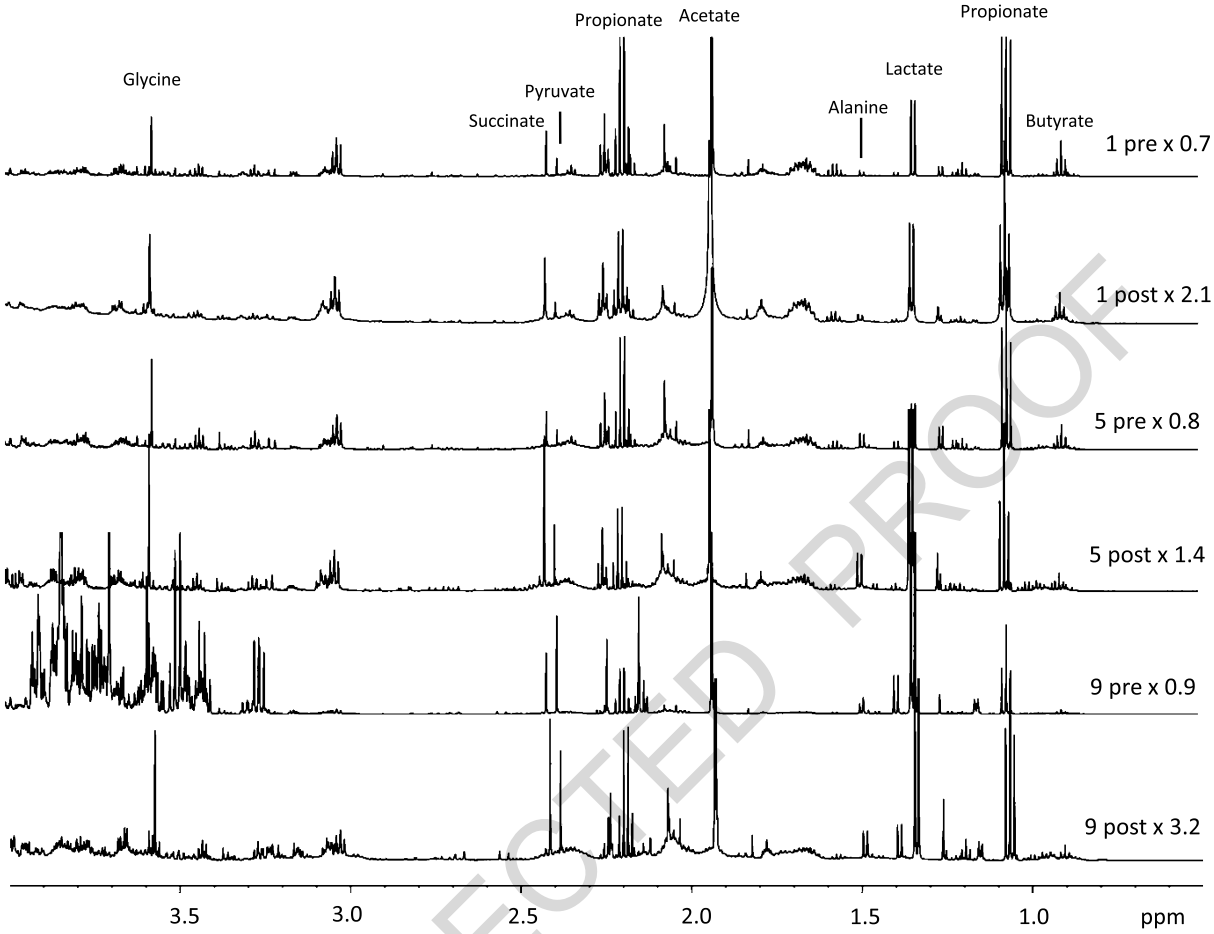

Fig. 1. Representative 600 MHz 1 H NMR spectra of saliva samples, both pre- and post-exercise. Some of the identified metabolite signals are labeled. In this plot no normalization was taken into account, thus no direct visual comparison of signal intensities across spectra is accurate. The normalization factor used in this study is indicated for each spectrum.

Over 70 compounds were identified in the spectra of saliva, including amino acids, soluble membrane precursors, carboxylic acids, nucleotides and related compounds, carbohydrates, and a series of other compounds like ethanol, methanol, urea, taurine and putrescine. Integral values were converted into the concentration for each metabolite using TSP as internal standard. Table 1 shows the average concentration of the metabolites identified in the two groups.

3.2. Multivariate analysis of pre- and post-exercise sample

Principal component analysis of the samples, excluding 9B, was performed in order to asses if there is some initial separation between the two groups in an unsupervised analysis using the complete spectrum obtained for each sample, with the only exclusion of the water signal region. Figure 2(A) and 2(B) show the separation using the first three components, which explain together 75% of the total variation. Although there is some degree of separation, especially along the first component, there is partial superposition between the two groups. A relevant observation is that for all the couples of samples, pre and

Table 1
Concentrations (mM) of saliva metabolites as determined by ^{1}H NMR

	Average pre	Average post
Amino acids		
Alanine	0.319 ± 0.592	0.164 ± 0.066
Arginine	0.027 ± 0.053	0.087 ± 0.098
Aspartate	0.068 ± 0.043	0.098 ± 0.047
Glutamine	0.018 ± 0.055	0.069 ± 0.078
Glycine	0.640 ± 0.198	0.858 ± 0.285
Histidine	0.043 ± 0.044	0.068 ± 0.046
Isoleucine	0.008 ± 0.010	0.017 ± 0.007
Leucine	0.032 ± 0.014	0.078 ± 0.088
Phenylalanine	0.041 ± 0.019	0.069 ± 0.017
Proline	0.330 ± 0.320	0.593 ± 0.335
Serine	n.d.*	0.115 ± 0.160
Taurine	0.298 ± 0.105	0.158 ± 0.044
Threonine	n.d.*	0.035 ± 0.055
Tyrosine	0.084 ± 0.043	0.120 ± 0.031
Valine	0.024 ± 0.011	0.037 ± 0.016
Soluble mebrane precursor		
Choline	0.017 ± 0.010	0.019 ± 0.010
Ethanolamine	0.056 ± 0.072	0.123 ± 0.071
Glycerol	0.155 ± 0.046	0.371 ± 0.117
O-acetylcholine	0.013 ± 0.006	0.010 ± 0.009
O-phosphocholine	0.015 ± 0.013	0.008 ± 0.005
O-phosphoethanolamine	0.042 ± 0.065	0.087 ± 0.050
sn-glycero-3-phosphocholine	n.d.*	0.004 ± 0.003
Nucleotide and related compounds		
Hypoxanthine	0.024 ± 0.014	0.018 ± 0.009
Xanthine	0.025 ± 0.014	0.029 ± 0.015
Uracil	0.013 ± 0.014	0.014 ± 0.011
Carbohydrates		
Fucose	0.211 ± 0.087	0.230 ± 0.114
Galactose	0.163 ± 0.064	0.196 ± 0.068
Glucose	0.128 ± 0.073	0.085 ± 0.043
Carboxylate		
2-hydroxyisobutyrate	0.003 ± 0.005	0.005 ± 0.003
2-hydroxyisovalerate	n.d.*	0.002 ± 0.002
3-hydroxybutyrate	n.d.*	0.010 ± 0.017
3-phenylpropionate	0.015 ± 0.016	0.016 ± 0.014
4-hydroxyphenylacetate	0.021 ± 0.024	0.019 ± 0.016
4-hydroxyphenyllactate	0.002 ± 0.007	0.005 ± 0.009
Acetate	16.724 ± 3.566	13.847 ± 2.408
Acetoin	0.044 ± 0.014	0.032 ± 0.011
Acetone	0.185 ± 0.045	0.167 ± 0.040
Butyrate	0.162 ± 0.180	0.140 ± 0.148

Table 1
(*Continued*)

	Average pre	Average post
Carboxylate		
Citrate	0.031 ± 0.060	0.020 ± 0.044
Formate	0.292 ± 0.572	0.470 ± 0.708
Fumarate	n.d.*	0.002 ± 0.003
Isocaproate	0.007 ± 0.013	0.018 ± 0.011
Isopropanol	0.003 ± 0.003	0.003 ± 0.001
Isolvalerate	0.029 ± 0.018	0.031 ± 0.011
Lactate	0.565 ± 0.310	2.138 ± 2.508
Malonate	0.011 ± 0.013	0.018 ± 0.009
Phenylacetate	0.034 ± 0.014	0.026 ± 0.015
Propionate	2.127 ± 0.835	1.627 ± 0.367
Propylene glycol	0.023 ± 0.022	0.023 ± 0.014
Pyruvate	0.159 ± 0.090	0.221 ± 0.086
Succinate	0.094 ± 0.062	0.181 ± 0.110
Other compounds		
Agmatine	0.028 ± 0.049	0.064 ± 0.078
Allantoin	0.009 ± 0.015	0.011 ± 0.015
Carnitine	n.d.*	0.005 ± 0.004
Carnosine	n.d.*	0.007 ± 0.014
Creatine	0.050 ± 0.017	0.033 ± 0.011
Creatinine	0.016 ± 0.003	0.014 ± 0.008
Dimethylamine	0.004 ± 0.002	0.005 ± 0.003
Ethanol	0.127 ± 0.059	0.074 ± 0.034
Histamine	0.017 ± 0.020	0.017 ± 0.015
Methanol	0.065 ± 0.040	0.038 ± 0.020
Methylamine	0.012 ± 0.007	0.011 ± 0.004
N-acetylglycine	0.096 ± 0.060	0.066 ± 0.054
N-acetylglucosamine	n.d.*	0.066 ± 0.073
Phenol	0.038 ± 0.026	0.018 ± 0.022
Putrescine	0.157 ± 0.108	0.267 ± 0.111
Pyroglutammate	n.d.*	0.039 ± 0.077
Sarcosine	0.019 ± 0.005	0.013 ± 0.004
Trimethylamine	0.008 ± 0.004	0.004 ± 0.002
Trimethylamine-noxide	0.004 ± 0.003	0.002 ± 0.001
Urea	0.973 ± 1.005	1.507 ± 1.099
Urocanate	0.015 ± 0.019	0.030 ± 0.024

*Not determined.

post, the former show in all cases a lower value for the first component, revealing a trend that can be discriminant for class separation.

To better accomplish the separation of the samples into the two groups, we applied a supervised analysis, namely an orthogonal projection of latent-structure discriminant analysis, OPLS-DA. The main idea of OPLS is to concentrate all the between-groups separation into the first predictive component (t_1) and within-group differences in orthogonal components (to_n). This separation results in improved

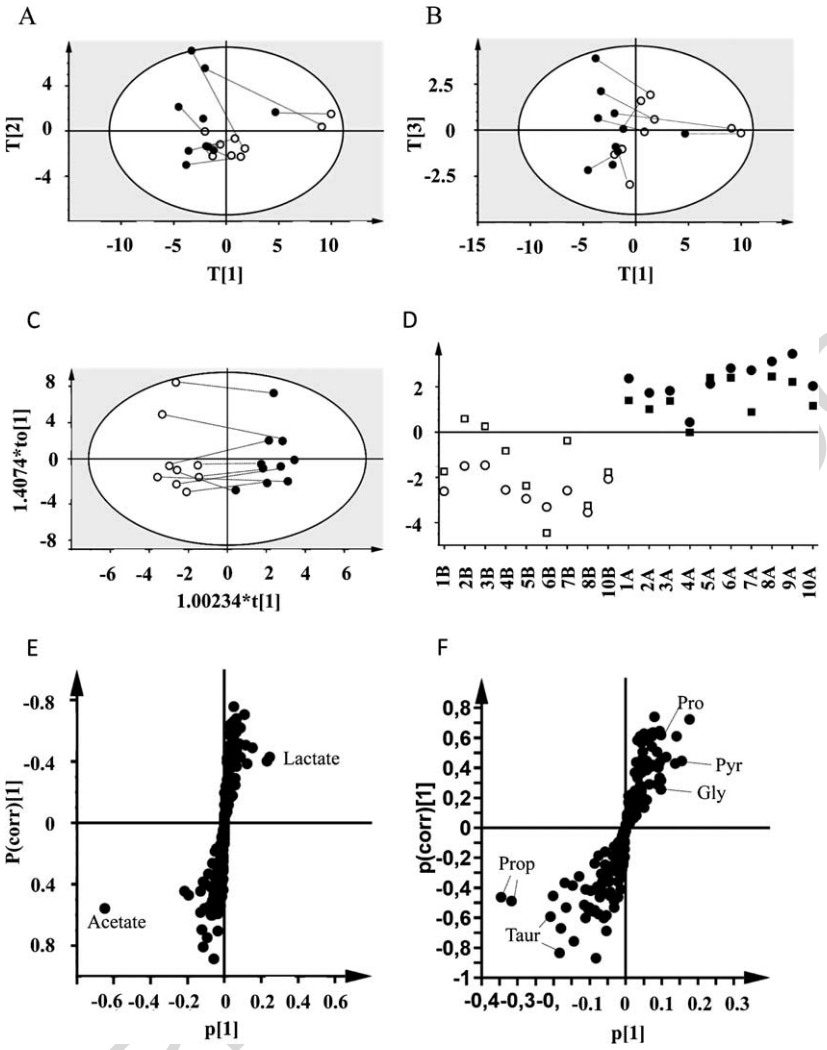

Fig. 2. Multivariate statistical analysis of data from the 1H NMR analysis of saliva. (A) and (B) PCA score plot excluding 9B (see text). PC1 accounts for 47% of the variation, PC2 for 21% and PC3 for 7%. Hotelling ellipse depicts a 95% confidence interval. (C) OPLS-DA score plot of all saliva samples excluding 9A and 9B. The pre exercise samples are in *white circles*, the post exercise samples are in *black circles*. (D) Cross validated score plot. *Circles* represent the calculated values of the predictive component, whereas rectangles represent the cross validated values. The smaller the difference for each sample between the calculated and predicted values is a measure of the accuracy of the model to predict if the saliva spectrum belongs to a pre- or post-exercise sample. (E) S-plot of all the samples, highlighting the differentiators of the groups, with signals on the upper right end being higher in post-exercise samples, and signals on the lower left end being higher in pre-exercise samples. (F) Same S-plot excluding acetate and lactate signals.

model transparency and interpretability [11]. *Calculation of this model was performed excluding also 9A, in view of the fact that it has no pre-exercise counterpart.* Figure 2(C) shows the result of applying the OPLS-DA analysis to the saliva spectra ($R^2X = 0.95$, $R^2X\,\mathrm{Pred} = 0.17$, $R^2X = 1$ and $Q^2 = 0.72$). R^2 *measures the goodness of fit while* Q^2 *measures the predictive ability of the model.* $R^2 = 1$ *indicates perfect description of the data by the model, whereas* $Q^2 = 1$ *indicates perfect predictability.* R^2 *increases monotonically with the number of components (NC) and will automatically approach 1 if*

NC approaches the rank of the X matrix. Q^2 *will not necessarily approach 1.*

Model validation was performed by analyzing the cross validated score plot (Fig. 2(D)). This plot visualizes the stability for each observation in the model. As can be observed, the model for pre-exercise samples is less stable than that for the post-exercise ones. The overall Q^2 value (*0.72*) is however acceptable for a model based on biological data [52].

Finding those metabolites that are at the basis of class separation is possible through the analysis of the S-plot. The S-plot, in which p (corr) and w^* are plotted, combines the information from loading plot and the column plot confidence limits. The variables centered in the middle are regarded as not related to class separation, while the ones residing on both ends of the "S"-shape are potential biomarkers. Figure 2(E) shows the corresponding S-plot, correlating to the loading plot of Fig. 2(C). From this analysis, it is clear that there are two metabolites that contribute most to the distinction between pre- and post-exercise saliva spectra: acetate, which decreases in the post-exercise samples, and lactate, that increases upon exercise.

To reveal the contribution of other discriminating metabolites, we then excluded the spectral regions containing the signals of acetate (1.92 ppm) and lactate (1.32, 4.10 ppm). The OPLS-DA score plot still showed a good group separation (data not shown) with parameters compatible with a robust model ($R^2X = 0.68$, $R^2X\,Pred = 0.14$, $R^2Y = 0.93$ and $Q^2 = 0.67$). From the corresponding S-plot (Fig. 2(F)), we could assign the following metabolites that are responsible for class separation: pyruvate, glycine, proline, succinate and putrescine that show increased concentration after exercise, and propionate, taurine, methanol, trimethylamine and O-phosphocholine that show the opposite trend. Variations in their concentration upon exercise, along with acetate and lactate, are indicated in Fig. 3 through the use of the binary logarithm of the fold change (FC).

3.3. *Correlation with hematic lactate levels*

We explored the possible correlation between the post-exercise saliva spectra and the levels of hematic lactate that were measured right *after* the training session (Table 2). First, we determined the concentration of lactate in all the post-exercise saliva spectra and correlated them with the observed value of hematic lactate (Fig. 4(A)). As it is clear from this plot, there is no evident correlation between the two

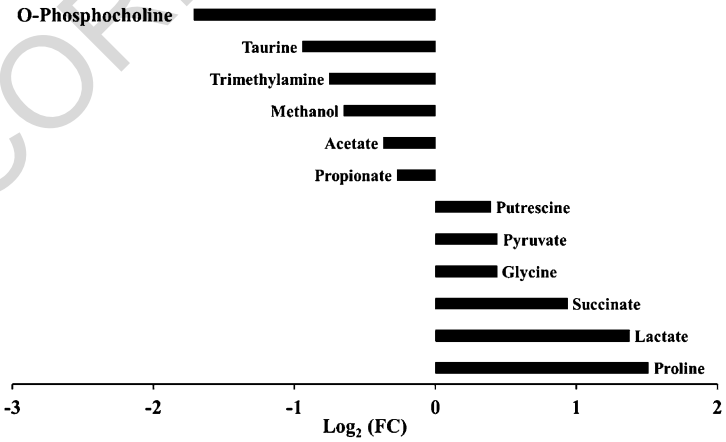

Fig. 3. Value of $\log_2$(FC) resulting from the comparison between pre and post samples. A positive value indicates a higher concentration in post samples, while a negative value indicates a lower concentration in post samples.

Table 2
Covered distance, serum and saliva lactate post-exercise concentrations

Athlete	Serum lactate [mM]	Saliva lactate [mM]	GPS value [m]
1	2.0	1.048	2770
2	2.4	0.486	n.d.*
3	4.3	0.676	2798
4	4.3	1.111	2584
5	2.3	6.711	2796
6	2.9	6.032	2854
7	1.8	0.491	3163
8	2.8	0.237	2570
9	3.6	1.529	2334
10	2.4	3.060	2589

*Not determined.

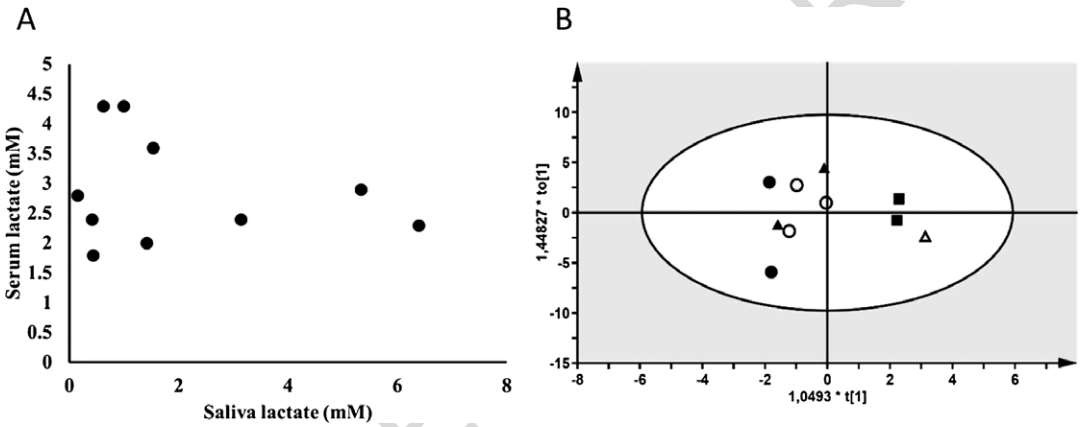

Fig. 4. (A) Correlation between post-exercise serum and saliva lactate concentrations in (mM). (B) OPLS score plot for the correlation. *Black circles* serum lactate $\leqslant$ 2 mM; *white circles* serum lactate between 2.3 and 2.4 mM; *black triangles* serum lactate between 2.8 and 2.9 mM; *white triangles* serum lactate = 3.6 mM; *black squares* serum lactate = 4.3 mM.

concentrations. We have further explored whether there was a change in the metabolic profile of the post-exercise saliva spectra that could be correlated with the hematic lactate concentrations. To this end, we performed an OPLS analysis using as Y-variable the concentration of lactate in plasma (Fig. 4(B)). Even in this case, we could not obtain a separation of the samples in the t_1 direction that reflects the plasma lactate concentrations, as evidenced by the mixed position of the samples. The statistical indicators are also in line with this lack of prediction ($R^2X = 0.44$, $R^2X\,Pred = 0.12$, $R^2Y = 0.78$ and $Q^2 = -0.45$).

3.4. Correlation with GPS-measured distances

A second parameter used to observe correlation with the salivary metabolic profile is the distance covered by each athlete during physical exercise (with exception of athlete 2 for which no data was available), measured with the GPS device. As in the case of hematic lactate, we performed an OPLS analysis using a Y matrix with the values reported in Table 2. Figure 5(A) shows the observed separation, which in this case correlates well with the distance values ($R^2X = 0.98$, $R^2X\,Pred = 0.07$, $R^2Y = 1$ and $Q^2 = 0.87$).

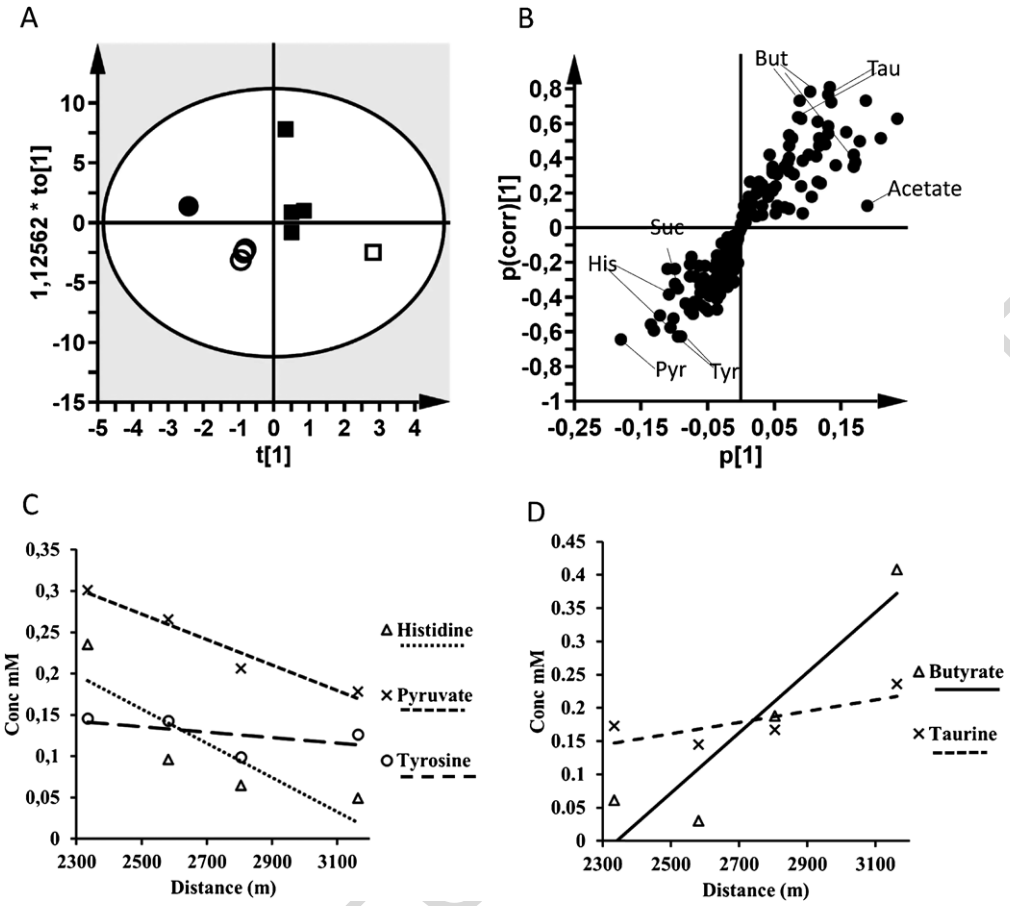

Fig. 5. (A) OPLS score plot for the correlation with covered distance. *Black circles* distance value lower than 2500 m, *white circles* distance values between 2500–2700 m, *black squares* distance values between 2700–3000 m and *white squares* distance value higher than 3000 m. (B) S-plot. (C), (D) Correlation between selected average metabolite concentration and covered distance value.

The S-plot (Fig. 5(B)) allowed the identification of metabolites that are responsible for the observed correlation. Butyrate, acetate and taurine were identified as metabolites which increase their concentration with increasing distance covered. Pyruvate, histidine and tyrosine show the opposite trend.

To better observe the correlation between the concentrations of these metabolites with the covered distance, we divided the athletes into four groups: 9A (2334 m); 4A, 8A and 10A (2400–2600 m); 1A, 3A, 5A and 6A (2600–2900 m) and finally 7A (3163 m). For each group the average post exercise concentration was determined using Chenomx and plotted against the distance (Fig. 5(C) and (D)).

4. Discussion

Soccer, as a high strength–endurance sport, is known to strongly stimulate the metabolic profile of athletes. To date studies on physical exercise metabolism have been developed to measure and compare the concentration of one or few metabolites in biofluids or directly into muscle tissue, in both exercise and recovery [4,29,41,54]. Most of the metabolomic studies have been developed in blood, serum,

plasma or urine samples, since they reflected the physical exercise-related changes occurring in the muscle [22,38,59]. Recently, the use of saliva has become very popular in the field of sports and exercise sciences. Saliva can provide a useful, non-invasive alternative biofluid, as it can be collected rapidly, frequently and without stress. Furthermore, saliva collection requires less medical training and can be performed on the sports field [37]. We previously showed that changes in salivary metabolome of professional soccer players were correlated with the performance obtained in level 1 Yo–Yo test [42]. In the present study we explored the profile of salivary metabolite changes in elite soccer players due to a session of small sided games (SSG), namely soccer specific training matches with fewer number of players in smaller field dimensions. To date the systemic metabolism contribution to the salivary metabolic profile is still debated. Factors like clinical features [9], dietary intake [56], age or gender [2] have little impact on the concentration distribution of metabolites observed in saliva. No significant correlation was found between body mass index (BMI), glycated hemoglobin and systolic and diastolic blood pressure [9]. Conversely, the degree of interindividual variation results to be significant [56]. Our data confirm this feature, as indicated by the lack of a clear separation in the PCA between pre- and post-exercise metabolic profile probably due to a high intra-group variability.

Normalization of salivary spectra of pre- and post-exercise samples is of high importance for a metabolomic study that compares concentrations. We have observed an increase in the overall metabolites concentration of about 50% in the post-exercise samples. This is probably due to an altered secretion induced by hormonal stimuli [61]. The action of parasympathetic hormones stimulates the saliva secretion resulting in a hypoconcentrated compound with low concentrations of organic substances [3], while the sympathetic stimulation induces saliva secretion with higher concentration of organic substances, making the medium hyperconcentrated [3,30]. In physical exercises, an increase in the secretion of sympathetic hormones occurs, especially catecholamines [43], causing an increase in the sodium, potassium and lactate concentrations in saliva [8,13,14]. Although the level of salivary lactate was significantly enhanced in the post-SSG samples, we observed no relationship between concentrations of hematic and salivary lactate, and found no changes in the metabolic profiles that correlate with the blood lactate values. While measurement of salivary lactate was proposed as an alternative to its measurement in blood [44,45], it was demonstrated that this method is not accurate [19]. The main reason for the observed discrepancies between blood and salivary lactate was found in the lactate produced by bacteria living in the oral mucosa. This extra source of salivary lactate renders the method inaccurate for determining exercise-induced lactate productions. Together with lactate levels, we find increased salivary concentrations of succinate and pyruvate, which are interconnected. In fact, during exercise, the rate of pyruvate formation increases in muscle, which in part is converted into lactate by lactate dehydrogenase. Succinate is also related through the Krebs cycle. The increase of lactate, succinate, pyruvate and alanine is a well establish fact during exercise [25]. Other metabolites that exhibit significant changes between pre- and post-salivary metabolic profile have been found to be variable during day period: propionate, acetate, trimethylamine and methanol. A direct connection with systemic changes cannot be automatically invoked, as they are also associated to bacterial production or food consumption. For example, the level of acetate in saliva is suggested to reflect carbohydrate fermentation related to an earlier eating session [56]. Propionate and acetate levels are mainly regulated by microorganism metabolism in the oral cavity. For example, lactate can be metabolized to acetate and propionate by *H. parainfluenzae*, *N. sicca*, *Eubacterium*, *Propionibacterium*, *Arachnia* and *Veillonella* bacterial species [21,51]. Trimethylamine also has a dietary origin [47]. The origin of methanol in saliva is less clear. Methanol was reported to originate from inhalation of cigarette smoke [46,48] or by food intake, mainly fruits and alcoholic drinks. However, a secretion of methanol by the parotid glands is possible [48] and hence variations in

its concentration upon exercise may be a marker of a different gland activity. Another metabolite that we found to strongly decrease with the exercise is O-phosphocholine. Phosphocholine is an intermediary in the synthesis of phosphatidylcholine, a major constituent of the phospholipid monolayer part of lipoproteins. A decrease in phosphocholine and choline in lipids in plasma in mice that perform spontaneous exercise was observed [34]. These changes can be accompanied by a parallel variation in circulating cholesterol or in lipoprotein composition, but this information is missing. On the other hand, the decrease in phoshocholine in saliva can be related to an enhancement in phospholipid metabolism caused by the exercise [34], reaching levels that are observed in the younger population [17]. Putrescine increase in saliva is not unexpected, in view of the known effect of polyamine accumulation in the skeletal muscle after physical exercise [53]. This study found that endurance and resistance exercise provoked a three- and fourfold increase in serum testosterone levels, respectively, that was followed a few hours later by an increase in polyamine synthetic rate. Moreover, the fact that testosterone levels rose before the polyamines, strongly suggested that testosterone was responsible for triggering polyamine synthesis. This effect may represent one of the physiological mechanisms whereby testosterone promotes muscle anabolism. On the other hand, exhaustive exercise causes greater oxidative stress in slow muscle fibers due to their greater reliance of oxidative energy production. High increases in oxygen consumption, as seen during exercise, help the production of Reactive Oxygen Species (ROS) that can severely damage muscle. Muscle injury, in turn, stimulates the local proliferation of neutrophils and macrophages that literally eat away damaged muscle tissue in preparation for subsequent regeneration. Therefore, polyamine expression is very important in aiding slow muscle fibers recover from exhaustive exercise. A second source of polyamines is food, and this exogenous pool is also used by skeletal muscle [5]. Saliva content in putrescine may reflect both the endogenous and the exogenous contributions. It is thought that polyamines originating from food may substitute for endogenous polyamines if physical exercise is more prolonged and/or systemic.

Salivary amino acid concentrations seem to be in general unrelated to short-term changes in plasma concentrations, with the only exceptions of threonine and valine [10]. Remarkably, high concentrations of D-alanine, D-proline and D-aspartate were found in saliva [35]. A racemase of an aminotransferase located in the salivary glands are thought to produce these amino acids [35]. This fact can, at least in part, explain the lack of correlation with the plasma concentration of amino acids. We have detected 15 amino acid types, including taurine. NMR signals for the two enantiomers are coincident, so concentrations reported in the table are to be considered as the total amounts of L- and D-enantiomers. The presence of relatively high concentrations of D-amino acids could mask the metabolic changes in the salivary amino acid levels triggered by the physical effort. However, amino acids levels were already found to slightly increase in saliva with physical exercise [36]. Consistently, we found glycine and proline among the most relevant metabolites that increase their concentration in post-SSG samples.

An interesting observation is the decreased level of taurine that we find in the post-exercise samples. Taurine is considered a natural amino acid that may act as a powerful membrane stabilizer [16]. The effects of taurine related to physical activity have long been known [49,50,57], and a diminished concentration in rat skeletal muscles after exercise was also observed [33,60]. Furthermore, an appropriate level of intracellular taurine seems to be important to ensure muscle performance [18]. Taurine was also found to be decreased in blood in a group of mice that were spontaneous wheel runners, and was considered one of the markers of the metabolic effects of long-term exercise in tissues and organs [34]. A decrease of taurine was also found in athletes that covered a longer distance during the exercise. It is tempting to speculate that this reflects a higher resistance to fatigue and those players that are capable

of running longer distances keep taurine at higher levels. This is in line with the observation that taurine administration increases the running time to exhaustion in rats [60] and with the increase observed for taurine induced by training in human serum [39]. Taurine level in saliva is a potentially significant marker which needs further investigation to be correlated with physical effort.

5. Conclusions

Most of the metabolites that were found to change significantly their concentration in saliva after exercise are correlated with metabolic responses of muscle and may be linked to changes already observed in blood. Probably some extra discriminants we observed in saliva may be of microbial or dietary origin, namely acetate, methanol, trimethylamine and propionate. Noteworthy we identified metabolites that correlated with increasing distance covered by players during the SSGs. Altogether these results point to a potential use of saliva to follow metabolic changes during an athletic competition, and open the possibility of using this non-invasive biofluid for the study of athlete training state and performance. More studies are in progress to sustain this hypothesis.

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