Can salivary lactate be used as an anaerobic biomarker?

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Abstract

Background. ~~A non-invasive sampling method for the evaluation of athletes' anaerobic capacity may be useful. This study aims to evaluate whether salivary lactate can be used as an anaerobic biomarker.~~ Salivary lactate has been suggested as a non-invasive anaerobic biomarker in sport medicine for decades, yet has not been widely applied until now. This study aimed to explore possible issues related to its application and suggest directions for future method improvement.

Methods. A liquid chromatography - mass spectrometry (LC-MS) method~~, with~~ for the ~~relative standard deviation (RSD)~~determination of ~~19.70%,~~ salivary lactate was developed, validated and applied ~~to analyze lactate in saliva~~on samples collected from a group of professional athletes. The evaluation ~~criteria~~ included variation with mouth rinses, consistency at different sampling times, ~~variation~~change after trainings and association with blood lactate. One-way analysis of variance (ANOVA), intra-class correlation coefficients (ICC) and ~~RSD~~relative standard deviation (RSD) were used to evaluate data variances. Pearson correlation was applied to show correlation between salivary and blood lactate.

Results. The RSD of the LC-MS method was 19.70%. Salivary lactate concentration was affected by mouth rinse times before sampling (ANOVA p~~=~~ = 0.025, $\eta^2 = 0.40$, $1-\beta = 0.99$, ICC~~=~~ = 0.23, mean RSD of four sampling~~=~~ = 55.30%), and ~~reduced to bottom levels~~stabilized after mouth rinsing three times~~ of mouth rinses~~. The concentrations at resting state across three weeks were consistent at group level (ANOVA p~~=~~ = 0.~~572~~57, $\eta^2 = 0.03$, $1-\beta = 0.20$), but varied greatly ~~at individual levels~~individually (ICC~~=~~ = 0.22, mean RSD~~=~~ = 56.16%). ~~Notwithstanding the poor reproducibility of resting state, salivary~~Salivary lactate level ~~was found~~ significantly increased after treadmill and cycle ergometer trainings (ANOVA p~~=~~ = 0.0002, $\eta^2 = 0.46$, $1-\beta = 0.9999$ and ANOVA p = 0.0019, $\eta^2 = 0.40$, $1-\beta = 0.9993$, respectively), and displayed positive correlation with blood lactate concentration (r~~=~~ = 0.61, p~~=~~ = 0.0004, $1-\beta = 0.9596$).

Discussion. Salivary lactate ~~is~~ was found to be a potential anaerobic biomarker~~, but a~~. However, reproducible ~~sampling method needs~~methods for sample collection and analysis, as well as more knowledge on the secretion mechanism and pattern of salivary lactate are required to ~~be developed in advance~~make it a practical anaerobic biomarker.

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Introduction

Objective and timely evaluation of ~~athletes'~~an athlete's anaerobic capacity is of great significance in sports medicine ~~(Noordhof et al., 2010).~~(Noordhof et al., 2010). Biomarkers are substances in biospecimen that objectively reflect the physiological and pathological processes within the body. Blood lactate, whose ~~level~~concentration increases with the load of anaerobic exercise, is currently regarded as ~~a~~the "gold standard" a ~~to evaluate athletes' aerobic capacities (Zagatto et al., 2019).~~anaerobic biomarker (Billat, 1996). The measurement of blood lactate has been simplified with portable lactate analyzers ~~(EKF Diagnostics, 2022). Although the test only takes 0.2 μL of~~ (EKF Diagnostics, 2022). However, blood sampling ~~sample, it~~test is ~~after all~~ an invasive test, which brings pain, stress, and risk of infection~~.~~ (Segura et al., 1996). Therefore, a non-invasive test may be welcome if it has equivalent ~~value~~ accuracy in evaluating ~~the athletes'~~an athlete's anaerobic capacity.

~~Urine and saliva are two common non-invasive biological samples in clinical tests (Prasad et al., 2016). Urine samples reflect the metabolism in the body during a period of time ranging from a few minutes to a few hours, and the concentrations of urinary solutes are affect by multiple factors such as diet, water intake, sweating and breathing. Hence, urine samples are more often used in qualitative (positive/negative) analysis, such as doping tests. Besides, the collection of urine sample is often restricted by the site (for privacy considerations) and time (for urine accumulation). Unlike urine, saliva is continuously produced by the salivary glands and can be collected anytime, anywhere. Therefore, saliva samples may be useful in evaluating the athletes' metabolic status on daily basis.~~

~~In this study, we evaluated whether salivary lactate can be used as an anaerobic biomarker in short-time high-intensity trainings. The study was conducted on professional athletes. Since lactate analyzers are designed for whole blood rather than saliva samples (EKF Diagnostics, 2022), we developed and validated a liquid chromatography - mass spectrometry (LC-MS) method for the analysis of saliva samples in this study.~~

Saliva is a type of non-invasive biological sample that can be easily collected. Positive correlation between salivary lactate and blood lactate (Mendez et al. 1976; Ohkuwa et al., 1995; Santos et al., 2006; Bocanegra et al., 2012; Tekus et al., 2012), as well as elevated salivary lactate level after intensive physical exercises (Bardon et al., 1983; Ohkuwa et al., 1995; Santos et al., 2006; Tekus et al., 2012; Volodchenko et al., 2019) were observed in a number of studies.

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80 In 1996, Segura et al concluded that salivary lactate can be used as an alternative to blood lactate
81 after a series of validation work (Segura et al., 1996). In the following year, Calvo et al (1997)
82 further introduced the concept of salivary lactate threshold, and displayed high correlation
83 between blood lactate threshold and salivary lactate threshold ($r = 0.93$, $p < 0.001$). Despite these
84 promising data generated decades ago, salivary lactate is still not widely applied in sport
85 medicine nowadays. In this study, we aimed to explore what might have prevented salivary
86 lactate from being used in practice.
87 Specifically, factors that potentially affect the analysis of salivary lactate, including analytical
88 method, mouth rinses before sampling, and data consistency at different sampling days were
89 evaluated. Elevation of salivary lactate during and after two types of short-time high-intensity
90 trainings, and its correlation with blood lactate were also verified.
91 A customized liquid chromatography - mass spectrometry (LC-MS) method was developed and
92 validated for the analysis of salivary lactate. This method was chosen for its high throughput. In
93 fact, we also simultaneously measured 23 other potential salivary biomarkers of physical
94 activities, including creatinine, uric acid, urea, cortisol, testosterone, choline, 5-hydroxyindole
95 acetic acid, homovanillic acid and 15 essential amino acids together with lactate, with the hope
96 of discovering new salivary biomarkers in sports. However, these 23 compounds were either
97 undetected or unchanged after the exercise trainings, and hence the data were not shown in this
98 report.
99

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100 **Materials & Methods**
101 *Samples*
102 The participants were professional track and field athletes (mainly sprinters) who had been
103 engaged in training for three years or longer at a training center where the study was conducted.
104 Verbal informed consent from the athletes and their coaches were obtained prior to sampling.
105 ~~*Sample collection and analysis*~~
106 All the participants fulfilled the following criteria: (1) they were in good health, without sport
107 injuries or bleeding wounds in the mouths, nor underwent dental surgery in the last three months;
108 (2) they did not smoke, drink alcohol, or participate in strenuous physical activities 24 hours
109 prior to the study, and (3) they did not eat food or chew gum two hours prior to sampling. The
110 study was conformed to the Declaration of Helsinki and approved by the ethics committee of the

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111 Stomatological Hospital of Chongqing Medical University, with the approval number of
112 2021(061).

113 To obtain a saliva sample, a medical cotton ball weighing approximately 0.3g was chewed for
114 one minute, and spitted into a 10 ml centrifuge tube. The tube was immediately sealed and
115 temporarily stored in an ice box for no more than 2 hours before transported to the laboratory for
116 storageand stored at −‒20 °C.

117 The evaluation was consisted of five parts: (1) validation of method accuracy and precision, (2)
118 the influence of mouth rinses, (3) consistency at resting state, (4) variation during training, and
119 (5) correlation with blood lactate. Accordingly, the athlete participants were asked to provide
120 saliva samples as follows: (1) at resting state, before and after 1, 2, and 3 times of mouth rinses
121 with 10 ml of drinking water (4 females and 6 males); (2) at resting state, before breakfast
122 (approximately 7 am) in Monday mornings of three consecutive weeks and after three times of
123 mouth rinses (11 females and 9 males); and (3) five minutes before, immediately after and five
124 minutes after acute high-intensity treadmill training with increasing load or cycle ergometer
125 trainings (8 females and 8 males) after three times of mouth rinses.

126 As many athletes in the training center as possible were recruited into this study. Sample sizes
127 required were calculated based on the effect sizes of previous similar studies (Bardon et al.,
128 1983; Ohkuwa et al., 1995; Segura et al., 1996; Calvo et al., 1997; Bocanegra et al., 2012; Tekus
129 et al., 2012), the significant level of 0.05 and the power of 0.8, which were 6 (95% CI: 0 – 13)
130 for paired group comparisons and 11 (95% CI: 3 – 20) for Pearson correlation analysis,
131 respectively. Hence, the actual sample sizes were larger than the required sample sizes.

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132 The treadmill and cycle ergometer trainings started at approximately 9 am on two Thursday
133 mornings with a one-month interval. The athletes provided their information including sex, age,
134 height and body weight, and sat quietly for 20 minutes prior to the trainings. The acute high-
135 intensity treadmill training with increasing load was conducted with a Metalyzer 3B (Cortex,
136 Germany) spiroergometry system and a RUN 7410 treadmill (RUNNER, Italy) preheated for 30
137 min. The training included five-minute warm-up at 8 km/h and 0° grade, 8 min load-increasing
138 stage with increasing speed and slope every minute based on the participants' individual
139 capacities, and three-minute recovery stage at 5–7 km/h, 0° grade. The participants were
140 equipped with heart rate monitors and accompanied by a team doctor. They were encouraged to

141 complete the training but allowed to stop anytime when exhausted. The exhaustion criteria were
142 set as the heart rate remaining at no less than 180 beats/min for two minutes, and the rate of
143 perceived exertion (RPE) scale (Borg, 1982) reaching 18–20. The cycle ergometer training was
144 similar to the Wingate test, which was ~~based~~completed on a Monark 874E weight cycle
145 ergometer. Each participant was asked to ride on the cycle ergometer for 3×30 seconds with 3
146 min intervals at the load of 0.075 kg/kg body weight. Blood lactate was measured before and
147 immediately after the cycle ergometer training using a Lactate Scout Lactate analyzer (EKF
148 Diagnostics, UK), and transformed from mmol/L to µg/ml. Since the actual sample size was
149 more than double of the required sample size, no blood lactate test was conducted during the
150 treadmill training to reduce the participants' stress.

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151 *Instruments and procedures*

152 Prior to analysis, the cotton ball soaked with saliva was defrosted at room temperature and
153 transferred into a 4 ml Eppendorf tube with a small hole drilled at the bottom. The 4 ml tube was
154 then put into a clean 10 ml Eppendorf tube whose cap was cut off. The inner diameter of the 10
155 ml Eppendorf tube was just between the outer diameters of the body and edge of the 4 ml
156 Eppendorf tube, enabling the 4 ml tube to be stuck at the top of the 10 ml tube. After that, the 10
157 ml tube was centrifuged with an Eppendorf Centrifuge 5920 R (Hamburg, Germany) at 4 °C and
158 3000 rpm for 3 min~~. The 4 ml Eppendorf tube and~~ , so that the saliva in the cotton ~~were discard,~~
159 ~~and~~ball was collected in the 10 ml Eppendorf tube. 100 µl of the saliva sample ~~in the 10 ml~~
160 ~~Eppendorf tube~~ was transfer ~~to~~into a 1.5 ml Eppendorf tube, and vortex-mixed with 20 µl of 1
161 µg/ml internal standard 2-Cl-phenylalanine. Then, 1 ml of acetonitrile was added and the mixture
162 vortexed again. After that, the 1.5 ml centrifuge tube was centrifuged with an Eppendorf
163 Centrifuge 5427 R (Hamburg, Germany) at 4 °C and 10,000 rpm for 10 min. The supernatant
164 was transferred to a new 1.5 ml Eppendorf tube, dried with a Labconco CentriVap vacuum dryer
165 (MO, USA) at 40 °C, and then reconstituted in 100 µl of 5% methanol. The solution was
166 centrifuged again at 4 °C, 10,000 rpm for 5 min and 80 µl of the supernatant was transfer to an
167 LC-MS vial for injection. Calibration samples were prepared in the same way (starting from
168 internal standard addition) as the saliva samples. A pool sample was prepared by mixing 100 µl
169 aliquots of all the test samples and used in method validation.

170 The LC-MS analysis was performed using ~~the~~an Agilent 1290-6495C Ultra High Performance
171 Liquid Chromatography-Triple Quadrupole Tandem Mass Spectrometry (CA, USA). LC
172 ~~separations were~~separation was performed using a Waters HSS T3 (100×2.1 mm, 1.7 µm)
173 column with a Waters ACQUITY UPLC HSS T3 VanGuard (2.1×5 mm, 1.8 µm) pre-column.
174 Mobile phase A was aqueous solution of 0.1% formic acid, and mobile phase B was acetonitrile.
175 The gradients were: 0–0.5min: 5% B, 0.5–1min: 5-50% B, 1–7.5min: 50% B, 7.5–8min: 50–5%
176 B, 8–10min: 5% B. The flow rate was 0.3 ml/min, the injection volume was 2 µl, and the column
177 temperature was 40 °C. The ion source parameters for the mass spectrometry were: Gas Temp:
178 200 °C, Gas Flow: 14 L/min, Nebulizer: 35 psi, Sheath Gas Temp: 300 °C, Sheath Gas flow: 11
179 L/min, Capillary: –3000 V, Nozzle Voltage: 500 V, iFunnel H: –150 V, L: –60 V. Multiple
180 reaction monitoring (MRM) mode was used to analyze lactate and the internal standard 2-Cl-
181 phenylalanine. Q1(m/z), Q3(m/z) and CV(eV) were set as 89.0, 43.2 and 13, respectively, for
182 lactate~~,~~; and 198.0, 181.0 and 9, respectively, for 2-Cl-phenylalanine. Lactate standard was
183 purchased from Bidepharm (Shanghai, China), and 2-Cl-phenylalanine was from
184 MedChemExpress (Shanghai, China).

185 The method was validated for accuracy and precision. Accuracy was determined by adding
186 ~~known amount~~10 µl/ml of lactate into a ~~quality control saliva~~pool sample and calculating the
187 recovery, ~~namely~~which was the ratio of measured concentration increase to the theoretical
188 concentration increase~~.~~ (10 µl/ml). Precision was determined by analyzing seven 100 µl sub-
189 aliquots of the ~~quality control~~pool sample twice, and ~~calculate~~calculating the relative standard
190 deviation (RSD) of the fourteen measured concentrations. ~~It was calculated that the method~~
191 ~~recovery was 95.9%, and the method RSD was 19.7%.~~

192 ~~*Participants*~~

193 ~~Participates were professional track and field athletes (mainly sprinters) who had been engaged~~
194 ~~in training for three years or longer. Verbal informed consent from the athletes and their coaches~~
195 ~~were obtained prior to sampling. All the participants fulfilled the following criteria: (1) they were~~
196 ~~in good health, without sport injuries or bleeding wounds in the mouth, and did not undergo~~
197 ~~dental surgery in the last three months; (2) they did not smoke, drink alcohol, or participate in~~
198 ~~strenuous physical activities 24 hours prior to the study, and (3) they did not eat food or chew~~
199 ~~gum two hours prior to sampling. The study was compliant to the Declaration of Helsinki and~~

~~has been approved by the ethics committee of the College of Stomatology, Chongqing Medical University, with the approval number 2021(061).~~

~~The evaluation of salivary lactate was consisted of four parts: (1) the influence of mouth rinse, (2) consistency at resting state, (3) variation after training and (4) correlation with blood lactate. Accordingly, the athlete participants were asked to provide saliva samples as follows: (1) at resting state, before and after 1, 2, and 3 times of mouth rinse with 10 ml of drinking water (4 females and 6 males); (2) at resting state, before breakfast in Monday mornings of three consecutive weeks (11 females and 9 males); and (3) five minutes before, immediately after and five minutes after acute high-intensity treadmill training with increasing load (8 females and 8 males) and bicycle ergometer trainings (8 females and 9 males).~~

~~*Training programs*~~

~~The trainings for the third and fourth parts started after the athletes provided their basic information including sex, age, height and body weight, and sit quietly for 20 minutes. The acute high-intensity treadmill training with increasing load was conducted with a Metalyzer 3B (Cortex, Germany) spiroergometry system and a RUN 7410 treadmill (RUNNER, Italy) preheated for 30 min. The treadmill training included five-minute warm-up at 8 km/h and 0°, 8 min load-increasing stage with increasing speed and slope every minute based on the participants' individual capacities, and three-minute recovery stage at 5–7 km/h, 0°. The participants were equipped with heart rate monitors and accompanied with a team doctor. They were encouraged to complete the training but can stop anytime when exhausted. The exhaustion criteria were set as the heart rate remaining at no less than 180 beats/min for two minutes, and the rate of perceived exertion (RPE) scale (Borg, 1982) reaching 18–20. The bicycle ergometer training was similar to Wingate test, which was based on a Monark 874E weight bicycle ergometer. Each participant was asked to ride on the bicycle ergometer for 3×30 seconds with 3 min intervals at the load of 0.075 kg/kg body weight. Blood lactate was measured before and immediately after the bicycle ergometer training using a Lactate Scout Lactate analyzer (EKF Diagnostics, UK), and transformed from mmol/L to μg/ml.~~

~~*Data analysis*~~

Each set of data is expressed as mean ± standard deviation~~.~~ (SD). Paired one-way analysis of variance (ANOVA) and Tukey' post-hoc multiple comparisons test were used to compare data

230 among different time points of the same group of participants., and multiplicity adjusted P value
231 for each comparison with family-wise significance and confidence level set at 0.05 was reported.
232 Intra-class correlation coefficients (ICC) and relative standard deviation (RSD)RSD of multiple
233 sampling times were used to evaluate data variances of each participant. Pearson correlation
234 coefficient (r) was calculated for salivary lactate and blood lactate of the same
235 participantparticipants at the same time point. The level of significance for all tests
236 was $p < 0.05$.points. Effect size and power were calculated following ANOVA and correlation
237 analyses. ANOVA, RSD and Pearson correlation were calculated using GraphPad Prism 8 for
238 mac (GraphPad Software, Boston, MA, USA), while ICC was calculated with IBM SPSS
239 Statistics software, version 20.0.0 (IBM Corporation, Armonk, NY, USA). Effect sizes of
240 ANOVA (η^2) were calculated from ANOVA F values, using the equation $\eta^2 = F \times (k-1) / [F \times$
241 $(k-1) + (n-k)]$ (Cohen, 1988), while thatose of Tukey's post-hoc tests (Cohen's d) and t-tests
242 wasere calculated with R for Mac OS X GUI 1.70 (R Core Team, 2020) and package "esc"
243 (Lüdecke, 2019). Power ($1-\beta$) and sample sizes of all analyses were calculated with R software
244 and package "pwr" (Champely et al., 2020).

245

246 **Results**

247 We first investigated the effect of mouth rinse times (0, 1, 2, 3) on the salivary lactate
248 concentrations. It was calculated that the method accuracy was 95.9% and the RSD was 19.7%.
249 Using this method, it was found that the times of mouth rinse had rinses (0, 1, 2, 3) significant
250 effects onaffected salivary lactate levels (Fig. 1,). ANOVA p= was 0.00250025, effect size (η^2)
251 was 0.40, power ($1-\beta$) was 0.99, ICC= was 0.23, and mean RSD of four sampling= was
252 55.30%), and the%. The average concentration decrease with the increaseincreasing times of
253 mouth rinse timesrinses. After three times of mouth rinserinses, the salivary lactate concentration
254 was significantly lower than that without mouth rinse and that after one rinse, so three times of
255 mouth rinses prior to sampling was applied in the following studies.evaluations.

256 We then investigated whether the salivary lactate concentration was consistent at different
257 sampling timedays via analyzing samples collected once a week (Fig. 2). ANOVA showed that
258 the average concentration of the 20 participants did not differ from week to week (p= = 0.57).,
259 effect size (η^2) = 0.03, power ($1-\beta$) = 0.20). However, marked individual variance was observed,

260 which was shown by ~~the broken lines in the figure,~~ an ICC ~~value~~ of 0.22 and ~~an~~a mean RSD of
261 56.16%.

262 ~~We then explored if the~~Next, we verified whether salivary lactate increased after training.
263 Despite large inter-individual variances, it was clear that the salivary lactate levels significantly
264 increased after the treadmill (Fig. 3A) and ~~bicycle~~cycle ergometer (Fig. 3B) trainings. ANOVA p
265 value was 0.0002, effect size (η^2) was 0.46, and power (1–β) was 0.9999 for treadmill trainings;
266 while ANOVA p value was 0.0019, effect size (η^2) was 0.40, and power (1–β) was 0.9993 for
267 cycle ergometer trainings. There was no significant difference between immediately and 5 min
268 after training.

269 ~~For practical reasons, we only obtained~~ One participant missed the blood lactate ~~concentration~~
270 ~~before~~test, and ~~immediately after the bicycle~~ another participant missed one time point of the
271 blood lactate test on the day of cycle ergometer training ~~from 15 athletes.~~. Consequently, 14 out
272 of the 16 participants underwent the blood lactate before and immediately after the cycle
273 ergometer training, and one out of the 16 participants only underwent blood lactate test at one
274 time point, which provided a total of 14×2+1=29 pairs of blood-salivary lactate data ~~were~~
275 ~~obtained~~. The ~~correlation test~~29 pairs of salivary-blood lactate data showed significant and
276 positive correlation ~~between salivary lactate and blood lactate concentrations.~~(Fig. 4). The 95%
277 confidence interval of correlation coefficient was 0.32- – 0.80 ~~(Fig. 4). For all tests in the study,~~
278 ~~no significant~~. Significant difference between male and female participants was observed in none
279 of the tests conducted in this study.

280

281 ~~**Discussion**~~

282 ~~In this study, we evaluated whether salivary lactate can be used as an anaerobic biomarker. A~~
283 ~~LC-MS method with an RSD of 19.70% was first developed to analyze salivary lactate. Using~~
284 ~~the method, an RSD of 55.30% was generated from saliva samples collected after different~~
285 ~~mouth rinse times. This indicated that the mouth rinse had significant impact on salivary lactate,~~
286 ~~which was also supported by the ANOVA and ICC data. Previous studies applied no mouth rinse~~
287 ~~(Hough et al., 2013), one mouth rinse (Liu et al., 2017) or tooth brushing (Volodchenko et al.,~~
288 ~~2019) before sample donation. Based on our data, three times of mouth rinses prior to sample~~
289 ~~collection was sufficient to stabilize salivary lactate, which was applied in the following studies.~~

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290 Less mouth rinses may not be sufficient, and tooth brushing may not be practical for quick
291 sampling.

292 The RSD of weekly variation was also as high as 56.16%. This meant even the participants
293 rinsed their mouth for three times prior to sampling, the salivary lactate concentration was still
294 inconsistent. Saliva is secreted at the rate of approximately 0.5 ml/min by three major pairs of
295 salivary glands and a number of minor mucous glands (Dawes, 1974). The salivary glands are
296 tubuloalveolar structures which contain acini and a branching system, controlled by cholinergic
297 nerves and sympathetic adrenergic nerves, and supplied by arteries (Chicharro et al., 1998). Only
298 a small proportion of blood lactate is secreted into the saliva, as shown in Fig. 4. In addition,
299 microbes in the oral cavity such as *Lactobacillus* produce lactate (Selle & Klaenhammer, 2013).
300 Hence, the concentration of salivary lactate is determined by various factors, including the
301 structure of salivary glands, nervous system (including stimuli), blood supply and oral
302 microbiome, which may vary from person to person and from time to time. Indeed, we noticed
303 the volumes of saliva samples differed greatly among the participants when all of them were
304 given the same one minute to chew the cotton balls. Unstimulated sampling methods such as
305 passive drool were also used in saliva studies (Kaufman & Lamster, 2002), and were reported to
306 produce more consistent data (Kawanishi et al., 2019). However, these methods take longer than
307 stimulated ones, and was not investigated in this study due to inconvenience for the participants.

308 Despite the large variances of salivary lactate concentration at resting state, it was interesting to
309 see that salivary lactate increased significantly during the two forms of short-term high-intensity
310 exercises (Fig. 3), and there was significant positive correlation between salivary and blood
311 lactate (Fig. 4). A number of previous studies have investigated salivary lactate in different forms
312 of exercise and the association with blood lactate. The salivary lactate was found correlated with
313 the blood lactate after maximum intensity exercise (Mendez et al., 1976; Segura et al., 1996;
314 Tekus et al., 2012), 400-m run (Ohkuwa et al., 1995) and marathon (Santos et al., 2006), but
315 could not be used as determinants of anaerobic threshold in a maximal graded exercise test on
316 cycle and ergometer (Zagatto et al., 2004). These studies and the present study together suggest
317 that salivary lactate increase during different forms of exercise and correlated well with blood
318 lactate, but is currently not as reliable as blood lactate, possibly due to the influencing factors of
319 saliva secretion. In addition, there was no significant difference in salivary lactate between

~~immediately and 5 min after training, suggesting the saliva samples may be taken within five minutes after training.~~

Discussion

In this study, we evaluated whether salivary lactate can be used as an anaerobic biomarker. Significant increase of salivary lactate during and after two forms of short-time high-intensity exercises (Fig. 3), as well as positive correlation between salivary and blood lactate (Fig. 4), were observed. These observations were consistent with previous studies (Mendez et al., 1976; Bardon et al., 1983; Ohkuwa et al., 1995; Santos et al., 2006; Bocanegra et al., 2012; Tekus et al., 2012; Volodchenko et al., 2019). However, the correlation was overall moderate, which did not support the replacement of blood lactate with salivary lactate. Based on the displayed data, this may be due to three reasons.

Firstly, the production of saliva lactate was influenced by multiple factors. Even with the same sampling procedures, at the same time of day and day of week, saliva samples were found to vary, with the RSD as high as 56.16% (Fig. 2). It should be noted that this RSD may be overestimated, because the samples were obtained by the participants ~~themselves~~ without supervision, and hence the sampling instructions m~~ay~~ight not be strictly followed. Chicharro et al (1998) summarized that the saliva composition is regulated by nervous system, salivary flow rate, stimuli and other factors such as age, circadian rhythm, circannual rhythms and reflex. A recent study suggested that body fat, body water content and skeletal muscle mass index are associated with salivary lactate levels after exercise (Okano et al., 2022). Each of these factors may vary among individuals and change ~~with~~over time. Moreover, microbes in the oral cavity such as *Lactobacillus* produce lactate (Selle & Klaenhammer, 2013), which can be disolved in saliva.

Secondly, saliva collection methods may affect lactate concentration. Saliva is secreted at the rate of approximately 0.5 ml/min by three major pairs of salivary glands and a number of minor mucous glands, and the flow rate may increase with stimuli (Dawes, 1974). ~~Hence~~Thus, saliva samples can be obtained either in an unstimulated way, such as passively drool (Kawanishi et al., 2019), or a stimulated way such as that in this study. Unstimulated methods are reported to produce more consistent data but take~~s~~ longer to accumulate the required volume, and stimulated

methods take less time but ~~the~~ generate~~d~~ ~~data are~~ less consistent data (Kawanishi et al., 2019). We chose stimulated method in this study because the participants may have dry mouths after the trainings, so unstimulated methods may take too long. The RSD of 55.30% and ICC of 0.23% of salivary lactate were observed in the saliva samples collected after different mouth rinse times (Fig. 1). This indicated that sampling method and preparation prior to sampling had significant impact on salivary lactate. Our data also suggested that three times of mouth rinses prior to sample collection was able to stabilize salivary lactate. Previous studies applied different strategies for mouth cleaning prior to sampling, such as no mouth rinse (Hough et al., 2013), one mouth rinse (Liu et al., 2017) or tooth brushing (Volodchenko et al., 2019), which indicated that a standardized sampling protocol may be needed for future studies.

Finally, the precision of the analytical method was not satisfactory. In this study, an LC-MS method with an RSD of 19.70% was developed to analyze salivary lactate. In contrast, enzymatic methods for salivary lactate measurements normally produce an RSD of approximately 2% or less (Segura et al., 1996; Artisan Technology Group, 2000). This ~~was probably becaus~~may be due to ~~-~~the saliva samples ~~had~~having to be prepared for LC-MS analysis, and the process included multiple steps such as pipetting, centrifugation, evaporation and reconstitution. Besides, no deuterated internal standard of lactate was applied in this method. All of these factors may have contributed to the relatively large RSD of the LC-MS method. In the future, simplified sample preparation procedures and application of a deuterated internal standard are recommended in the LC-MS analysis of salivary lactate.

Conclusions

To summarize, salivary lactate is a promising biomarker for ~~evaluating~~ the evaluation of anaerobic capacities ~~in short-term high-intensity trainings~~, but ~~issues related to its inconsistent secretion from~~reliable sampling and analytical methods, together with comprehensive knowledge on the ~~salivary glands need to be resolved. Future studies is required to investigate the factors that affects~~mechanism and pattern of salivary lactate secretion ~~and establish a method to stabilize~~, are crucial elements to make it practical.

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