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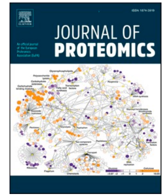

Salivary proteomics and metabolic responses to resistance training with and without blood flow restriction in young adults

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ABSTRACT

Blood flow restriction (BFR) has been applied as alternative strategy to reproduce the effects of conventional high-load intensity training (HI) while training with low-loads (LI). Therefore, the aim of this research was to evaluate the salivary proteomic and metabolic responses to different resistance training in young adults.Ten participants were selected and underwent to training with LI (30 %1RM – one repetition maximum) plus BFR vs. HI (70 %1RM) without BFR, at 48 h-interval. Stimulated saliva was collected before and immediately after the sessions, while breath-by-breath oxygen uptake (VO_2) was measured during and after each session. Arterial blood samples for lactate concentration measurement (in $EqO_{2[La]}$) were taken at 1st minute of resting between each exercise. For HI, there was an increase in two actin cytoplasmic isoforms and two immunoglobulin isoforms and a decrease of six hemoglobin isoforms. For LI-BFR, there was an increase in two hemoglobin isoforms, and the same immunoglobulin isoforms (*t*-test, $p < 0.05$). No differences were significant between HI and LI + BRF training regarding the total energy demand (in mlO_2), absolute oxygen values (mlO_2) for oxidative response (VO_{2ON}), glycolytic ($EqO_{2[La]}$), and oxygen debt (VO_{2OFF}) ($p > 0.05$). Both HI and LI + BFR protocols modulated immune system activity and exhibited divergent hemoglobin patterns.

Significance: The current study, which identified protein from saliva samples, a non-invasive method, and analyzed physiological markers, enabled the comparison of different resistance exercise protocols. Although no significant differences were observed in the metabolic responses to each protocol, which highlights the potential of LI + BRF to reproduce a high-intensity training stimulus, the changes in salivary protein profiles indicate specific functional adaptations that may become evident over time.

1. Introduction

Salivary secretion is regulated by the autonomic nervous system; so sympathetic stimuli, such as those induced by physical exercise, can increase the production of a series of proteins as amylase [1,2]. Therefore, different training protocols can lead to the presence of different proteins as shown by Ventura et al. (2022) [3], who found Epoxide hydrolase 3 in saliva in individuals with Parkinson's disease after practicing interval physical exercise. Another study, by Missaglia et al. (2023) [4] found changes in the protein irisin in saliva, induced by exercise. Specifically involving the molecular responses to resistance training (RT), the study of Bang (2023) [5] showed that RT practice lasting 12 weeks increased the levels of BDNF (Brain-derived neurotrophic factor) and NGF (Nerve Growth Factor) in obese middle-aged women, which are molecules responsible for the plasticity of the nervous system, highlighting the neuroprotective role of RT practice.

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On the other hand, studying a single protein may be insufficient to understand the benefits of physical exercise, and more extensive studies on these molecules and their analysis of physiological exercise indices are needed [6]. Thus, studies related to the identification of protein biomarkers from saliva samples, which have a non-invasive, painless and easy way of sampling, are desirable [7–9]. Previous study using proteomic analysis in exercise, showed that high-load intensity resistance training (HI) increased the presence of proteins associated with muscle metabolism, oxidative stress, and systemic benefits such as anti-atherosclerotic effects [10]. In contrast, low-load intensity training (LI) with blood flow restriction (LI + BFR) increased presence of proteins related to angiogenesis, tissue remodeling, and neuroprotection [10]. These findings are advantageous, as proteomic analysis provides a comprehensive overview of protein presence in the sample, based on pre-established comparisons [10]. However, this previous study gave only a preliminary insight since the low sample size and the lack of physiological variables constrained a comprehensive analysis.

Regarding physiological parameters, the intensity of resistance training directly influences serum lactate concentration [11]. Higher lactate blood values have been reported at 70 %1RM without BFR (one-repetition maximum) compared to 50 %1RM without BFR during a training session involving full-body movements [12]. Despite RT planed with HI have been associated with higher values of blood lactate when compared to training LI, the RT planed with LI + BFR might likely produce high stimuli on blood lactate as HI [13,14]. The assumptions of similarities in the metabolic responses when training with HI and LI + BFF were supported by mechanical stress during HI and the stimulation of fast-twitch fiber, which may also happen while exercising with LI + BFR due to the ischemic condition [15].

While the information on metabolic differences between HI and LI + BFR protocols is scarce, a recent study evidenced that metabolism during RT (i.e., the amount of O_2 consumed) is higher in LI than for HI, which might be due to exercise duration differences [16]. Indeed, for practical purposes, training with LI + BFR has been considered an alternative to traditional HI training when the goal is to perform exercises that demand a high level of exertion, which has often been associated with improvements in muscle strength and mass, since greater metabolic stimulation is considered a reasonable signal for the activation of anabolic metabolism [17]. However, there is a lack of information about proteins signalization during LI + BFR training.

In the current study, the metabolic demand during different RT protocols were assessed from the responses of oxygen uptake (VO_2) during exercise and rest, in addition to the oxygen equivalent (EqO_2) of blood lactate concentrations, and to changes in the salivary proteome. Therefore, the current study aimed to compare HI vs LI + BFR protocols by analyzing salivary proteomics and metabolic responses to explore whether the physiological adjustments during each RT protocol correspond to similar or different molecular stimuli, and therefore suggesting a similar or different adaptations to exercise.

2. Material and methods

2.1. Ethical aspects

This study was submitted to the Research Ethics Committee of São Paulo State University "Júlio de Mesquita Filho" as an amendment to the project "Energy Response in Resistance Exercise with Blood Flow Restriction," under approval number 3.572.379, CAAE no. 19824719.3.0000.5398, which was duly approved on September 12, 2019. The research participants received a detailed explanation of the study's objectives through the reading of the Clarification Letter and signed the Informed Consent Form. Additionally, they were provided with written instructions and schedules.

A total of 10 young male adults, aged 18 to 35 years, residing in Bauru-SP or surrounding areas, were selected for participation following broad dissemination through UNESP-Bauru's media channels. The participants should have good general and oral health, so they could not have ischemic diseases, diabetes, arrhythmias, hypertension, obesity (BMI $\geq$ 30 kg/m^2), smoking habits, active dental caries and periodontitis.

2.2. Resistance training protocols and metabolic assessments

The 1RM test was applied to assess the reference of maximal load in each exercise planned for both experimental training protocols. Test procedures followed previous recommendations [18]. Two different protocols were planned with eight exercises for both upper- and lower-limbs to be performed on different days (48 h apart) and in randomized order: (1) HI – 70 %1RM, 3-sets, 12-repetitions, 60 s of rest between sets and 120 s between exercises; and (2) LI + BFR – 30 %1RM, 3-sets, 15-repetitions, 30 s of rest between sets and 180 s between exercises. The exercises were performed following the same order in both protocols, being: (Ex.1) flat bench press, (Ex.2) seated row, (Ex.3) triceps curl, (Ex.4) bicep curl, (Ex.5) single leg extension, (Ex.6) prone leg curl machine, (Ex.7) horizontal leg press, and (Ex.8) rack calf raises. The protocols of training were planned according to the guidelines proposed for muscle mass and strength improvements involving intermediate individuals training with HI, and for resistance training with LI + BFR aiming to stimulate high-intensity physiological demand [14].

The blood lactate ($[La^-]$) was analyzed from blood samples (25 μL of capillary blood) of the earlobe, using heparinized and calibrated capillaries. The samples were stored in Eppendorf tubes with 50 μL of sodium fluoride (NaF at 1 %), and analyzed by an enzymatic method (Yellow Spring 2500-STAT). The samples were taken at rest (i.e., before starting the first exercise), immediately after the 3-sets of each exercise, and at 1, 3, 5, and 7 min during the recovery period of each protocol. At the end of each RT protocol, saliva was also collected for proteomic analysis. The ($[La^-]$) was determined in oxygen equivalent ($EqO_{2[La]}$ in mlO_2) from the values in $mmolxL^{-1}$, according to the Eq. (1) [19]:

$$EqO_{2[La]} = [(\beta \times \Delta[La^-]) \times BM] \tag{1}$$

where, β is the O_2 equivalent for each 1.0 $mmol \times L^{-1}$ $[La^-]$ of variation above the baseline value corresponding to ~3.0 $mlO_2 \times kg^{-1}$; $\Delta[La^-]$ is the variation of the $[La^-]$ above the resting value ($\Delta[La^-] = [La^-]_{peak}$ - $[La^-]_{rest}$); and BM is the whole-body mass in kg.

The breath-by-breath VO_2 was continuously sampled during each trial and recovery for 420 s, using a portable gas analyzer ($K4b^2$, Cosmed, Rome, Italy). The data were time aligned, followed by noises exclusion (coughing, sighing and sneezing), which were defined as three standard-deviation from the local mean of five breaths and, finally, the data were interpolated second-by-second [20]. During each RT protocol, the VO_2 demand was obtained from the net VO_2 (in $mlO_2 \times s^{-1}$) curve time integral (Eq. (2)) of each exercise set (VO_{2ON} in mlO_2), and during two-minute recovery phase after three sets of each exercise (VO_{2OFF} in mlO_2)

$$Cost\ O_2 = \left(\int_{x=i.ON}^{x=f.ON} VO_{2ON} \times t_{ON} - (VO_{2Base} \times t_{ON}) \right) + \left(\int_{x=i.OFF}^{x=f.OFF} VO_{2OFF} \times t_{OFF} - (VO_{2Base} \times t_{OFF}) \right) \tag{2}$$

where "t_{ON}" is the time duration of each set, "t_{OFF}" is the isotime of VO_2 analysis in recovery phase after the three sets of each exercise (120 s), and "VO_{2Base}" is the VO_2 measured sited at rest (ten minutes) previously protocol execution.

Thus, the total net VO_2 ($VO_{2T(net)l}$ in mlO_2) demanded for each RT protocol is the result of Eqs. (1) and (2), according to the Eq. (3):

$$VO_{2\text{Total}} = \left(\int_{x=i.ON}^{x=f.ON} VO_{2ON} \times t_{ON} - (VO_{2Base} \times t_{ON}) \right) + \left(\int_{x=i.OFF}^{x=f.OFF} VO_{2OFF} \times t_{OFF} - (VO_{2Base} \times t_{OFF}) \right) + EqO_{2[La]} \quad (3)$$

Thus, total gross VO_2 ($VO_{2T(gross)}$ in mlO_2) demanded for each RT protocol added the resting values of VO_2 and [La^-].

2.3. Saliva collection and preparation

Saliva was collected 10–15 min before and 10–15 min after the end of training during the morning period, as performed in a previous study by our research group [10]. All participants were instructed to perform oral hygiene and remain for 45 min without ingesting any type of food or liquid or using any object to chew. After this period, the participants had their mouths washed with 20 mL of distilled water and remained at rest for 10 to 15 min. Stimulated whole saliva, using Parafilm, was collected in a 50 mL Falcon tube immersed in ice for 5–10 min, to obtain at least 10 mL of saliva per participant. The supernatant was collected after centrifugation at 4500 xg for 15 min at 4 °C. The sample was stored in cryogenic tubes at −80 °C until the samples were processed for proteomic analysis [10].

The protein extraction protocol was based on a previous study [10]. For extraction, 1 mL of each collected sample was separated into tubes. The aliquots were diluted 1:1 with an extraction solution containing 6 M urea, 2 M thiourea and 50 mM NH_4HCO_3, pH 7.8. After dilution, the samples were shaken for 10 min at 4 °C, then placed in an ultrasonic bath for 5 min and then centrifuged at 14,000 xg at the same temperature for 10 min. This step was repeated twice more. After extraction, the samples were concentrated to 150 μL using Amicon Ultra-0.5 Centrifugal Filter Units with a 3 kDa MWCO (Molecular Weight Cutoff) (MilliporeSigma, Catalogue No. UFC8003). In this step, 1 μL of each sample was diluted in 149 μL of deionised water, and 150 μL of Bradford solution was added for protein quantification by Bradford method (Bio-Rad®, Hercules, CA, EUA) and 100 μg/protein was standardized for each sample prior to digestion for the differential expression analysis. This protein quantification was performed in duplicate on a 96-well plate and the analysis conducted by BioTek Gen5 software (Agilent) using spectrophotometry. To reduce the disulfide bridges, 5 mM dithiothreitol (DTT) was used for 40 min at 37 °C and, subsequently, 10 mM iodoacetamide was added for 30 min in the dark to prevent the cysteine residues from forming new disulfide bonds. For the digestion of proteins was used 2 % (*w*/w) trypsin (Thermo Scientific Pierce Trypsin Protease, Rockford, Illinois, USA) for 14 h at 37 °C. Then, 10 μL of 5 % trifluoroacetic acid was added.

The samples were purified and desalted using Spin C18 columns (Thermo Fisher Scientific®, Waltham, MA, USA) and 1 μL of each sample was used for peptide quantification (Pierce Quantitative Colorimetric Peptide Assay, 500 Assays, ThermoScientific™). The samples were resuspended in the solution containing 3 % acetonitrile and 0.1 % formic acid and subjected to analysis by mass spectrometer coupled to chromatography (Nano Liquid Chromatography Electron Spray Ionization Tandem Mass Spectrometry – LC-ESI-MS/MS, Waters, Wilmslow, United Kingdom).

2.4. Proteomic analysis

The analysis of typical peptides was performed on a nanoACQUITY UPLC system (Waters, Milford, MA, USA) coupled to a Xevo Q-TOF G2 mass spectrometer (Waters, Milford, MA, USA) controlled by MassLynx v.4.1 (Waters). Data collection was in data-independent acquisition mode (LC-MSE), and the mass range from 50 to 2000 *m/z* [10]. The ACQUITY UPLC nano system was equipped with an HSS T3 column (Acquity UPLC HSS T3 column 75 μm × 150 mm; 1.8 μm) (Waters, Milford, MA, USA), previously equilibrated with 7 % mobile phase B (100 % acetonitrile +0.1 % formic acid). The peptides were separated by a linear gradient from 7 to 85 % of mobile phase B for 70 min with a flow rate of 0.35 μL/min at 45 °C. The mass spectrometer was operated in positive ion mode, with 75 min of data acquisition time. All samples were analyzed individually, thus totaling 10 analyses for each group. The final protein quantification (after data acquisition) was normalized in silico using Enolase (Enolase MassPREP Digestion Standard Yeast Enolase, #186002325, Waters) added to the samples during the resuspension step, after preparation and before sending to the mass spectrometer, as the reference protein (Housekeeping). So the quantified intensity of all proteins in each sample was corrected based on the detected intensity of Enolase.

Data was then processed using ProteinLynx Global Server (PLGS) version 3.03 software (Waters, Milford, MA, USA). Proteins were identified through the software's ion counting algorithm by comparing the spectra against the *Homo sapiens* reviewed database from UniProt (UniProtKB/Swiss-Prot, available at http://www.uniprot.org/). The use of the human database precludes the identification of bacterial proteins that may be present in saliva. Each protein was examined based on its respective accession number, while redundant sequences, reverse matches, and fragments were excluded from analysis. The False Discovery Rate (FDR) value was applied in our results, and it was at maximum 4 %.

Proteomic data derived from the salivary samples were analyzed using the PLGS and a comprehensive summary of the dataset metrics was compiled for the entire cohort. On average, 9967 MS/MS spectra (HE ions) were acquired per sample. On average, 175,938 homologous peptides were detected, while 80,512.5 non-homologous peptides were identified. Protein identification revealed an average of 20.98 homologous proteins and 12.9 non-homologous proteins per sample. The average sequence coverage among the identified proteins was 34.99 %, reflecting reliable fragmentation and database alignment. To assess analytical depth, only proteins that were curated as "green" (high confidence) and supported by at least two matched peptides were considered. This resulted in an average analytical depth of 11.98 proteins per sample. All samples were analyzed once, with no technical or analytical replicates performed. This should be considered a limitation with regard to reproducibility metrics. However, stringent filtering criteria were applied throughout the analysis to enhance confidence in peptide and protein identification.

2.5. Quality control and normalization

To ensure instrument stability and retention time control, mass spectrometry calibration was performed weekly. In addition, we used a commercial standard from Thermo Fisher, Pierce™ HeLa Digest/PRTC Standard (Cat. A47996), injected regularly to monitor LC/MS system performance between runs and over time.

In terms of data normalization, protein quantification was performed using the Bradford method prior to desalination (C18 column) and padronized by 100 μg/protein, and peptide quantification was done after this procedure, before drying in SpeedVac. Furthermore, in silico normalization was used for the final quantification of proteins, based on the intensity of enolase detection (after data acquisition). The presence of PRTC peptides in the QC standard was used to correct and normalize variations in peak intensities between different chromatographic runs, which helps reduce the impact of instrumental variations in the runs.

2.6. Statistical analysis

All proteins identified with a confidence score higher than 95 % were included in the quantitative statistical analysis incorporated into the PLGS software. The identical peptides from each sample were pooled according to mass accuracy (<10 ppm) and the retention time tolerance <0.25 min, using the clustering software included in the PLGS. Normalization was automatically implemented by the software (default parameters). The change in presence between groups (with different RT

protocols and before vs. after exercise) was calculated using a *t*-test, represented as 1-$p > 0.95$ for proteins present in greater abundance, and at $p < 0.05$ for proteins present in lower abundance, when the groups were compared or "before" was compared to "after" physical exercise. Proteins were analyzed by their UNIPROT accession number.

Statistical analyses and data visualization were performed using R software (version 4.4.1) within the RStudio environment. Data manipulation and tidying were conducted using the "tidyverse" package. Initially, proteomic data were filtered to exclude common contaminants and reverse (decoy) sequences. Intensity values were log_2-transformed and row-scaled to minimize technical variability. Proteins with zero variance were excluded from the downstream analysis. Principal Component Analysis (PCA) was conducted using the "FactoMineR" package, and the results were visualized using "factoextra" package to generate 95 % confidence ellipses and variable contribution plots. The statistical significance of global proteomic profile shifts ('before' vs 'after') was assessed via Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations, based on Euclidean distances [21] using the "vegan" package. Hierarchical clustering heatmaps were generated with the "pheatmap" package to visualize differential expression patterns. Statistical significance was set at $p < 0.05$.

2-way ANOVA/Bonferroni was used to estimate significant differences in salivary flow before and after both RT. Independent *t*-test compared the average responses of each metabolism component between HI and LI + BFR protocols. For both analysis, $p < 0.05$ was considered as significant (Graph Pad Prism, USA).

3. Results

The total amount of peptides recovered varied, with mean values (per group) of 47.9 μg (Pre-HI) and 64.9 μg (Pre-LL-BFR) before training, and 237.3 μg (Post-HI) and 198.8 μg (Post-LL-BFR) after training.

Table 1 presents the participants' stimulated salivary flow, showing similarity in salivary flow between RT protocols and between before and after training, regardless of the protocol. Thus, the blood restriction did not affect saliva production in quantitative terms.

When comparing the proteomic profile of saliva stimulated before and immediately after the conventional high-intensity resistance training session (HI), 34 proteins were found common to both comparisons. Furthermore, it was observed that the proteins more abundant after exercise were: Actin_ cytoplasmic 1, Immunoglobulin kappa constant, Actin_ cytoplasmic 2 and Immunoglobulin kappa light chain. In contrast, the less abundant proteins were: Hemoglobin subunit alpha, Hemoglobin subunit beta, Hemoglobin subunit delta, Hemoglobin subunit epsilon, Hemoglobin subunit gamma-1, Hemoglobin subunit gamma-2 and Basic salivary proline-rich protein 2 (Table 2).

For low-intensity resistance training associated with blood flow restriction (LI + BFR), after versus before, 51 proteins were found common to both comparisons. Furthermore, an overpresence of the following proteins was observed after the activity: Immunoglobulin kappa constant, Immunoglobulin kappa light chain, Hemoglobin subunit gamma-2, Statherin and Hemoglobin subunit delta. In contrast, the less abundant proteins were: Acidic proline-rich phosphoprotein 1/2, Basic salivary proline-rich protein 2 and Basic salivary proline-rich protein (Table 3).

In the comparison between ALI + BFR x AHI, 50 proteins common to both comparisons were found. Furthermore, the most abundant protein in the protocol with flow restriction was Statherin. In contrast, the less abundant proteins were: Prolactin-inducible protein and Basic salivary proline-rich protein 2. However, the reduction was less than 50 % (Table 4).

Fig. 1 depicts the average response of each metabolism during each protocol. No differences were observed (in absolute oxygen values, mlO_2) for oxidative response (VO_{2ON}), glycolytic ($EqO_{2[La]}$), and oxygen debt (VO_{2OFF}) between training protocols ($p > 0.05$). Neither was

Table 1
Mean and standard deviation of salivary flow in mL/min for stimulated saliva before (BHI) and after (AHI) high-intensity resistance training, and before (BLI + BFR) and after (ALI + BFR) blood flow restriction low intensity resistance training.

		Mean ± Standard Deviation
HI	BHI	1.06 ± 0.40aA
	AHI	1.06 ± 0.34aA
LI + BFR	BLI + BFR	1.16 ± 0.38aA
	ALI + BFR	1.34 ± 0.32aA

Two-way ANOVA/Bonferroni (LI + BFR vs. HI $p = 0.364$, before vs. after $p = 0.935$, interaction $p = 0.969$). Identical lowercase letters indicate similarity in salivary flow values before and after each type of training; identical uppercase letters indicate similarity between HI and LI + BFR at each time point (before and after).

Table 2
Differential presence of proteins in stimulated saliva for the conventional high-intensity resistance training (HI) group after (AHI) and before (BHI).

[a]Access Number	Protein Name	PLGS Score	[b]Ratio AHI:BHI	P
P60709	**Actin cytoplasmic 1**	**253**	**5.26**	**0.04**
P01834	**Immunoglobulin kappa constant**	**287**	**4.90**	**<0.01**
P63261	**Actin cytoplasmic 2**	**253**	**4.76**	**<0.01**
P0DOX7	**Immunoglobulin kappa light chain**	**166**	**4.26**	**0.01**
P04280	Basic salivary proline-rich protein 1	470	1.58	0.02
P12273	Prolactin-inducible protein	610	1.35	0.02
P09228	Cystatin-SA	186	1.17	0.9
P02808	Statherin	907	1.21	0.85
P68032	Actin_ alpha cardiac muscle 1	30	2.92	0.82
P19961	Alpha-amylase 2B	2366	1.02	0.82
P68133	Actin_ alpha skeletal muscle	30	2.86	0.81
P62736	Actin_ aortic smooth muscle	30	3.10	0.77
Q96DR5	BPI fold-containing family A member 2	253	1.72	0.73
P04746	Pancreatic alpha-amylase	1652	1.01	0.72
P63267	Actin_ gamma-enteric smooth muscle	30	2.29	0.71
P01876	Immunoglobulin heavy constant alpha 1	97	1.06	0.71
P0DTE7	Alpha-amylase 1B	3288	1.01	0.69
P0DUB6	Alpha-amylase 1 A	3288	1.01	0.66
P0DTE8	Alpha-amylase 1C	3288	1.01	0.63
P23280	Carbonic anhydrase 6	107	1.06	0.53
P15516	Histatin-3	1139	1.07	0.51
P01037	Cystatin-SN	317	0.95	0.32
P01036	Cystatin-S	1303	0.94	0.28
P02768	Albumin	297	0.93	0.26
Q96DA0	Zymogen granule protein 16 homolog B	132	0.81	0.11
P02810	Salivary acidic proline-rich phosphoprotein 1/2	302	0.81	0.01
P02814	Submaxillary gland androgen-regulated protein 3B	1378	0.61	<0.01
P69905	**Hemoglobin subunit alpha**	**994**	**0.20**	**<0.01**
P68871	**Hemoglobin subunit beta**	**2064**	**0.18**	**<0.01**
P02042	**Hemoglobin subunit delta**	**923**	**0.18**	**<0.01**
P02100	**Hemoglobin subunit epsilon**	**485**	**0.12**	**<0.01**
P69891	**Hemoglobin subunit gamma-1**	**485**	**0.12**	**<0.01**
P69892	**Hemoglobin subunit gamma-2**	**485**	**0.12**	**<0.01**
P02812	**Basic salivary proline-rich protein 2**	**277**	**0.04**	**<0.01**

The proteins highlighted in bold are increased or decreased by at least more than 2-fold.

[a] Identification based on the protein ID from the UniProt protein database, reviewed entries only (http://www.uniprot.org/).

[b] Proteins with significantly altered presence are organized according to their ratio.

Table 3
Differential presence of proteins in stimulated saliva for the blood flow restriction low intensity resistance training group (LI + BFR) after (ALI + BFR) and before (BLI + BFR).

[a]Access Number	Protein Name	PLGS Score	[b]Ratio ALI + BFR:BLI + BFR	P
P01834	**Immunoglobulin kappa constant**	**598**	**2.61**	**<0.01**
P0DOX7	**Immunoglobulin kappa light chain**	**427**	**2.46**	**0.01**
P69892	**Hemoglobin subunit gamma-2**	**136**	**2.25**	**0.02**
P02808	**Statherin**	**3572**	**2.01**	**<0.01**
P02042	**Hemoglobin subunit delta**	**136**	**2.01**	**0.02**
P01833	Polymeric immunoglobulin receptor	53	1.63	<0.01
P02768	Albumin	820	1.52	<0.01
P01876	Immunoglobulin heavy constant alpha 1	219	1.32	0.01
P01877	Immunoglobulin heavy constant alpha 2	45	1.31	0.02
P0DOX2	Immunoglobulin alpha-2 heavy chain	34	1.30	0.03
P68871	Hemoglobin subunit beta	136	1.60	0.94
P69905	Hemoglobin subunit alpha	111	2.61	0.92
P02100	Hemoglobin subunit epsilon	136	2.27	0.92
P69891	Hemoglobin subunit gamma-1	136	2.16	0.92
P60709	Actin_ cytoplasmic 1	241	1.82	0.86
P68133	Actin_ alpha skeletal muscle	67	2.08	0.85
P63261	Actin_ cytoplasmic 2	241	1.86	0.85
P68032	Actin_ alpha cardiac muscle 1	67	2.27	0.84
P62736	Actin_ aortic smooth muscle	67	1.82	0.82
P63267	Actin_ gamma-enteric smooth muscle	67	1.93	0.81
Q562R1	Beta-actin-like protein 2	93	1.93	0.78
A0M8Q6	Immunoglobulin lambda constant 7	629	1.88	0.77
A5A3E0	POTE ankyrin domain family member F	24	1.80	0.77
P02814	Submaxillary gland androgen-regulated protein 3B	3795	1.06	0.75
Q6S8J3	POTE ankyrin domain family member E	24	1.97	0.73
P0DTE8	Alpha-amylase 1C	6826	1.01	0.69
Q9BYX7	Putative beta-actin-like protein 3	24	1.86	0.69
P0DUB6	Alpha-amylase 1 A	6826	1.01	0.67
P01591	Immunoglobulin J chain	977	1.13	0.65
P19961	Alpha-amylase 2B	4464	1.01	0.63
P01857	Immunoglobulin heavy constant gamma 1	107	1.48	0.63
P0DTE7	Alpha-amylase 1B	6826	1.01	0.62
P0DOX5	Immunoglobulin gamma-1 heavy chain	107	1.28	0.62
Q6P5S2	Protein LEG1 homolog	282	0.98	0.56
P04746	Pancreatic alpha-amylase	3920	1.00	0.53
P0DOY2	Immunoglobulin lambda constant 2	629	0.93	0.46
P0DOX8	Immunoglobulin lambda-1 light chain	629	0.96	0.44
P0CG04	Immunoglobulin lambda constant 1	629	0.83	0.42
B9A064	Immunoglobulin lambda-like polypeptide 5	629	0.84	0.39
P01037	Cystatin-SN	137	0.96	0.35
P28325	Cystatin-D	711	0.55	0.34
P0CF74	Immunoglobulin lambda constant 6	629	0.84	0.34
P0DOY3	Immunoglobulin lambda constant 3	629	0.79	0.33
P23280	Carbonic anhydrase 6	130	0.58	0.16
Q96DA0	Zymogen granule protein 16 homolog B	755	0.84	0.06
P12273	Prolactin-inducible protein	608	0.76	0.02
P01036	Cystatin-S	387	0.70	<0.01
P09228	Cystatin-SA	344	0.55	<0.01
P02810	**Salivary acidic proline-rich phosphoprotein 1/2**	**2527**	**0.40**	**<0.01**
P02812	**Basic salivary proline-rich protein 2**	**1481**	**0.17**	**<0.01**
P04280	**Basic salivary proline-rich protein 1**	**1686**	**0.12**	**<0.01**

The proteins highlighted in bold are increased or decreased by at least more than 2-fold.
[a] Identification based on the protein ID from the UniProt protein database, reviewed entries only (http://www.uniprot.org/).
[b] Proteins with significantly altered presence are organized according to their ratio.

different the total energy demanded (in absolute oxygen values, mlO_2) in each protocol (HI = 22,216.9 vs. LI + BFR = 20,890.0 mL O_2).

Visual evidence regarding data variance and quality is included below, starting with the Principal Component Analysis (PCA) (Fig. 2). PCA results suggest for the LI + BFR group a salivary proteomic difference between before and after exercise. The 95 % confidence ellipses reveal that, although there is expected individual variability after the stimulus (yellow ellipse), the overall proteomic profile underwent a significant shift along PC1, which is corroborated by the *p*-value ($p = 0.015$) obtained in the PERMANOVA analysis. In contrast, the HI group ($p = 0.768$) did not show a statistically significant change, despite the high variance (66.7 %) explained by the PCA components, as shown by the drawing of the ellipses, one superimposed on the other.

4. Discussion

First, it is important to note that, according to the PCA and Heatmap results, our findings suggest distinct proteomic behavior between protocols. LI + BFR exercise appears to trigger a homogeneous and directional proteomic pattern, as evidenced by the significant spatial segregation of the 95 % confidence ellipses ($p = 0.015$). This suggests that the physiological stress of blood flow restriction may lead to a dominant stimulus that overrides individual baseline variability. On the other hand, the HI group exhibited significant overlap between the "before" and "after" states ($p = 0.768$), indicating that although high-intensity exercise is a potent stimulus, its impact on the overall salivary proteome is more susceptible to interindividual biological variation. This suggest that while HI induces individual protein changes, the overall final proteomic remains closer to the baseline when compared to the physiological stress induced by the LI + BFR protocol (before vs. after).

4.1. Comparison AHI X BHI

Carbon dioxide transport associated with oxygen transport, in the comparison AHIxBHI, is associated with genes (HBA, HBB, HBD, HBE1, etc.) that encode hemoglobin subunits (ClueGo®). The proteins hemoglobin subunit alpha, hemoglobin subunit beta and hemoglobin subunit delta are involved in the transport of oxygen from the lungs to peripheral tissues and, to a lesser extent, of carbon dioxide from peripheral tissues to the lungs, that is, in the opposite direction [22]. Therefore, the predominance of this functional category in resistance exercise practice highlights the body's work in intensifying the gas exchange process in order to meet the metabolic demands generated by muscle stress. Furthermore, the significant decrease ($p < 0.01$) in the alpha, beta and delta subunits may be related to another function already recorded in the literature: elimination of oxidizing agents and the reduction of reactive oxygen species (ROS) [23,24]. This may be a way in which the

Table 4
Differential presence of proteins in stimulated saliva for the group after blood flow restriction low intensity training (ALI + BFR) versus after high intensity resistance training (AHI).

[a]Access Number	Protein Name	PLGS Score	[b]Ratio ALI + BFR:AHI	P
P02808	**Statherin**	**2443**	**2.16**	**<0.01**
P02814	Submaxillary gland androgen-regulated protein 3B	2281	1.70	<0.01
Q96DA0	Zymogen granule protein 16 homolog B	412	1.43	0.02
P02768	Albumin	1732	1.25	<0.01
P01037	Cystatin-SN	385	1.17	0.02
P09228	Cystatin-SA	182	1.17	0.91
P01833	Polymeric immunoglobulin receptor	159	1.22	0.9
P02100	Hemoglobin subunit epsilon	373	1.73	0.89
P69891	Hemoglobin subunit gamma-1	373	1.52	0.84
P69892	Hemoglobin subunit gamma-2	373	1.54	0.83
P02042	Hemoglobin subunit delta	528	1.31	0.77
Q6P5S2	Protein LEG1 homolog	152	2.18	0.75
P01834	Immunoglobulin kappa constant	211	1.19	0.67
P68871	Hemoglobin subunit beta	866	1.09	0.65
P01876	Immunoglobulin heavy constant alpha 1	922	1.05	0.65
P69905	Hemoglobin subunit alpha	437	1.11	0.64
P0DOX2	Immunoglobulin alpha-2 heavy chain	464	1.05	0.63
P01877	Immunoglobulin heavy constant alpha 2	487	1.04	0.63
P01036	Cystatin-S	413	1.02	0.62
P04280	Basic salivary proline-rich protein 1	788	0.99	0.51
P0DOX7	Immunoglobulin kappa light chain	79	1.05	0.51
P01591	Immunoglobulin J chain	266	1.02	0.5
Q9BYX7	Putative beta-actin-like protein 3	33	0.84	0.41
P62736	Actin_ aortic smooth muscle	76	0.77	0.35
Q562R1	Beta-actin-like protein 2	33	0.79	0.35
P01857	Immunoglobulin heavy constant gamma 1	94	0.58	0.32
Q6S8J3	POTE ankyrin domain family member E	44	0.73	0.29
A5A3E0	POTE ankyrin domain family member F	44	0.73	0.29
P0DOX5	Immunoglobulin gamma-1 heavy chain	94	0.63	0.28
P68032	Actin_ alpha cardiac muscle 1	76	0.77	0.27
P63267	Actin_ gamma-enteric smooth muscle	76	0.67	0.27
P23280	Carbonic anhydrase 6	157	0.76	0.26
P0DTE8	Alpha-amylase 1C	5088	0.98	0.23
P68133	Actin_ alpha skeletal muscle	76	0.66	0.19
P0DUB6	Alpha-amylase 1 A	5088	0.98	0.18
P0DOY2	Immunoglobulin lambda constant 2	331	0.67	0.17
P0DOX8	Immunoglobulin lambda-1 light chain	331	0.67	0.17
B9A064	Immunoglobulin lambda-like polypeptide 5	331	0.63	0.17
P0CF74	Immunoglobulin lambda constant 6	331	0.64	0.16
P0CG04	Immunoglobulin lambda constant 1	331	0.65	0.15
P0DOY3	Immunoglobulin lambda constant 3	331	0.67	0.15
P19961	Alpha-amylase 2B	3030	0.97	0.14
A0M8Q6	Immunoglobulin lambda constant 7	265	0.61	0.14
P60709	Actin_ cytoplasmic 1	200	0.60	0.13
P02810	Salivary acidic proline-rich phosphoprotein 1/2	2918	0.84	0.13
P04746	Pancreatic alpha-amylase	2826	0.96	0.12
P0DTE7	Alpha-amylase 1B	5088	0.98	0.08
P63261	Actin_ cytoplasmic 2	200	0.55	0.06
P12273	Prolactin-inducible protein	1067	0.75	0.03
P02812	Basic salivary proline-rich protein 2	902	0.67	<0.01

The proteins highlighted in bold are increased or decreased by at least more than 2-fold.

[a] Identification based on the protein ID from the UniProt protein database, reviewed entries only (http://www.uniprot.org/).

[b] Proteins with significantly altered presence are organized according to their ratio.

metabolism compensated for the gradual increase caused by high-intensity exercise, since resistance training with intense strength can cause an increase in oxidative stress, leading to the production of ROS at higher systemic levels [23,24]. In healthy skeletal muscle, ROS generation induced by muscle contraction can lead to the regulation of cellular signaling pathways and control of several redox-sensitive transcription factors [25]. Moreover, physiological levels of ROS are closely linked to the generation of muscle strength, which indicates the relevant role of these hemoglobin subunits in redox balance, preventing cellular damage and aiding in muscle remodeling [25].

The hemoglobin alpha binding category is represented by genes that encode proteins that perform antioxidant activities and participate in muscle metabolism [26]. The action of proteins, especially stabilizing proteins, as binding of hemoglobin alpha, is essential to avoid the generation of ROS and to prevent their precipitation when exposed to oxidative stress [26,27]. In this sense, aiming to promote the reduction of toxic free radicals in the body and also develop protective effects against various pathologies, the practice of resistance training can be a great ally in muscle remodeling and respiratory efficiency.

Overpresence of the actin cytoplasmic 1 protein was observed after conventional resistance training (Table 2). Actin is a cytoplasmic protein that forms reticular networks in the cytoplasm of cells [27]. This protein participates in numerous cellular functions, ranging from organelle trafficking and pathogen motility to cell migration and regulation of gene transcription [28]. Therefore, the increase in salivary levels of this molecule suggests a more intense activity in cellular metabolism and cytoskeleton remodeling, however, it is not known from which cell(s) this protein originates from. Also, as demonstrated by Balasubramanian et al. (2010) [29], actin cytoplasmic 1, through its polymerization/depolymerization functioning, can govern lateral or membrane-parallel tension generated by the actin filament network during cytoskeletal reorganization processes and the cytoskeletal recruitment of signaling elements for hypertrophic cellular responses in adult cardiomyocytes. So then, this protein can be associated to hypertrophy.

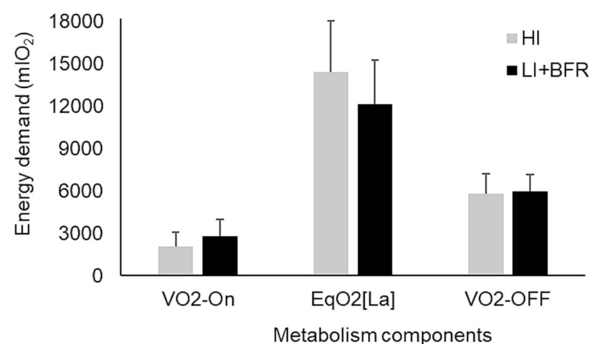

Fig. 1. The response of each component of energy demand during each protocol of training. The acronyms VO_{2On}, $EqO_{2[La]}$, and VO_{2OFF} are representing the oxidative, glycolytic and oxygen debt during training, respectively.

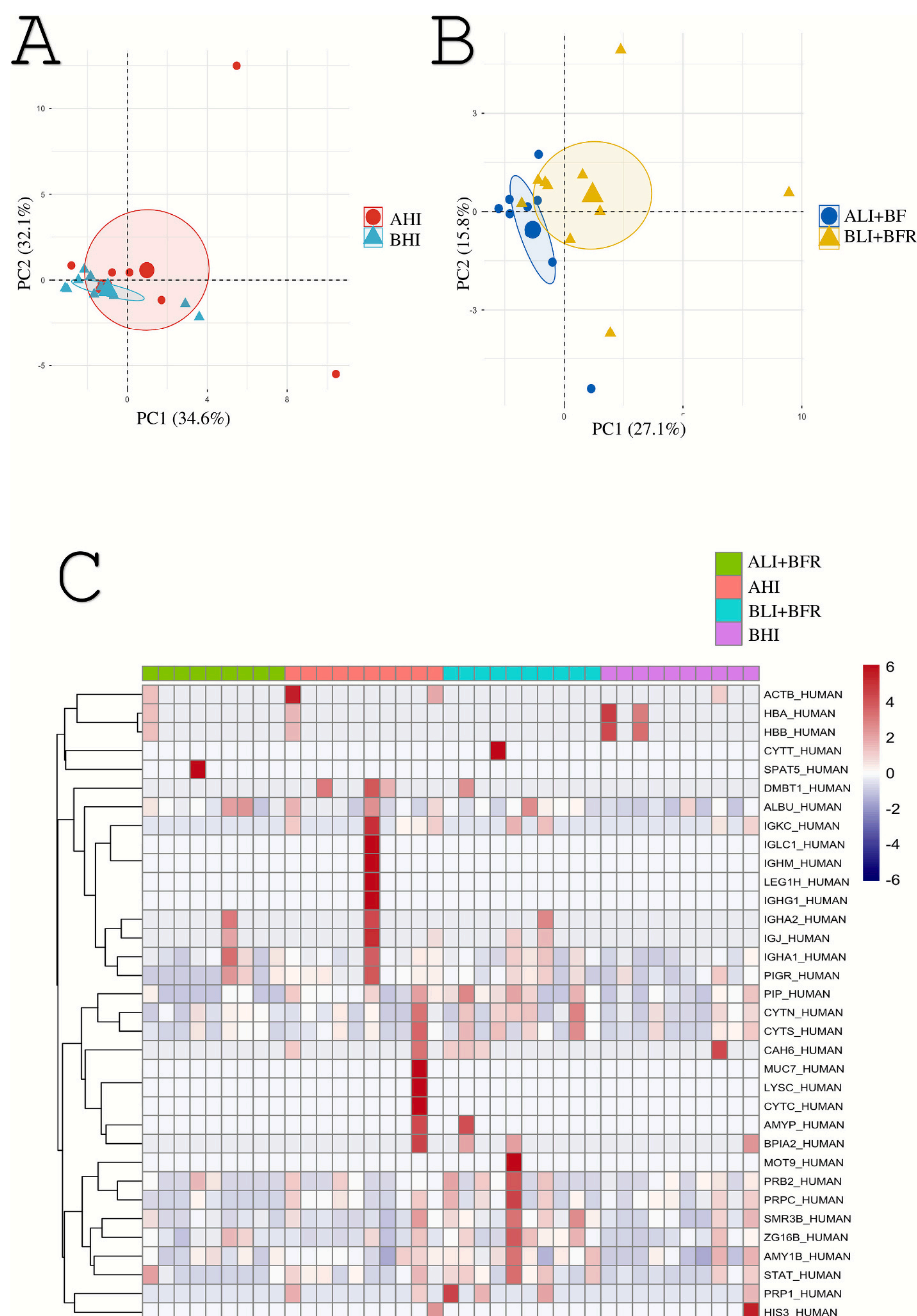

Fig. 2. A) PCA by HI: Principal Component Analysis shows the samples grouped according to collection time (before/azul vs. after/red). The first and second components (PC1 and PC2) explain 34.6 % and 32.1 % of the total variance, respectively. Although the 95 % confidence ellipses overlap, the PC1 and PC2 explain a high proportion of the total variance (66.7 %). Multivariate analysis by PERMANOVA indicated that there was no statistically significant change in the overall proteomic profile for this specific protocol ($p = 0.768$), suggesting a greater dominance of interindividual variability over the acute effect of exercise. (B) PCA by LI + BFR: Principal Component Analysis shows the samples grouped according to collection time (before/yellow vs. after/blue). The ellipses represent the 95 % confidence interval for each condition. The total variance explained by the first two dimensions is 42.9 % (PC1: 27.1 %; PC2: 15.8 %). PERMANOVA analysis confirmed a statistically significant difference between the group centroids ($p = 0.015$). (C) Heatmap of salivary proteins identified in both exercise protocols. The columns represent individual samples, organized by experimental group (green, pink, blue, purple), and the rows represent 34 proteins (identified by Uniprot Entry code). The color scale (blue to red) indicates the relative abundance of proteins (z-score of log-transformed intensities). Hierarchical clustering (dendrogram on the left) highlights protein clades with similar expression patterns. Reverse sequences and common contaminants were removed to ensure the biological reliability of the data. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Additionally, there was an overpresence in two species of immunoglobulins: immunoglobulin kappa constant and immunoglobulin kappa light chain. Immunoglobulins, also known as antibodies, are secreted or membrane-bound glycoproteins produced by B lymphocytes (Uniprot). In the recognition phase of humoral immunity, membrane-bound immunoglobulins serve as receptors that, upon binding to a specific antigen, trigger clonal expansion and differentiation of B lymphocytes into plasma cells (immunoglobulin-secreting plasma cells) (Uniprot). Secreted immunoglobulins mediate the effector phase of humoral immunity, which results in the elimination of bound antigens (Uniprot). Elevated levels of immunoglobulin kappa constant, that is the kappa light chain constant region, have been identified as robust markers of

plasma cell infiltration and are correlated with improved clinical outcomes in solid tumors such as breast, lung, and colorectal cancers [30–32] and autoimmune and infectious conditions [33,34]. Taken together, the overpresence of those immunoglobulins species strongly supports a context of heightened humoral immunity, potentially reflecting both recruitment and functional antibody-mediated immune mechanisms.

The AHI vs. BHI comparison aligns with the results of our previous study [10], which also found a reduction in the alpha, beta, and delta species of hemoglobins following HI training, along with an increased presence of other proteins, such as S100-A8 and S100-A9. These proteins are considered important for immune system responses, including antimicrobial, pro-inflammatory, and apoptotic responses (Uniprot).

Despite the results shown in Table 2 regarding hemoglobin and immunoglobulin subunits, PCA analysis for the AHI vs. BHI comparison showed that individuals presented important individual characteristics in response to the training stimulus (red ellipse superimposed on blue, but a high value of 66.7 %). This is confirmed by the AHI and BHI ranges in the heatmap (Fig. 2.C), where the proteins range HBA_HUMAN, HBB_HUMAN, IGKC_HUMAN, IGLC1_HUMAN, and ACTB_HUMAN appear with different colours (red to blue) between the samples (columns), despite the association between them, as shown in the dendrogram on the left. Although the sample size is a significant limitation, the results found are fundamental to guiding future research in the area.

4.2. Comparison ALI + BFR x BLI + BFR

The IgA immunoglobulin complex comprises numerous genes associated with the action and functionality of immunoglobulins in the human body (Uniprot). There are 5 main classes of heavy chain constant domains from immunoglobulins [35]. Each class defines the isotypes IgM, IgG, IgA, IgD and IgE [35]. The IgA isotype, of greatest relevance in the study in question, is essential in the protection of mucosal surfaces against toxins, viruses and bacteria, by promoting direct neutralization or preventing the binding of these antigens to the mucosal surface [35]; and also acts as an important enhancer of the immune response of intestinal tissue, through the capture of antigens for dendritic cells [35]. Furthermore, IgA receptors are present on neutrophils, which can be activated to mediate ADCC (antibody-dependent cellular cytotoxicity) locally [35]. Thus, the predominance of the IgA immunoglobulin complex after muscle stimulation reinforces the idea that low-intensity resistance training with flow restriction has also an effective role in promoting immunity.

In agrement, we also showed overpresence of the immunoglobulin kappa light chain and immunoglobulin kappa constant proteins in Table 3, as well as immunoglobulin heavy constant alpha 1, immunoglobulin heavy constant alpha 2, immunoglobulin alpha-2 heavy chain. As discussed in the AHI x BHI comparison, this result can be correlated to an enhanced humoral immune response.

Differently from AHI x BHI, in ALI + BFR x BLI + BFR happened an increase in the presence of some hemoglobin species in saliva. They were hemoglobin subunit gamma-2 and hemoglobin subunit delta (Table 3). We propose that during BFR, hemoglobins are less recruited to active muscle tissue due to restricted blood flow, resulting in these proteins “remaining” in or re-entering the saliva, where they are more readily detected. This hypothesis aligns with observation of elevated total and deoxygenated muscle hemoglobin during BFR training, suggesting altered hemoglobin redistribution [36]. At the same time, PCA (Fig. 2B) further confirms significant changes ($p < 0.05$) following the BFR protocol. The non-overlapping ellipses demonstrate a distinction between the ‘before’ and ‘after’ states, highlighting the effectiveness of the protocol in promoting systemic changes.

4.3. Comparison ALI + BFR x AHI

When analyzing Table 4, comparison of ALI + BFR with AHI, it was possible to identify the overpresence of two proteins in saliva after flow restriction training (ALI + BFR), namely statherin and submaxillary gland androgen-regulated protein 3B. Statherin is a specific salivary protein secreted by the parotid and submandibular salivary glands, which stabilizes saliva supersaturated with calcium salts, inhibiting the precipitation of calcium phosphate salts, and also modulates the formation of hydroxyapatite crystals on the tooth surface, contributing to the biomineralization process [37]. Furthermore, the C-terminal fragments of statherin bind to *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, both cause periodontal infection and play a role in the initiation of periodontal disease [37]. Thus, statherin inhibits the growth of anaerobic bacteria and contributes to selective bacterial colonization on the supragingival and subgingival surfaces of teeth [37]. Thus, the results suggest that the level of statherin in saliva increases and may provide a protective effect against gingival inflammation [37].

Submaxillary gland androgen-regulated protein 3B (SMR3B) has been identified as a lipopolysaccharide (LPS)-binding protein from *Porphyromonas gingivalis* in human saliva [38]. Lipopolysaccharides (LPSs) are known to be involved in bacterial adhesion and colonization and in the modulation of immune responses [38]. According to a study by Ren et al. (2004) [39], LPS-binding proteins were significantly elevated in a healthy population compared with patients with periodontitis, suggesting that the presence of SMR3B in saliva may contribute to periodontal homeostasis, since SMR3B, through the neutralization of LPSs, are crucial in the host’s cellular responses to bacterial challenge. Therefore, the increase in SMR3B after physical activity with flow restriction suggests that a healthier periodontal condition may be favored in individuals who perform this training protocol overtime. Therefore, this result points to the need to assess the oral condition of participants (with or without gingivitis) before and after physical activity (using gingival bleeding and plaque index) with further follow up.

Finally, when checking Tables 1, 2 and 3, basic salivary proline-rich protein 2 was present at lower levels after both training protocols, which is also visualized in the heatmap on the PRB2_HUMAN line. The most heterogeneous family of human salivary proteins is represented by proline-rich proteins (PRPs), which are divided into acidic, basic and basic glycosylated (aPRPs, bPRPs, gPRPs) [40]. In humans, PRPs or proline-rich domains are very common and participate in almost all biological processes, such as RNA splicing and processing, modulation of signaling pathways, misfolded protein binding, collagen fibril organization and chondrocyte development [41].

In the oral cavity, oral surfaces can be coated by salivary pellicles formed by the selective adsorption of components of whole saliva, among which proline-rich basic proteins stand out, which constitute more than 30 % of the proteins secreted by the parotid glands [41,42]. Pellicle proteins provide a range of receptors for the adhesion of microorganisms, such as the fungus *Candida albicans* and the bacterium *Streptococcus gordonii* [42]. *Candida albicans* is part of the normal human oral microbiota, but under conditions of host immunosuppression, can cause a variety of mucosal infections [42]. *Streptococcus gordonii* has been shown to play a beneficial role in the maintenance of oral microbial balance and potentially contributing to caries prevention, by inhibiting biofilm formation and helping to maintain a less acidogenic environment on tooth surfaces [43,44]. Furthermore, PRPs contribute to mineralization by providing alternative nucleation sites on dental surfaces [45]. In summary, the functional roles of PRPs are far from being completely elucidated; however, it can be inferred that the practice of both resistance training protocols can negatively affect the level of this molecular family, which may impact their protective effects.

4.4. Energy demand

Regarding the energy demand, no significant differences were observed between the contribution of the oxidative, glycolytic and oxygen debt in the response to the training protocols ($p < 0.05$), in spite of

the proteomic specificities.

VO_2 and lactate are indicators of the systemic metabolic stimuli and energetics balance, whose full manifestation is inherently time and intensity dependent. During high-intensity short-term intermittent exercise, such as that employed in this study (i.e., resistance exercise), the VO_2 response is influenced by the limited ability of muscle fibers to adjust their oxidative metabolic rate sufficiently rapidly (due to temporal limitations in blood perfusion, gas diffusion and mitochondrial function) to match the energy requirements of the active fibers. This deficit of O_2 availability to the muscle will require the contribution of the finite reserves of anaerobic energy (e.g., high-energy phosphates and muscle glycogen) to maintain contractile activity [46]. Consequently, the anaerobic energy contribution scales with the accumulated oxygen deficit (AOD). This relationship, in turn, influences the post-exercise VO_2 kinetics, as the rate and magnitude of oxygen recovery are closely associated with ATP-CP (creatine phosphate) resynthesis and lactate clearance [47].

Despite the lack of statistical significance between protocols, the current findings provide a reliable estimate of the metabolic demand for a full resistance training session. This is supported by previous data reporting energy costs of 14, 33, and 49 kJ for aerobic, anaerobic, and EPOC components during two sets in bench press [48]. While direct comparisons are limited by differences in VO_2 (aerobic) sampling and EPOC duration, an extrapolation to 24 sets (yielding 8, 19, and 28 L of O_2 equivalent) aligns closely only with the anaerobic (lactate) responses observed in the current HI protocol.

The lack of difference in energetic responses between HI and LI + BFR protocols aligns with the assumption that mechanical stress during HI stimulates fast-twitch fiber recruitment, similar to what is expected during low-load exercise under ischemic conditions. However, the mechanisms underlying blood lactate accumulation likely differ between protocols due to distinct intramuscular dynamics. In the HI protocol, glycolysis is activated at high rates via the early recruitment of type II fibers; however, significant lactate accumulation is attenuated by reuptake and oxidation in type I fibers (favoring lactate clearance) [49]. Conversely, in the LI protocol, the lower rate of lactate production may accumulate over time due to the delayed recruitment of type II fibers, restricted venous outflow at the occlusion site, and longer exercise duration [49]. This extended duration is expected to elicit a greater aerobic response (VO_2) during LI without BFR, but not necessarily a higher EPOC [50].

In contrast, the salivary proteome demonstrated superior sensitivity and faster response kinetics in differentiating acute stressors. Salivary proteins reflect immediate molecular modulation (e.g., salivary amylase) as well as cellular (e.g., actin, hemoglobins) responses that are activated at the onset of the adaptive cascade. The literature supports that proteomic panels are promising tools for diagnosing and monitoring responses to acute stressors due to their capacity to capture specific molecular processes [51].

Therefore, the salivary proteome exhibits superior temporal sensitivity for discriminating molecular stress pathways induced by short and intense resistance protocols (saliva was collected after 15 min of RT compared to 1–7 min for blood colection). Our findings suggest that while VO_2 and lactate represent markers of the final systemic metabolic state, the salivary proteome serves as a robust indicator of the acute cellular processes that precede and signal subsequent physiological adaptations.

4.5. Limitations

The present study is subject to some limitations that should be considered when interpreting the results. Firstly, the absence of significant differences in physiological markers (VO_2 and lactate) between training protocols may be in part due to an artifact of the sampling procedures compared to previous studies, since the experimental protocols lack similar designs for direct comparison. In fact, the absence of a low-intensity control protocol precludes an assertive conclusion regarding BFR effects on energy demand during resistance training. However, for practical purposes, the current findings suggest that low-intensity training with BFR may be a viable alternative to traditional high-load resistance training when aiming for exercise that demands high exertion levels, potentially leading to improvements in muscle strength and mass. Secondly, a limitation regarding reproducibility metrics is that all proteomic samples were analyzed only once, without the inclusion of technical or analytical replicates. Finally, the different proteins profiles found between the protocols must be confirmed by using ELISA assay or Western Blot in the future.

Consequently, although the comparison between HI and LI + BFR demonstrates similar systemic metabolic demands, along with slightly different salivary proteomic profiles (as observed in the PCA analysis and heatmap), the study design does not yet enable us to draw definitive conclusions about the specific contribution of blood flow restriction, load intensity, or their interaction to the observed responses. However, the results do allow us to speculate. Observing the PCA analysis and heatmap, one might speculate that LI + BFR promotes a standardization of biological adaptations, while HI promotes more heterogeneous responses dependent on the individual phenotype. However, this is a speculation that needs to be confirmed by a study with a considerable sample size and longitudinal follow-up.

5. Conclusion

Both protocols presented similar energy demand under short-term response. Furthermore, HI and LI-BRF influenced immune system activity while showed distinct hemoglobin redistribution profiles in saliva, despite, HI promotes more heterogeneous responses dependent on the individual phenotype compared to LI-BRF. Further studies are needed to validate these findings and their clinical implications under longitudinal follow up.

CRediT authorship contribution statement

Gustavo Zanete Alencar: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dalton Muller Pessôa Filho:** Writing – review & editing, Supervision, Resources, Project administration, Investigation. **Karina Oliveira Santos:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anderson Geremias Macedo:** Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Henrique Basso Vitti:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Guilherme Neves Gasparino:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Larissa Tercília Grizzo Thomassian:** Software, Formal analysis. **Murilo Henrique Faria:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Ana Carolina Magalhães:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2025.105587.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD070541.

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