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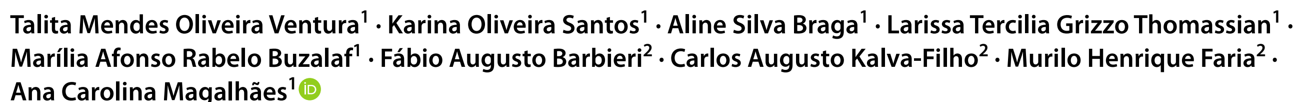
ORIGINAL ARTICLE

Salivary proteomic profile of young adults before and after the practice of interval exercise: preliminary results



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Abstract
The practice of exercise can change biological markers. Thus, the use of saliva to define possible biomarkers has increased with the advances in "omics" techniques. Therefore, this study analyzed the salivary proteomic profile in young adults before and after interval exercise. Five adults, without comorbidities and with normal salivary flow, aged between 20 and 35 years (3 men and 2 women) were selected. The collection of stimulated (SS) and non-stimulated (NSS) saliva was done before (BE) and after (AE) exercise, which consisted of interval exercise with 5 min walking on a treadmill and 5 min at rest, during a total of 40 min. The proteins were extracted individually and processed by label-free proteomics. There was no significant difference between BE and AE salivary flows. For SS, 69 and 60 proteins were found BE (12 exclusives) and AE (3 exclusives), respectively. Ten subunits of immunoglobulins (8 more than twofold), as well as *Lysozyme C* and *Cystatin-S*, were increased AE compared to BE. Six hemoglobin's isoforms were decreased AE compared to BE. For NSS, 76 and 65 proteins were found BE (18 exclusives) and AE (7 exclusives), respectively. *Albumin* was increased AE, while *Basic salivary proline-rich protein 1* and *2* decreased. *Apolipoprotein A-I* was identified exclusively AE compared to BE. The salivary proteome after interval exercise suggests increase of immunity.

Keywords Salivary flows · Saliva · Proteins · Proteomics · Interval exercise · Biomarkers

Introduction

Regular physical activity is associated with several general health benefits, including a protective action against metabolic diseases, such as obesity, diabetes mellitus and hypertension [1, 2]. The practice of exercise produces reactive oxygen species (ROS) in cells and body fluids. To neutralize these molecules, the body is stimulated to respond with the antioxidant system, which includes some enzymatic and non-enzymatic mechanisms, uric acid, vitamins and minerals [3]. Some studies also suggest that the variation in the duration, type and intensity of the exercise can affect, for example, oxidative stress markers and the levels of pro-inflammatory biomarkers in urine, blood or/and saliva [4, 5, 6].

In the study of Hajizadeh Maleki et al. [4], participants walked on a treadmill for 25–30 min, 3 days a week for 12 weeks. In the next 12 weeks, the time was increased for 40–45 min, during 3 days per week as well. Blood samples were collected before the beginning of the research and at different periods of follow-up. The authors observed that low and moderate-intensity training decreased the levels of IL-1β, IL-6, IL-8 and TNF-α in the blood for a period of 12–24 weeks; however, their concentrations return to the baseline values after 30 days without exercise [4].

Body fluids play a fundamental role as a tool for the analysis of the biomarkers. One of the main fluids applied in sports medicine is saliva, since the collection is

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non-invasive, without pain or discomfort, differently from what occurs in blood analysis [7]. Salivary secretion is controlled by the autonomic nervous system; so sympathetic activation results in the secretion of saliva rich in proteins, while parasympathetic activation is responsible for water and ionic content [8]. Therefore, the secretion and composition of saliva may vary throughout the day and by different stimuli [9, 10].

Studies have shown that the practice of exercise alters the expression of several salivary biomarkers, due to the activation of the sympathetic nervous system [6, 8, 11]. The concentration of salivary protein may increase with the sympathetic stimulation of the salivary glands by catecholamines [12], which are more expressed above the anaerobic threshold [11, 13]. Cycling for 15 min increases the saliva viscosity immediately after exercise [13], as a result of increase in the concentration of proteins, such as MUC5B [13, 14]. Other proteins—amylase, lactoferrin, lysozyme—also increase due to exercise, with a reduction of the level 30 min after the end of the activity [10, 15]. These proteins are known for their antimicrobial effect and role in the formation of the acquired enamel pellicle [16]. In addition, studies have shown that exercise may alter the immune system, depending on its volume, intensity, and nature of the activity [17, 18]. Exercise at moderate intensity increases IgA secretion after the immediate practice; however, these values are reduced to baseline levels after 2–24 h [19].

Considering the aforementioned parameters, it is expected that exercise may modulate the innate and acquired immune system and the response to neuroendocrine stress, which is represented by the hypothalamic–pituitary–adrenal axis (HHA) and the adreno-medullary sympathetic axis (SAM) [20, 21]. One of the main markers for their activation is cortisol [21, 22]. Cortisol can directly impact immune cells, since it induces lymphopenia under high-intensity physical exercises. In contrast, at low intensity, there is no change or, in some cases, a reduction in the concentration of cortisol [23]. Therefore, cortisol tends to combat the stress of exercise, from the axes HHA, modulating the immune system [24]. Instead of analyzing salivary cortisol, the proteomic determination of saliva may show proteins involved in the immune system after exercise, which will help to disseminate the importance of the exercise and to standardize biomarkers able to confirm the benefits of exercise on the oral and general health.

Accordingly, there are no data on quantitative and comparative protein changes in stimulated (SS) and unstimulated (NSS) saliva from the collection before and after exercise under controlled conditions. In view of this, the present study compared the main proteins present in stimulated and unstimulated saliva of young adults, before and after 40 min of interval exercise (low to moderate intensity), using a proteomic approach, to find possible quantitative and qualitative changes in biomarkers expressed in the saliva that reflect the effects of exercise on oral and general health.

Materials and methods

Ethical aspects

This study was submitted to the local Institutional Ethics Committee (No. 29182720.3.0000.5417). SS and NSS, before (BE) and after (AE) interval exercise, were collected after approval and the signature of the informed consent form. The study was carried out in accordance with the Declaration of Helsinki.

Volunteers' information

Five young adults, aged between 20 and 35 years, from both genders (3 men and 2 women), with good oral and general health, non-smokers, non-pregnant and without any systemic condition, were included. Volunteers presenting gingivitis, active caries or erosive lesions were excluded [25]. SS and NSS were collected BE and AE. In addition, all volunteers practiced aerobic exercise twice a week.

Saliva collection and exercise

The volunteers were previously instructed to have the last meal and brush their teeth at least 30 min (the mean was 40–60 min) before saliva collection. The collection of SS and NSS saliva was performed strictly as previously described [26, 27]. Saliva collection was done between 4:00 pm and 6:00 pm The volunteers rinsed their mouths with 5 mL of deionized water before collection. For the collection of NSS, the volunteers spat out all the saliva in tubes immersed in ice for 10 min. For the collection of SS, the volunteers chewed a *Parafilm*® (5 cm) and all saliva produced by the stimulation was spat out in tubes immersed in ice for 5 min.

The volunteers were submitted to exercise protocol, under interval exercise method, on a treadmill (Inbramed ATL, Inbrasport, Porto Alegre, Brazil) with a preferred speed for 5 min walking (medium speed of 4.6 ± 0.4 km/h) and 5 min at rest, during a total period of 40 min. To determine the preferred speed, the volunteers performed three efforts on a linear 10-m circuit 5 min before the first saliva collection. The mean speed of these efforts was assumed as the exercise intensity (4.6 ± 0.4 km h^{-1}). The time of each effort was assessed through a stopwatch (Timex, model 85103). After the exercise, the volunteers remained seated for 5 min and the saliva collection was performed by repeating the same steps described above for SS and NSS. In total, saliva was collected twice (before the first cycle of exercise and after the last cycle of exercise).

After collection, the salivary flow was measured and the saliva samples were centrifuged at 4500×*g* for 15 min at 4 °C to remove possible debris such as insoluble material, cells and food debris. The supernatant from each sample was frozen at − 80 °C until proteomics analysis. These procedures were based also on previous studies [26, 27, 28].

Shotgun label-free quantitative proteomic analysis

SS and NSS, BE and AE, were extracted and prepared individually. For this, 1 mL of saliva was transferred to new tubes and an extraction solution containing 6 M urea, 2 M thiourea in 50 mM Ammonium Bicarbonate, pH 7.8, in a volume similar to the samples (1:1) was used. Samples were vortexed for 10 min at 4 °C, sonicated for 5 min and centrifuged at 20.817×*g* for 10 min at 4 °C for twice. The procedures for the preparation of saliva samples and shotgun proteomic analysis were performed exactly as described [27].

Briefly, after extraction, the samples were concentrated with Amicon tubes (Amicon Ultra-15 Centrifugal Filter Units—Merck Millipore®, Tullagreen, County Cork, Ireland) and centrifuged at 4.500×*g* at 4 °C to a volume of approximately 150 µL. The total protein quantification was performed by Bradford method (Bio-Rad Bradford Assays, Hercules, California, USA) and the samples were then reduced (5 mM dithiothreitol, Bio Rad Laboratories, Canada) and alkylated (10 mM iodoacetamide, GE Healthcare, Little Chalfont, Buckinghamshire UK). In sequence, the samples were digested for 14 h at 37 °C by the addition of 2% (w/w) trypsin (Thermo Scientific Pierce Trypsin Protease, Rockford, lL, USA). Digestion was stopped by the addition of 5% formic acid solution. Samples were desalted and purified using C18 spin columns (Thermo Scientific, Rockford, Illinois, USA). A 1-µL aliquot of each sample was removed for protein quantification by the Bradford method (Bio-Rad Bradford Assays, Hercules, California, USA). Samples were then resuspended in 3% acetonitrile and 0.1% formic acid for nano LC–ESI–MS/MS.

Data acquisition was performed by Xevo G2 QTof mass spectrometer coupled to the nanoACQUITY Ultra Performance Liquid Chromatography (both from Waters, Manchester, UK) controlled by MassLynx v.4.1 (Waters, Manchester, UK). Data collection was in data-independent acquisition mode (LC–MS^E), the mass ranges from 50 to 2000 m/z, and assays were performed exactly as previously described [25, 27]. All samples were analyzed individually, thus totalizing 5 analyses for each condition. The proteins were identified using the software's ion counting algorithm, and a search was performed on the *Homo sapiens* database (revised only, UniProtKB/Swiss-Prot) downloaded in December 2020 from UniProtKB (http://www.uniprot.org). Maximum missed cleavages by trypsin allowed up to 1, variable modifications by oxidation and a false discovery rate value at maximum 4%.

For the label-free quantitative analysis, PLGS was used for analyzing nine raw MS files from each group. Proteins identified with a confidence score higher than 95% were included. The identical peptides from each duplicate by 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 difference in expression between the groups was analyzed by dependent *t* test ($p < 0.05$). The following relevant comparisons were performed: SS AE vs BE; NSS AE vs BE; BE SS vs NSS; AE SS vs NSS.

Bioinformatics analysis

The proteins were analyzed by their access number by UNIPROT, and repeated proteins, reverse proteins and fragments were excluded. Gene ontology was evaluated according to the ClueGo® plugins of the Cytoscape® 3.8.2 Software. The functional distribution of proteins identified with differential expression in the comparison SS, AE vs BE, was done. Protein categories was based on gene ontology (GO) annotation of the broad biological process, molecular function, immune system process and cell component. Terms of significance ($\kappa = 0.04$) and distribution were according to the percentage of the number of associated genes. The number of access of the proteins was provided by UNIPROT. The mass spectrometric proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier (PXD028085).

Results

All participants (ages between 20 and 35 years), 3 male and 2 females, with a mean body mass of 74.6 ± 12.4 kg and mean height of 1.68 ± 0.08 m, completed the study protocol. The mean of the individual body mass index was 26.60 ± 5.15 kg/ cm^2 (2 normal, 2 overweight and 1 obese). Table 1 shows the mean (± SD) of NSS and SS flows, BE and AE.

Table 1 Means ± standard deviations (SD) of NSS and SS saliva flows, before (BE) and after (AE) exercise

Conditions		Mean ± SD (mL/min)
BE	Non-stimulated saliva (NSS)	0.85 ± 0.48
	Stimulated saliva (SS)	1.26 ± 0.41
AE	Non-stimulated saliva (NSS)	0.83 ± 0.36
	Stimulated saliva (SS)	1.24 ± 0.29

The total mean amounts of protein recovered from NSS for BE and AE were 31.9 and 28.6 µg, respectively, while from SS for BE and AE were 28.7 and 28.0 µg, respectively. For the quantitative proteomics analysis, all samples were standardized to 28.0 µg of proteins.

For the comparison of SS, AE vs BE, the total number of proteins identified were 60 and 69 proteins, respectively, among which 57 proteins were common to both conditions, such as *Statherin* and *Histatin-1* and *-3* (Fig. 1A). Twelve proteins were identified exclusively BE, such as *Neutrophil defensin 1* and *-3*. Three proteins were identified exclusively AE such as *Haptoglobin* and *Lactotransferrin*. In the differently expressed proteins, 32 and 13 proteins were increased and decreased, respectively, in AE compared to BE. Among the down-regulated proteins AE were: 4 isoforms of amylase, *Salivary acidic proline-rich phosphoprotein ½*, *Immunoglobulin kappa constant* and 6 isoforms of hemoglobin. On the other hand, 10 isoforms of immunoglobulins, *Lysozyme C*, *Albumin*, *Mucin-7*, *Cystatin-S*, *Submaxillary gland androgen-regulated protein 3B*, *Carbonic anhydrase 6*, 5 isoforms of cystatins and 2 isoforms of proline-rich protein were up-regulated AE compared to BE (Table 2).

Figure 2 shows the functional analysis according to the biological process by Gene Ontologies (GO) with the most significant term, for the comparison of SS, AE vs BE. The categories with the high percentages of genes were retina homeostasis (31.9%) and phagocytosis, engulfment (21.3%) (Fig. 2). In the functional analysis according to the molecular function process by Gene Ontologies (GO) with the most significant term, for the comparison of SS, AE vs BE, it was shown the highest percentage of genes was hemoglobin alpha binding (55.6%) (Fig. 3). The functional analysis according to the cell component process by Gene Ontologies (GO) with the most significant term was also done in this comparison. The category with the highest percentage of genes was immunoglobulin complex, circulating secretory (66.7%) (Fig. 4). In the functional analysis according to the immune system process by Gene Ontology (GO) with the most significant term, for the comparison of SS, AE vs BE, it was shown that the category with the highest percentage of genes was immunoglobulin complex, circulating (46.2%) (Fig. 5).

When NSS was analyzed, AE vs BE, the total number of proteins identified were 65 and 76, respectively, with 58 proteins common to both conditions such as *Salivary acidic proline-rich phosphoprotein ½*, *Carbonic anhydrase 6*, 15 isoforms of immunoglobulins, 5 isoforms of cystatins, *Histatin-1* and *-3*, *Lactotransferrin*, *Neutrophil defensin 1* and *-3*, 4 isoforms of amylase, *Mucin-7*, *Lysozyme C*, *Statherin* and *Submaxillary gland androgen-regulated protein 3B* (Fig. 1B). Eighteen proteins were identified exclusively BE, such as *BPI proteins fold-containing family B member 1* and *Torsin-2A*, while 7 proteins were identified exclusively AE, such as *Apolipoprotein AI*, *Proline-rich protein 4* and *Serotransferrin*. In the differentially expressed proteins, only *Albumin* was increased AE, while 3 proteins were decreased, such as *Basic salivary proline-rich protein 1* and *-2* (Table 3).

For the comparison BE, SS vs NSS, the total number of proteins identified were 70 and 76, respectively, with 54 proteins common to both conditions such as *Albumin*, *Carbonic anhydrase 6*, *Histatin-1* and *-3*, *Cystatin-D* and

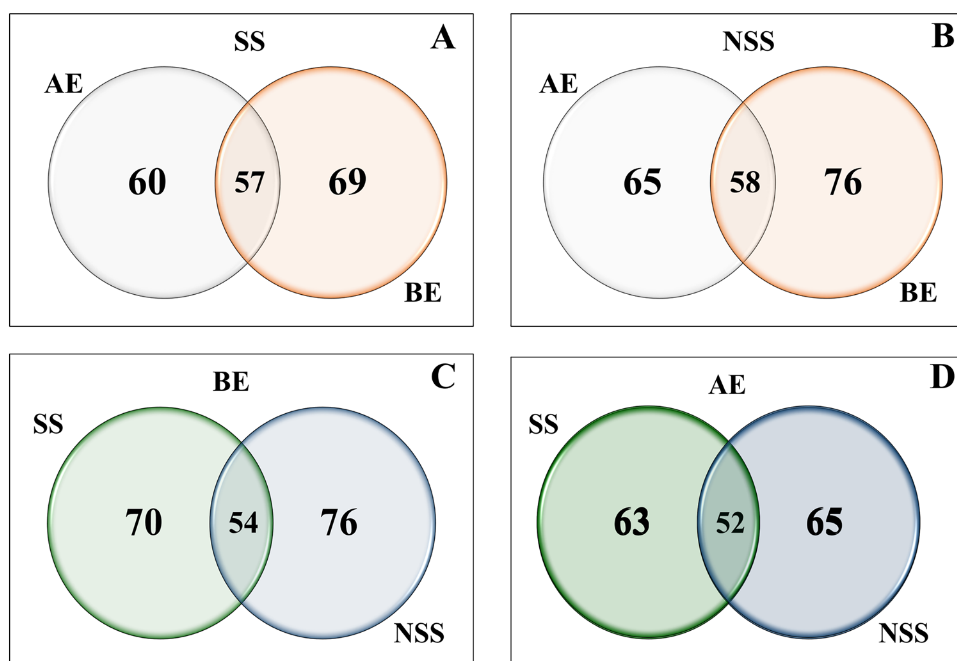

Fig. 1 Venn diagram with the total number of proteins identified in stimulated (SS) and non-stimulated (NSS) saliva before interval exercise (BE) and after interval exercise (AE). **A** comparison between AE vs BE in the stimulated salivary flow (SS). **B** comparison between AE vs BE in non-stimulated salivary flow (NSS). **C** comparison between SS vs NSS, BE. **D** comparison between SS vs NSS, AE

Table 2 Differential protein expression in the stimulated salivary flow (SS) before (BE) and after (AE) of exercise

[a]Access number	Protein name	*PLGS* Score	[b]Ratio SS AE:BE	*p*
P0DOY2	**Immunoglobulin lambda constant 2**	**158.33**	**3.22**	**< 0.01**
P0CG39	**POTE ankyrin domain family member J**	**49.28**	**3.19**	**< 0.01**
P0CF74	**Immunoglobulin lambda constant 6**	**158.33**	**3.06**	**< 0.01**
P0DOY3	**Immunoglobulin lambda constant 3**	**158.33**	**2.77**	**< 0.01**
P0CG04	**Immunoglobulin lambda constant 1**	**158.33**	**2.61**	**< 0.01**
P61626	**Lysozyme C**	**460.04**	**2.61**	**< 0.01**
P02768	**Albumin**	**712.78**	**2.44**	**< 0.01**
B9A064	**Immunoglobulin lambda-like polypeptide 5**	**158.33**	**2.36**	**< 0.01**
Q8TAX7	**Mucin-7**	**157.66**	**2.36**	**< 0.01**
P63261	**Actin_ cytoplasmic 2**	**166.61**	**2.34**	**< 0.01**
P60709	**Actin_ cytoplasmic 1**	**174.62**	**2.27**	**< 0.01**
P01876	**Immunoglobulin heavy constant alpha 1**	**568.29**	**2.16**	**< 0.01**
P01877	**Immunoglobulin heavy constant alpha 2**	**193.05**	**2.10**	**< 0.01**
P02814	Submaxillary gland androgen-regulated protein 3B	1377.11	1.95	< 0.01
P01036	Cystatin-S	1604.13	1.93	< 0.01
P01833	Polymeric immunoglobulin receptor	338.42	1.92	< 0.01
P23280	Carbonic anhydrase 6	1614.57	1.88	< 0.01
P01591	Immunoglobulin J chain	253.27	1.84	< 0.01
P12273	Prolactin-inducible protein	1339.51	1.84	< 0.01
P01857	Immunoglobulin heavy constant gamma 1	48.55	1.79	< 0.01
P31025	Lipocalin-1	611.49	1.73	< 0.01
P01034	Cystatin-C	229.61	1.70	< 0.01
Q96DA0	Zymogen granule protein 16 homolog B	213.86	1.65	< 0.01
Q5VSP4	Putative lipocalin 1-like protein 1	283.55	1.63	< 0.01
P09228	Cystatin-SA	430.26	1.58	< 0.01
P28325	Cystatin-D	1071.55	1.57	< 0.01
P01037	Cystatin-SN	1517.98	1.57	< 0.01
P01871	Immunoglobulin heavy constant mu	131.06	1.57	0.01
P04746	Pancreatic alpha-amylase	1766.53	1.43	< 0.01
Q96DR5	BPI fold-containing family A member 2	465.41	1.32	< 0.01
P04280	Basic salivary proline-rich protein 1	347.76	1.23	< 0.01
P02812	Basic salivary proline-rich protein 2	683.18	1.14	< 0.01
P0DTE7	Alpha-amylase 1B	2350.97	0.90	< 0.01
P0DTE8	Alpha-amylase 1C	2350.97	0.89	< 0.01
P19961	Alpha-amylase 2B	2022.82	0.88	< 0.01
P0DUB6	Alpha-amylase 1A	2383.53	0.87	< 0.01
P02810	Salivary acidic proline-rich phosphoprotein 1/2	2354.27	0.81	< 0.01
P01834	Immunoglobulin kappa constant	211.52	0.78	< 0.01
P0CG38	POTE ankyrin domain family member I	49.28	0.73	< 0.01
P68871	Hemoglobin subunit beta	2780.86	0.34	< 0.01
P69905	**Hemoglobin subunit alpha**	**2874.68**	**0.33**	**< 0.01**
P69891	**Hemoglobin subunit gamma-1**	**736.88**	**0.29**	**0.01**
P02042	**Hemoglobin subunit delta**	**906.38**	**0.29**	**0.02**
P69892	**Hemoglobin subunit gamma-2**	**736.88**	**0.28**	**< 0.01**
P02100	**Hemoglobin subunit epsilon**	**736.88**	**0.27**	**< 0.01**
P02647	Apolipoprotein A-I	96.59	BE*	–
Q4G163	F-box only protein 43	55.04	BE*	–
P00367	Glutamate dehydrogenase 1_ mitochondrial	41.48	BE*	–
P49448	Glutamate dehydrogenase 2_ mitochondrial	41.48	BE*	–
A0M8Q6	Immunoglobulin lambda constant 7	103.95	BE*	–

Table 2 (continued)

[a]Access number	Protein name	*PLGS* Score	[b]Ratio SS AE:BE	*p*
P13349	Myogenic factor 5	118.34	BE*	–
P59665	Neutrophil defensin 1	201.41	BE*	–
P59666	Neutrophil defensin 3	184.87	BE*	–
Q14207	Protein NPAT	29.99	BE*	–
Q8N392	Rho GTPase-activating protein 18	43.27	BE*	–
Q9UJT2	Testis-specific serine kinase substrate	45.54	BE*	–
Q6VVX0	Vitamin D 25-hydroxylase	66.29	BE*	–
P61769	Beta-2-microglobulin	69.31	AE*	–
P00738	Haptoglobin	61.64	AE*	–
P02788	Lactotransferrin	29.72	AE*	–

Differential expression among the conditions was expressed as $p<0.05$. Proteins highlighted in bold are increased or decreased more than twofold

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

[b]Proteins with significantly altered expression are organized according to the ratio

*Indicates unique protein in alphabetical order

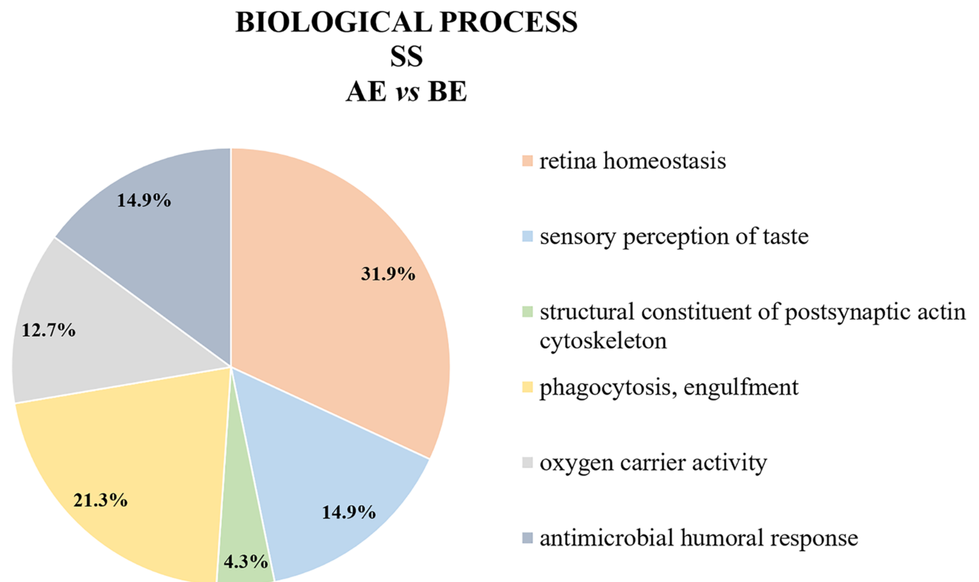

Fig. 2 Graphic of the functional distribution of proteins identified with differential expression in the SS after interval exercise (AE) vs. before interval exercise (BE). Protein categories based on the Gene Ontology (GO) annotation of the broad Biological Process. Terms of significance ($\kappa=0.04$) and distribution according to the percentage of the number of associated genes. The access number for the proteins was provided by UNIPROT. Genetic ontology was evaluated according to the ClueGo® pluggins of the Cytoscape® 3.8.2 software

5 isoforms of immunoglobulins. Sixteen proteins were identified exclusively SS, such as *Apolipoprotein A-I* and 6 isoforms of hemoglobin. Twenty-two proteins were identified exclusively NSS, such as *BPI fold-containing family B member 1*, 3 isoforms of immunoglobulins and *Lactotransferrin* (Fig. 1C). In the differentially expressed proteins, 10 proteins were increased in the SS, such as *Salivary acidic proline-rich phosphoprotein ½, Neutrophil defensin 1* and *-3*, *Basic salivary proline-rich protein 2* and 4 isoforms of amylases, while 27 proteins were decreased, such as 4 isoforms of cystatins, 7 isoforms of immunoglobulins, *Submaxillary gland androgen-regulated protein 3B*, *Statherin*, *BPI fold-containing family A member 2*, *Prolactin-inducible protein*, *Basic salivary proline-rich protein 1*, *Mucin-7* (decreased nearly fivefold) and *Lysozyme C* (decreased more than fivefold) (Table 4).

As for the comparison AE, SS vs NSS, the total number of proteins identified were 63 and 65, respectively, with 52 proteins being identified in common to both conditions, such as *Prolactin-inducible protein*, *Lactotransferrin*, *Basic salivary proline-rich protein 2*, *Histatin-1*, *Alpha-amylase 1C* and *Cystatin-D*. Eleven proteins were identified exclusively in the SS, such as 7 isoforms of hemoglobin. On the other hand, 13 proteins were identified exclusively in the NSS, such as *Apolipoprotein A-I*, 4 isoforms of immunoglobulins, *Proline-rich protein*

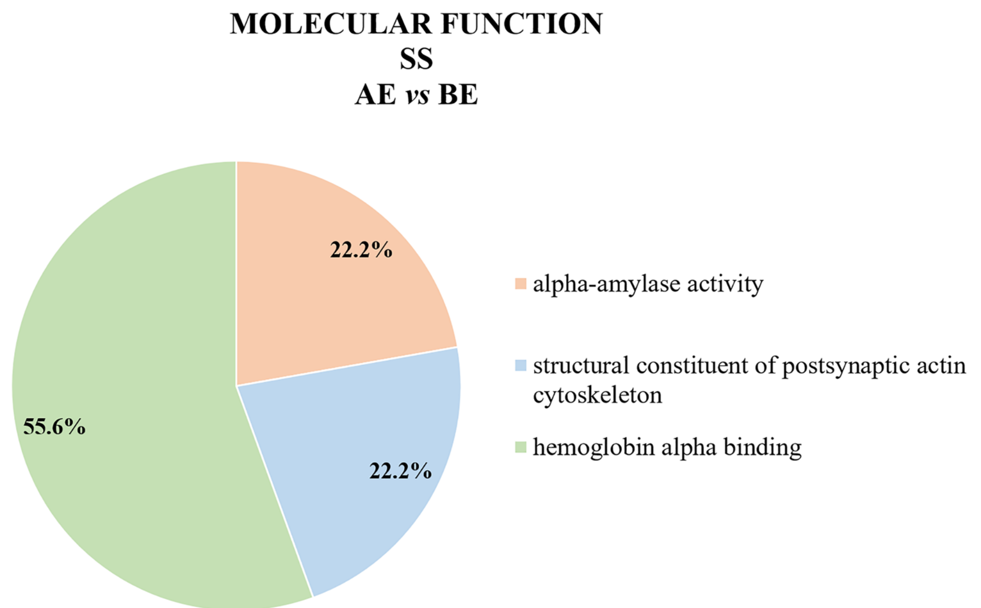

Fig. 3 Graphic of the functional distribution of proteins identified with differential expression in the SS after interval exercise (AE) vs. before interval exercise (BE). Protein categories based on the Gene Ontology (GO) annotation of the broad Molecular Function. Terms of significance ($\kappa = 0.04$) and distribution according to the percentage of the number of associated genes. The access number for the proteins was provided by UNIPROT. Genetic ontology was evaluated according to the ClueGo® pluggins of the Cytoscape® 3.8.2 software

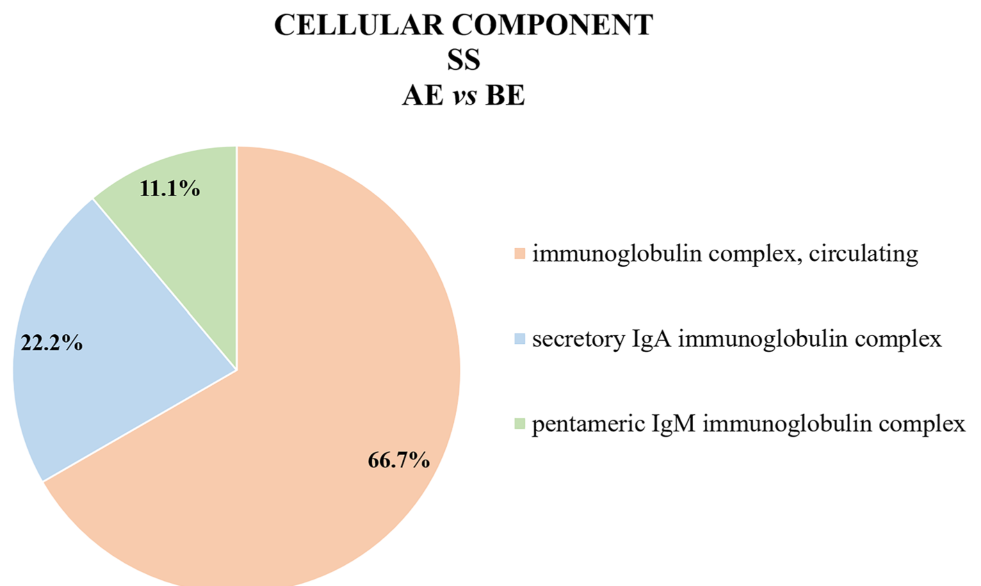

Fig. 4 Graphic of the functional distribution of proteins identified with differential expression in the SS after interval exercise (AE) vs. before interval exercise (BE). Protein categories based on the Gene Ontology (GO) annotation of the broad Cellular Component. Terms of significance ($\kappa = 0.04$) and distribution according to the percentage of the number of associated genes. The access number for the proteins was provided by UNIPROT. Genetic ontology was evaluated according to the ClueGo® pluggins of the Cytoscape® 3.8.2 software

4, *Serotransferrin* and *Neutrophil defensin 1* and *-3* (Fig. 1D). In the quantitative analysis, 10 proteins were increased in the SS, such as *Salivary acidic proline-rich phosphoprotein ½*, *Basic salivary proline-rich protein 1*, 2 isoforms of immunoglobulins, *Statherin*, *Carbonic anhydrase 6* and *Alpha-amylase 2B*, while 27 proteins were decreased, such as 6 isoforms of immunoglobulins, *Alpha-amylase 1A* and *-1B*, *Mucin-7*, *Albumin*, *Lysozyme C*, *Submaxillary gland androgen-regulated protein 3B*, *Histatin-3* and 4 isoforms of cystatins (Table 5).

Discussion

The search for markers through saliva has numerous advantages, mainly because its collection is not invasive, as in blood analysis, for example. In addition, saliva provides the identification of an arsenal of proteins and consequently biomarkers typical for the oral cavity and related to systemic health or disease either [29, 30]. Little is known about the difference in protein profile between

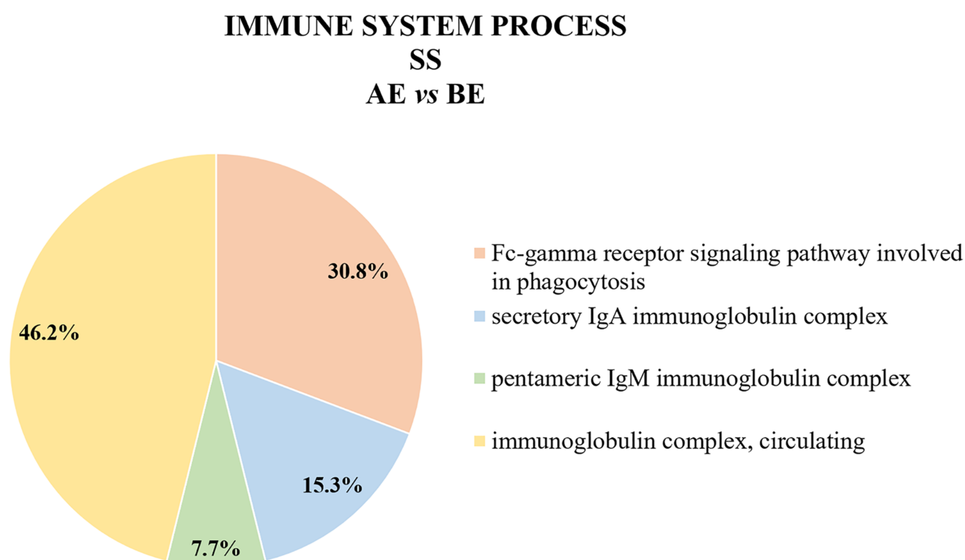

Fig. 5 Graphic of the functional distribution of proteins identified with differential expression in the SS after interval exercise (AE) vs. before interval exercise (BE). Protein categories based on the Gene Ontology (GO) annotation of the broad Immune System Process. Terms of significance ($\kappa = 0.04$) and distribution according to the percentage of the number of associated genes. The access number for the proteins was provided by UNIPROT. Genetic ontology was evaluated according to the ClueGo® pluggins of the Cytoscape® 3.8.2 software

the different salivary flows (stimulated and unstimulated) before and after exercise. Our study highlights important information about changes in salivary proteomic profile after interval exercise (low to moderate intensity), which may have an impact on oral and general health.

SS brings more information than NSS with this respect. When BE and AE were compared in the SS, it was observed an increase in many proteins involved with responses to the immune system. Among these findings, various immunoglobulins were increased AE compared to BE, among which 8 were increased more than twofold (Table 2). It is known that the practice of exercise, in general, provides great health benefits [31]. Although our results were not from a longer follow-up after exercise, our data suggest that the practice of exercise, under interval method, even if it is just a walk (as done in the present study), may provide important changes in immunity. This finding was supported by the results of the most affected genes. In the biological process, 15% of these proteins were involved with humoral antimicrobial response and 21.3% were related to phagocytosis activity. Checking the great interaction of these proteins involved with immune responses, almost 50% were involved in immunoglobulin complex circulating (Fig. 5). The immunoglobulin complex has the function of binding to the antigen if a suitable antigen is available. In addition, this complex is usually found in areas of mucosa, blood or lymph [32]. It is important to bear in mind that the increase of immunity was seen under the interval exercise tested in the present study, since it has been shown that the practice of exercise under high intensity for prolonged periods can decrease immunity [18], while the regular exercise of moderate intensity is beneficial, improving immunity throughout life [33] and stimulating immune functions [34]. On the other hand, longer follow-up after physical exercise shall be evaluated in future studies.

The practice of exercise also has a beneficial influence on oral health. *Mucin-7*, *Lysozyme C, Albumin,* cystatins and proline-rich proteins (PRPs) were increased in stimulated saliva AE in comparison to BE (Table 2). *Mucin-7* is an acid-resistant protein, which was identified in the acquired pellicle after episodes of erosive challenges [35]. In addition, it participates in important processes related to immune response, such as antimicrobial activity, and is involved with essential processes for mucosal protection and wound healing [36]. This protein has a protective capacity, promoting the elimination of bacteria in the oral cavity, assisting chewing, speech and swallowing [37]. In this perspective, *Lysozyme C,* also increased AE, has a bacteriological function, mainly in tissues and body fluids associated with the monocyte–macrophage system, which increases the activity of immune agents; in addition, it is related to antimicrobial humoral response (defense responses to gram-positive and gram-negative bacteria) and participates in inflammatory responses [37]. In addition, in a previous study, *Lysozyme* was increased in saliva after 30 min of steady-state cycling exercise [38].

On the other hand, six isoforms of the hemoglobin (*Hemoglobin subunit alpha, subunit delta, subunit epsilon, subunit gamma-1* and *gamma-2*) were found reduced AE compared to BE (Table 2). The decrease in these proteins may be due to the vasoconstriction in response to the sympathetic stimulation caused by the practice of exercise [39]. Exercise increases sympathetic vasoconstrictive responsiveness through a nitric oxide-dependent mechanism. When the exercise is prolonged, there are additional constricting adjustments in the less active tissues [40]. This constricting action allows the correct redistribution of blood to satisfy the needs of active muscles [41]. Among the processes of the most affected genes evaluated from the bioinformatics

Table 3 Differential protein expression in the non-stimulated salivary flow (NSS) before (BE) and after (AE) of exercise

[a]Access number	Protein name	*PLGS* Score	[b]Ratio NSS AE:BE	*P*
P02768	Albumin	542.96	1.36	< 0.01
P02812	Basic salivary proline-rich protein 2	821.73	0.59	0.02
Q96DA0	**Zymogen granule protein 16 homolog B**	**200.9**	**0.40**	**< 0.01**
P04280	**Basic salivary proline-rich protein 1**	**519.44**	**0.18**	**< 0.01**
Q8TDL5	BPI fold-containing family B member 1	156.14	BE*	–
P54108	Cysteine-rich secretory protein 3	126.11	BE*	–
Q8ND71	GTPase IMAP family member 8	74.66	BE*	–
P49450	Histone H3-like centromeric protein A	95.42	BE*	–
P13646	Keratin_ type I cytoskeletal 13	144.2	BE*	–
P19013	Keratin_ type II cytoskeletal 4	94.98	BE*	–
P22079	Lactoperoxidase	27.58	BE*	–
Q8NHM5	Lysine-specific demethylase 2B	16.1	BE*	–
Q9H173	Nucleotide exchange factor SIL1	108.99	BE*	–
Q9UMX2	Ornithine decarboxylase antizyme 3	92.4	BE*	–
Q8N2E6	Prosalusin	80.65	BE*	–
Q6ZSV7	Putative uncharacterized protein FLJ45177	187.82	BE*	–
Q9NSD7	Relaxin-3 receptor 1	86.69	BE*	–
Q5JU69	Torsin-2A	86.29	BE*	–
Q9BXT4	Tudor domain-containing protein 1	21.12	BE*	–
Q8N1X5	Uncharacterized protein FLJ37310	130.76	BE*	–
Q8IZS8	Voltage-dependent calcium channel subunit alpha-2/delta-3	93.91	BE*	–
A6NE52	WD repeat-containing protein 97	22.32	BE*	–
P02647	Apolipoprotein A-I	89.42	AE*	–
P61769	Beta-2-microglobulin	166.24	AE*	–
Q14687	Genetic suppressor element 1	62.34	AE*	–
Q16378	Proline-rich protein 4	253.04	AE*	–
Q8N8G6	Putative uncharacterized protein C15orf54	88.95	AE*	–
P02787	Serotransferrin	28.75	AE*	–
Q8TAL5	Uncharacterized protein C9orf43	93.35	AE*	–

Differential expression among the conditions was expressed as $p < 0.05$. Proteins highlighted in bold are increased or decreased more than twofold

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

[b]Proteins with significantly altered expression are organized according to the ratio

*Indicates unique protein in alphabetical order

analysis, we verified that in the biological processes 12.8% of these proteins were involved with oxygen-carrying activity (Fig. 2). We believe that this condition is directly related to the identified hemoglobin, since the most affected processes of molecular function with the highest percentage (55.6%) were related to hemoglobin bonds, mainly of the alpha subunit (Fig. 3).

These six isoforms of hemoglobin have already been identified in saliva in previous studies, where the salivary proteome was evaluated [27]. It is important to consider that these proteins may also have come from the crevicular fluid, where the hemoglobin alpha, beta and delta subunits have also been previously identified [42]. Another important point is that hemoglobin was reduced in SS compared to NSS, which is expected, since during stimulation, the parasympathetic action is predominant [8, 16, 43].

Regarding NSS, it was observed a decrease in basic proline-rich proteins and an increase in albumin in the results. In addition, albumin has been increased in saliva after moderate exercise and high-intensity sports [44]. However, no data have been identified in the literature on the increase of albumin in saliva under low-intensity exercises. Thus, to the best of our knowledge, this is the first study to report this finding. The increase in albumin in saliva seems to be related to physical activity regardless of the intensity. Interesting, lipids used as energy source during exercise are included in

Table 4 Differential protein expression in the stimulated (SS) and non-stimulated (NSS) salivary flow before exercise (BE)

[a]Access number	Protein name	*PLGS* Score	[b]Ratio BE SS:NSS	*P*
P0CG39	**POTE ankyrin domain family member J**	**68.57**	**3.39**	**< 0.01**
P02810	**Salivary acidic proline-rich phosphoprotein 1/2**	**354.96**	**3.29**	**< 0.01**
P59665	**Neutrophil defensin 1**	**258.72**	**2.39**	**< 0.01**
P59666	**Neutrophil defensin 3**	**258.72**	**2.23**	**0.01**
Q9BYX7	Putative beta-actin-like protein 3	112.81	1.70	0.01
P02812	Basic salivary proline-rich protein 2	821.73	1.42	< 0.01
P0DTE8	Alpha-amylase 1C	1531.78	1.23	< 0.01
P19961	Alpha-amylase 2B	1476.86	1.21	< 0.01
P0DTE7	Alpha-amylase 1B	1531.78	1.17	< 0.01
P0DUB6	Alpha-amylase 1A	1531.78	1.07	< 0.01
P01037	Cystatin-SN	729.02	0.88	< 0.01
P63261	Actin_ cytoplasmic 2	96.75	0.80	0.03
P02814	Submaxillary gland androgen-regulated protein 3B	2569.12	0.78	< 0.01
Q6S8J3	POTE ankyrin domain family member E	100.37	0.77	0.04
P68032	Actin_ alpha cardiac muscle 1	96.75	0.74	0.01
P68133	Actin_ alpha skeletal muscle	96.75	0.74	< 0.01
P62736	Actin_ aortic smooth muscle	96.75	0.73	0.01
P60709	Actin_ cytoplasmic 1	96.75	0.73	< 0.01
P63267	Actin_ gamma-enteric smooth muscle	96.75	0.73	< 0.01
P02808	Statherin	1459.30	0.71	0.04
P01034	Cystatin-C	257.34	0.70	0.02
Q96DR5	BPI fold-containing family A member 2	225.00	0.64	< 0.01
P01036	Cystatin-S	592.31	0.63	< 0.01
P01833	Polymeric immunoglobulin receptor	615.66	0.57	< 0.01
P0DOY3	Immunoglobulin lambda constant 3	207.25	0.55	< 0.01
P12273	Prolactin-inducible protein	649.70	0.55	< 0.01
P09228	Cystatin-SA	196.32	0.53	< 0.01
B9A064	Immunoglobulin lambda-like polypeptide 5	207.25	0.53	< 0.01
P0CF74	Immunoglobulin lambda constant 6	207.25	0.52	< 0.01
P0CG04	Immunoglobulin lambda constant 1	207.25	0.52	< 0.01
P0DOY2	Immunoglobulin lambda constant 2	207.25	0.52	< 0.01
P01876	**Immunoglobulin heavy constant alpha 1**	**471.10**	**0.45**	**< 0.01**
P01877	**Immunoglobulin heavy constant alpha 2**	**241.35**	**0.44**	**< 0.01**
P04280	**Basic salivary proline-rich protein 1**	**519.44**	**0.34**	**< 0.01**
Q8TAX7	**Mucin-7**	**341.37**	**0.33**	**< 0.01**
Q96DA0	**Zymogen granule protein 16 homolog B**	**200.90**	**0.21**	**< 0.01**
P61626	**Lysozyme C**	**625.56**	**0.20**	**< 0.01**
P02647	Apolipoprotein A-I	96.59	SS *	–
Q4G163	F-box only protein 43	55.04	SS *	–
P00367	Glutamate dehydrogenase 1_ mitochondrial	41.48	SS *	–
P49448	Glutamate dehydrogenase 2_ mitochondrial	41.48	SS *	–
P69905	Hemoglobin subunit alpha	2874.68	SS *	–
P68871	Hemoglobin subunit beta	2780.86	SS *	–
P02042	Hemoglobin subunit delta	906.38	SS *	–
P02100	Hemoglobin subunit epsilon	736.88	SS *	–
P69891	Hemoglobin subunit gamma-1	736.88	SS *	–
P69892	Hemoglobin subunit gamma-2	736.88	SS *	–
P13349	Myogenic factor 5	118.34	SS *	–
Q14207	Protein NPAT	29.99	SS *	–

Table 4 (continued)

[a]Access number	Protein name	*PLGS* Score	[b]Ratio BE SS:NSS	*P*
Q8N392	Rho GTPase-activating protein 18	43.27	SS *	–
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1	64	SS *	–
Q9UJT2	Testis-specific serine kinase substrate	45.54	SS *	–
Q6VVX0	Vitamin D 25-hydroxylase	66.29	SS *	–
P03973	Antileukoproteinase	113.66	NSS*	–
Q8TDL5	BPI fold-containing family B member 1	156.14	NSS*	–
P54108	Cysteine-rich secretory protein 3	126.11	NSS*	–
Q8ND71	GTPase IMAP family member 8	74.66	NSS*	–
P49450	Histone H3-like centromeric protein A	95.42	NSS*	–
P01859	Immunoglobulin heavy constant gamma 2	78.28	NSS*	–
P01860	Immunoglobulin heavy constant gamma 3	75.5	NSS*	–
P01861	Immunoglobulin heavy constant gamma 4	75.5	NSS*	–
P13646	Keratin_ type I cytoskeletal 13	144.2	NSS*	–
P19013	Keratin_ type II cytoskeletal 4	94.98	NSS*	–
P02788	Lactotransferrin	46.68	NSS*	–
Q8NHM5	Lysine-specific demethylase 2B	16.1	NSS*	–
Q9H173	Nucleotide exchange factor SIL1	108.99	NSS*	–
Q9UMX2	Ornithine decarboxylase antizyme 3	92.4	NSS*	–
Q8N2E6	Prosalusin	80.65	NSS*	–
Q6ZSV7	Putative uncharacterized protein FLJ45177	187.82	NSS*	–
Q9NSD7	Relaxin-3 receptor 1	86.69	NSS*	–
Q5JU69	Torsin-2[a]	86.29	NSS*	–
Q9BXT4	Tudor domain-containing protein 1	21.12	NSS*	–
Q8N1X5	Uncharacterized protein FLJ37310	130.76	NSS*	–
Q8IZS8	Voltage-dependent calcium channel subunit alpha-2/delta-3	93.91	NSS*	–
A6NE52	WD repeat-containing protein 97	22.32	NSS*	–

Differential expression among the conditions was expressed as $p < 0.05$. Proteins highlighted in bold are increased or decreased more than twofold

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

[b]Proteins with significantly altered expression are organized according to the ratio

*Indicates unique protein in alphabetical order

blood circulation bounded to albumin [45], which should be considered in future studies.

Based on our results, it is clear that the interval exercise did not have the same clear impact on NSS as it did for SS. Although NSS is considered the most important flow for the formation of the acquired pellicle and the protection of dental surface and mucosa [16, 43], during exercise the stimulated saliva seems to provide better data for assessing the protein profile of saliva and to correlate the findings with general health. One of the reasons is related to the time of collection, SS was collected 15 min after the end of the exercise, allowing those proteins from blood be found in saliva. Other important point to take into consideration is the role of glands in the production of SS and NSS, while parotid is very important under mechanical stimulation, submandibular has a great contribution for NSS (Proctor 2016), which may impact in its proteomic profile. These findings should be confirmed in future studies.

On the other hand, *Apolipoprotein A-I* was identified exclusively AE when compared to BE in NSS (Table 3) and when compared SS vs NSS AE (Table 5). This protein participates in the reverse transport of cholesterol from tissues to the liver for excretion, promoting the efflux of cholesterol from tissues and acting as a cofactor for lecithin cholesterol acyltransferase (information from UNIPROT). In addition, *Apolipoprotein A-I* binds to HDL (High-Density Lipoproteins), which removes excess cholesterol and takes it back to the liver, to be eliminated from the body [46]. Thus, even with low to moderate exercise, it is possible to observe that the lipid metabolism is activated, with possible increase in HDL level. *Apolipoprotein A-I* has also been identified in resistance training [47].

Table 5 Differential protein expression in the stimulated (SS) and non-stimulated (NSS) salivary flow after exercise (AE)

[a]Access number	Protein name	*PLGS* Score	[b]Ratio AE SS:NSS	*P*
P02810	**Salivary acidic proline-rich phosphoprotein 1/2**	**491.01**	**5.00**	**< 0.01**
P0CG38	**POTE ankyrin domain family member I**	**22.60**	**4.18**	**< 0.01**
Q9BYX7	**Putative beta-actin-like protein 3**	**127.58**	**2.61**	**< 0.01**
P04280	**Basic salivary proline-rich protein 1**	**403.94**	**2.48**	**< 0.01**
P01857	Immunoglobulin heavy constant gamma 1	238.32	1.75	< 0.01
P01871	Immunoglobulin heavy constant mu	219.17	1.62	0.01
P02808	Statherin	5095.56	1.57	0.02
Q6S8J3	POTE ankyrin domain family member E	149.93	1.22	0.04
P23280	Carbonic anhydrase 6	617.58	1.20	0.02
P19961	Alpha-amylase 2B	1282.61	1.07	0.01
P0DTE7	Alpha-amylase 1B	1314.24	0.97	0.01
P63261	Actin_ cytoplasmic 2	201.82	0.85	0.02
A5A3E0	POTE ankyrin domain family member F	146.58	0.84	0.03
P0DUB6	Alpha-amylase 1A	1329.86	0.84	< 0.01
Q8TAX7	Mucin-7	171.49	0.82	< 0.01
P02768	Albumin	361.14	0.79	< 0.01
P61626	Lysozyme C	242.92	0.79	< 0.01
P62736	Actin_ aortic smooth muscle	196.52	0.76	< 0.01
Q562R1	Beta-actin-like protein 2	179.58	0.67	< 0.01
P02814	Submaxillary gland androgen-regulated protein 3B	935.75	0.53	< 0.01
Q96DR5	BPI fold-containing family A member 2	307.32	0.52	< 0.01
P0CG04	**Immunoglobulin lambda constant 1**	**172.60**	**0.50**	**< 0.01**
P31025	**Lipocalin-1**	**153.08**	**0.50**	**< 0.01**
Q5VSP4	**Putative lipocalin 1-like protein 1**	**78.82**	**0.47**	**< 0.01**
P01037	**Cystatin-SN**	**670.05**	**0.46**	**< 0.01**
P0DOY3	**Immunoglobulin lambda constant 3**	**172.60**	**0.43**	**< 0.01**
P0DOY2	**Immunoglobulin lambda constant 2**	**172.60**	**0.42**	**< 0.01**
P0CF74	**Immunoglobulin lambda constant 6**	**172.60**	**0.42**	**< 0.01**
B9A064	**Immunoglobulin lambda-like polypeptide 5**	**172.60**	**0.42**	**< 0.01**
Q96DA0	**Zymogen granule protein 16 homolog B**	**103.68**	**0.42**	**< 0.01**
P01833	**Polymeric immunoglobulin receptor**	**230.84**	**0.41**	**< 0.01**
P01034	**Cystatin-C**	**199.46**	**0.36**	**< 0.01**
P15516	**Histatin-3**	**1985.98**	**0.36**	**< 0.01**
P01876	**Immunoglobulin heavy constant alpha 1**	**213.66**	**0.35**	**< 0.01**
P01877	**Immunoglobulin heavy constant alpha 2**	**169.30**	**0.34**	**< 0.01**
P01036	**Cystatin-S**	**1540.09**	**0.27**	**< 0.01**
P09228	**Cystatin-SA**	**429.62**	**0.27**	**< 0.01**
A0A1W2PQ73	ETS domain-containing transcription factor ERF-like	94.17	SS*	–
P00738	Haptoglobin	61.64	SS*	–
P69905	Hemoglobin subunit alpha	926.78	SS*	–
P68871	Hemoglobin subunit beta	1450.41	SS*	–
P02042	Hemoglobin subunit delta	158.03	SS*	–
P02100	Hemoglobin subunit epsilon	158.03	SS*	–
P69891	Hemoglobin subunit gamma-1	238.12	SS*	–
P69892	Hemoglobin subunit gamma-2	205.72	SS*	–
P22079	Lactoperoxidase	52.82	SS*	–
Q6IED9	Putative diacylglycerol	159.18	SS*	–
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1	66.9	SS*	–
P03973	Antileukoproteinase	119.85	NSS*	–

Table 5 (continued)

[a]Access number	Protein name	*PLGS* Score	[b]Ratio AE SS:NSS	*P*
P02647	Apolipoprotein A-I	89.42	NSS*	–
Q14687	Genetic suppressor element 1	62.34	NSS*	–
P01859	Immunoglobulin heavy constant gamma 2	84.35	NSS*	–
P01860	Immunoglobulin heavy constant gamma 3	51.87	NSS*	–
P01861	Immunoglobulin heavy constant gamma 4	51.87	NSS*	–
A0M8Q6	Immunoglobulin lambda constant 7	87.71	NSS*	–
P59665	Neutrophil defensin 1	416.78	NSS*	–
P59666	Neutrophil defensin 3	416.78	NSS*	–
Q16378	Proline-rich protein 4	253.04	NSS*	–
Q8N8G6	Putative uncharacterized protein C15orf54	88.95	NSS*	–
P02787	Serotransferrin	28.75	NSS*	–
Q8TAL5	Uncharacterized protein C9orf43	93.35	NSS*	–

Differential expression among the conditions was expressed as $p < 0.05$. Proteins highlighted in bold are increased or decreased more than two-fold

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

[b]Proteins with significantly altered expression are organized according to the ratio

*Indicates unique protein in alphabetical order

When we compared SS and NSS, BE, we observed that PRPs, amylases, *Neutrophil defensin 1* and *3* were increased in SS when compared to NSS. *Statherin*, cystatins isoforms, immunoglobulins isoforms, *Prolactin-inducible protein, Mucin-7, Lysozyme C* and *Basic salivary proline-rich protein 1* were reduced in SS when compared to NSS. *Statherin* is a salivary protein that stabilizes the calcium saturation by inhibiting the precipitation of calcium phosphate salts under neutral pH. It also modulates the formation of hydroxyapatite crystals on the tooth surface [37]. Regarding proline-rich proteins (PRPs), these are a heterogeneous group of proteins that are exclusively secreted by the parotid gland. The acidic PRPs inhibit the formation of hydroxyapatite and the basic PRPs strongly bind to the diet's tannins, avoiding their toxic effects by blocking their intestinal absorption [48]. In addition, PRPs act as highly potent inhibitors of the growth of calcium phosphate crystals under neutral pH, providing a protective and repairing environment for tooth enamel [37].

In general, proteins involved with antimicrobial and acid-resistant protection were reduced in SS when compared to NSS before exercise, which is expected, since in SS, the parasympathetic stimulation is predominant, with consequent high release of water and ions [8, 49] (Table 4).

The same findings were shown, when we compared the different salivary flows AE. We observed that isoforms of immunoglobulins, PRPs and *Statherin* were increased in the SS compared to NSS. On the other hand, amylases, *Albumin, Mucin-7, Lysozyme C,* cystatins, *Histatin-3* and isoforms of immunoglobulins were decreased in SS compared to NSS. In addition, *Apolipoprotein A-I,* isoforms of immunoglobulins, *Neutrophil defensin 1* and *3,* PRPs and *Serotransferrin* were identified exclusively in the NSS. Therefore, regardless of performing exercise (BE or AE), it is expected to find a low number of proteins in SS compared to NSS. Finally, the identification of amylases is very important, because these proteins are known as biomarkers of stress, anxiety and depression [50]. In our study, these proteins were reduced in saliva under stimulated flow after physical exercise, which demonstrates the benefits of physical activity. Furthermore, variations of amylase levels in saliva may be a response, especially in cases of intense training, to the need of individual ingests food energy after exercise [51, 52].

In our study, SS seems to be better at differentiating the effect of exercise on the salivary proteomic profile compared to NSS. On the other hand, in real life NSS is predominant over SS, which only happens during stimulation induced by meals (less than 1–2 h day^{-1}) [49, 53, 54]. This should be considered in the interpretation of the findings and in planning new studies. The stimulation of salivary flow after exercise should be considered in future studies. Moreover, it is interesting to further evaluate the effect of interval exercise for long-term periods, to verify how long the increased immunity-related proteins remain in saliva and their impact on oral and systemic conditions. In addition, these findings can still be evaluated in future studies with a larger number of participants.

From the results of this preliminary study, we can conclude that proteomic changes are more evident for stimulated than non-stimulated saliva and that the tested exercise protocol induced an increase of proteins related to

immunity, which shall be confirmed in future studies with higher number of participants.

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Author contributions TMOV and KOS: participated in the data acquisition, methodology, formal analysis, interpretation of the data and wrote the original draft. ASB and LTGT: participated in the data acquisition and methodology. MARB, FAB, CAK-F and MHF: participated in the methodology and collaboration with the interpretation of the data and draft revision of the manuscript. ACM: designed the study, participated in the data acquisition, methodology, formal analysis, interpretation of the data, wrote the original draft and supervision. All authors read and approved the manuscript.

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Availability of data and materials The mass spectrometric proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier (PXD028085).

Code availability Data set identifier PXD028085 in the PRIDE.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval This study was approved to the local Institutional Ethics Committee (No. 29182720.3.0000.5417). The study was carried out in accordance with the Declaration of Helsinki.

Consent to participate The collection was done after approval and the signature of the informed consent form.

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