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ARTICLE

TIME-DEPENDENT CHANGES IN THE EARLY SALIVARY PROTEOME AFTER THE ORAL STIMULATION WITH WINE DIFFERS BY INDIVIDUAL 6-n-PROPYLTHIOURACIL (PROP) TASTER STATUS

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Differences in the oral responsiveness to the bitter compound 6-n-propylthiouracil (PROP) between taster (Ts) and non-taster (NTs) individuals has also been related to differences in the long-lasting wine astringency perception, which could be linked to differences in the dynamics of salivary protein profile upon wine stimulation, depending on individual PROP taste status (PTS). To check this, the time-course changes in the early protein salivary profile (30 and 60 seconds) after the oral stimulation with a red wine (CRW) and with the same tannin-enriched wine (TRW) in Ts and NTs individuals (young women) was tested by using an untargeted proteomic approach. Results showed that Ts exhibited more pronounced protein changes (measured as the ratio of protein abundance before and after wine stimulation), compared to NTs, including proteins such as cystatins (SN, S, SA and D), α-amylase, prolactin (PIP), carbonic anhydrase VI (CA-VI) and acid proline-rich proteins (aPRP). These changes were more evident 30s (t1) than 60 s (t2) after the oral exposure to the wine and they were of higher magnitude after the exposure to TRW wine. These results suggest that differences in the salivary proteome profile induced by the oral stimulation with wine depending on PTS, might contribute to explain individual variations in wine astringency perception over time.

1. Introduction

Individual variations in taste perception are of outmost importance for explaining food preferences and food choice [1]. The higher or lower sensitivity to the bitter compound 6-n-propylthiouracil (PROP) has been widely used to classify individuals depending on their taste sensitivity [2].

PROP taster status (PTS) is the phenotypical manifestation of polymorphisms in the TAS2R38 gene, which codes for the bitter taste receptors TAS2R38 [3]. These are G-protein coupled receptors, localized in the oral cavity in taste buds embedded in the epithelium of the gustatory papillae on the tongue and palate [4]. Individuals with homozygous dominant alleles for this gene (PAV/PAV) experience greater bitterness from food and beverages and are called super-tasters (STs). In contrast, individuals who are recessive for this gene (AVI/AVI), are phenotypically non-tasters (NTs) and experience little PROP bitterness in comparison to STs. Heterozygotes individuals perceive moderate bitterness from PROP and are classified as medium tasters (MTs) [3].

PROP taster individuals have also shown higher intense sensitivity for different prototypical tastes (acid, sweet, salty) and other oro-sensory qualities including astringency [5], fat [6] and olfactive stimuli [7]. However, greater PROP bitterness does not always associate with heightened sensations for all oral stimuli [8,9]. Recently, Norden and co-authors suggested that the suprathreshold intensity of PROP is a confounded phenotype that captures both genetic variation specific to N–C=S chemical compounds (such as PROP) and overall oro-sensation [10]. This is why in spite of the correlation of PROP bitterness and TAS2R38 genotype, this genotype does not always correlate with the intensity of stimuli of different nature (burning of capsaicin, sweetness of sucrose, etc.) [10].

In any case, differences in PROP responsiveness have also been associated to other individual traits, such as the number and density of fungiform papillae or the polymorphism in the CA-VI gen (gustin) [11,12], although some other studies did not found this association [13,14]. On top of this, some studies have provided evidences on the effect of PROP phenotype in oral tactile sensations [15,16] and in some saliva proteins [17,18], which seems of interest to relate this phenotype with astringency perception.

Astringency is a tactile sensation perceived on the human palate and has been defined as a complex group of sensations involving dryness, tightening and shrinking of the oral surface and puckering sensations of the oral cavity [4]. The most established mechanism for astringency involves the interaction between polyphenols (mostly tannins) and salivary proteins (acidic PRPs, histatines, cystatins, etc.) to form insoluble aggregates that when size increased, precipitate [19]. Other higher molecular weight salivary proteins mostly adsorbed onto oral surfaces (basic PRPs, mucins) can also interact with polyphenols and form large aggregates damaging the protective lubricating layers or mucosal pellicle also contributing to astringency [4].

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The relationship of PROP phenotype and wine astringency has been studied yielding conflicting results, with some authors showing a positive relationship [5], while others not [20–22]. Besides the use of different methodologies, all of these studies rated astringency at one single point. However, as previously shown, this sensation evolves over time [23] giving rise to the perception of different astringency sub-modalities [24] and pointing out the importance of using Time-Intensity (T-I) methods for better assessing it [24,25]. In this way, recently, Velázquez and co-authors, using T-I, found significant differences in overall wine astringency depending on PTS, with taster individuals showing significantly higher astringency perception than NTs [26].

Whether changes in astringency (or astringency sub-modalities) over time could be related to time-dependent changes in salivary proteins (SPs) has been scarcely investigated. Additionally, most previous studies trying to elucidate the role of SPs were bioassays performed with selected SPs rather than considering whole saliva samples in which many different types of proteins are present [4]. Nonetheless, pioneers' studies on this topic provided interesting findings, showing that subjects who can maintain relatively constant SP levels after exposure to astringent stimuli (tannic acid) experience less astringency, whereas the inability to replenish these levels was associated with higher experience of astringency, especially upon repeated sampling [27,28]. In a further study, using a more complex polyphenol beverage (cranberry juice), Melis and co-authors showed that saliva of PROP STs had higher levels of acidic PRPs and Cystatins in comparison to that of NTs one minute after stimulation [29]. Using the same type of stimuli, in a more recent study Yousaf and colleagues, showed that STs had higher levels of salivary α-amylase than NTs after stimulation. They also found a gender effect with men STs exhibiting higher levels of basic PRPs after oral stimulation when compared to women STs, which was not observed in NTs individuals [30].

These previous studies arise the hypothesis that recent differences observed between PROP phenotypes in wine astringency perception using dynamic sensory methods (T-I) [26] could be related to differences in the salivary protein profile induced by wine, and that these changes could be different depending on PTS. Therefore, the main objective of this study was to compare the time-course changes in the early protein salivary profile (30 and 60 seconds) after the oral stimulation with a red wine depending on the individual taste PROP phenotype (Taster and Non-Taster individuals) by using an untargeted proteomic approach. Additionally, since the effect of tannins in the specificity of certain salivary proteins to complex and precipitate has been proven with other polyphenol-rich beverages [80], a second objective, was to check if the observed effect (changes in the early salivary proteome) was the same when oral stimulation was with the same wine spiked with a hydrolysable tannin (gallotanin) widely used in winemaking to improve wine quality.

2. Materials and Methods

2.1. Wine Samples

A red wine from the Tempranillo red grape variety was industrially produced at the IMIDRA experimental winery in Alcalá de Henares (Madrid, Spain). This wine was considered as a control wine (CRW). The chemical composition was: ethanol concentration: 13,9 %, pH 3.5, titratable acidity: 5,09 g/L, volatile acidity: 0.25 g/L, tartaric, malic and lactic acids: 2,84, 0,03 and 0,62 g/L respectively. This wine was spiked with a commercial hydrolysable oenotannin (gallic tannin with 1.4 mg/L gallic acid equivalent of total polyphenols) [26] provided by Laffort Ibérica S.A. This tannin was added to the control wine before bottling at a concentration of 300 mg/L (usual dosage recommended by the provider) to have a second wine type named as tannin-enriched wine (TRW).

2.2. Participants

For this study, 12 female individuals between 18 and 35 years old (average 23 y.o) belonging to two different PROP taste phenotypes (6 individuals Tasters and 6 Non-Taster) were selected by their responsiveness to the bitter compound 6-n-propylthiouracil [22]. As indicated in this study, depending on the intensity scores provided in the gLMS scale, individuals were classified in three groups (tertiles): non-tasters (NTs, belonging to the first tertile), medium tasters (MTs) (belonging to the second tertile) and supertasters (STs) (belonging to the third tertile). In the present study, all the individuals classified as tasters (T) belonged to the third tertile, while Non-Tasters (NT) were individuals from the first tertile. Inclusion criteria also were to be healthy, non-pregnant adults with a minimum Total salivary Protein Concentration (TPC) of 2 mg/mL which was determined using the commercial Pierce™ BCA Protein Assay Kit (Pierce ThermoScientific, Rockford, IL, USA) (Thermo Scientific). In addition, all volunteers completed a food allergy screening document, including allergies/intolerances to wine or any of its components. All participants were informed of the nature of this study and gave written consent to participate. The Ethics Committee of the Spanish National Research Council (CSIC, 008/2021) approved this work.

2.3. Saliva collection

Two saliva collection sessions were conducted on the same day. At the beginning of each session, volunteers rinsed their mouths with 30 mL of water. Saliva was collected for 5 minutes to obtain (stimulated) basal saliva (t0). The term basal saliva will be used along this study to referring the initial point before wine stimulation, being aware that usually basal saliva denotes unstimulated (resting) saliva that is not the case. Five minutes later, 15 mL of control wine (CRW) was served. Volunteers rinsed their mouths with the wine for 30 seconds and expectorate it. After a resting time of 15 seconds, saliva was then collected for 15 seconds (t1=30s). Then, volunteers rested for 15-seconds and another saliva sample was collected for other 15 seconds (t2=60s) (Figure1). Once the procedure was completed, volunteers were instructed to rinse their mouths with a water and pectin solution to clean the palate from any rest of wine polyphenols as previously recommended [24]. Following a 15-minutes resting period (time for total salivary proteins previously found to return to their basal values) [31], participants were given the polyphenol-enriched wine

(TRW) and they carried out the same procedure previously described for the CRW and shown in Figure 1.

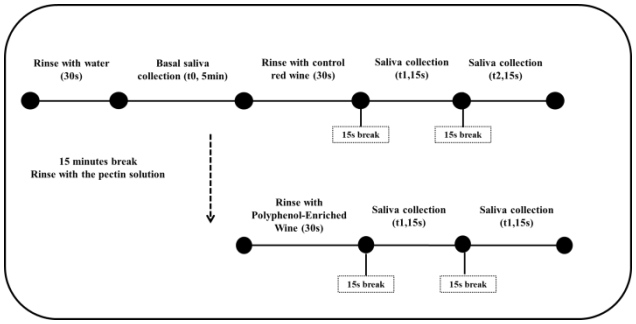

Figure 1.- Schematic representation of the general procedure followed for saliva collection.

Saliva samples from six individuals of the same taste phenotype, Taster (T) or Non-Taster (NT) were pooled to obtain five saliva mixtures for each taste phenotype. These samples corresponded with basal saliva (t0), saliva collected after the oral exposure to the control wine (CRW) at two different times, t1 (30 s) and t2(60 s), and saliva from the intervention with the tannin-spiked wine (TRW) collected at the same times (t1 and t2). Each saliva mixture was stored at -80 °C until analysis. Thawed samples were treated with a protease inhibitor cocktail (cOmpleteTablets EASYpack, Roche, Mannheim, Germany) before conducting the analysis. Table 1 shows the different saliva samples used for this study.

Table 1.- Saliva samples collected from individuals of both PROP taste phenotypes (T and NT) and average ± SD values of the total protein content (TPC) (mg/mL) of each saliva mixture.

PROP Phenotype Group	TPC (mg mL^{-1}) Basal saliva	TPC (mg mL^{-1}) Saliva after the oral exposure to the wine		
	Saliva t0	Wine Type	Saliva t1	Saliva t2
Taster	2.71 ± 0.79	CRW	3.34 ± 1.92	3.54 ± 1.09
		TRW	4.14 ± 0.85	3.76 ± 0.78
Non-Taster	2.19 ± 0.93	CRW	3.23. ± 1.64	3.77 ± 1.40
		TRW	4.18± 0.66	3.99 ± 1.44

t0 (basal saliva collected before the stimulation with the wine), t1 and t2 (saliva collected at 30s and 60s after stimulation with the wine).

2.5. Proteomic analysis

2.5.1. Protein digestion

The saliva samples (10µg), were suspended in a volume up to 50 µl of sample buffer (standard run buffer of SDS-PAGE gels), and then applied onto 1.2-cm wide wells of a conventional SDS-PAGE gel (0.75 mm-thick, 4% stacking, and 10% resolving) (Figure 1S). Then, run was stopped as soon as the front entered 3 mm into the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie staining, excised, cut into cubes (2 x 2 mm), and placed in 0.5 ml microcentrifuge tubes [32]. The gel pieces were destained in acetonitrile:water (ACN:H_2O, 1:1). They were reduced and alkylated (disulfide bonds from cysteinyl residues were reduced with 10 mM DTT for 1 h at 56 ºC, and then thiol groups were alkylated with 10 mM iodoacetamide for 30 min at room temperature in darkness) and digested in situ with sequencing grade trypsin (Promega, Madison, WI) as described by Shevchenko with minor modifications [33]. The gel pieces were shrunk by removing all liquid using sufficient ACN. Acetonitrile was pipetted out and the gel pieces were dried in a speedvac. The dried gel pieces were re-swollen in 100 mM Tris-HCl pH 8, 10mM $CaCl_2$ with 60 ng/µl trypsin at 5:1 protein: enzyme (w/w) ratio. The tubes were kept in ice for 2 h and incubated at 37°C for 12 h. Digestion was stopped by the addition of 1% TFA. Whole supernatants were dried down and then desalted onto OMIX Pipette tips C18 (Agilent Technologies) until the mass spectrometric analysis.

2.5.2. TMT Labeling and high pH fractionation

The resultant peptide mixture from desalted proteins tryptic digest (50 µg) was labeled using chemicals from the TMT sixplex Isobaric Mass Tagging Kit (Thermo Fisher Scientific, MA, USA). Two TMT labels were performed, as shown in Table 2, and the reagents were used essentially as described by the manufacturer. Briefly, peptides were dissolved in 50 μL of 100 mM triethylammonium bicarbonate (TEAB), adjusted to pH 8. For labeling, each TMT reagent was dissolved in 41 μL of acetonitrile and added to the respective peptide mixture and then incubated at room temperature for one hour [34]. Labelling was stopped by the addition of 8 μL 5% hidroxilamine. Whole supernatants were dried down and the six samples were mixed to obtain the "6plex-labeled mixture" TMT-mix 1 and TMT-mix 2 (as shown in Table 2). The mixtures were analysed by RP-LC-MS/MS to check the efficiency of the labelling [35].

Table 2. Conditions used in Tandem Mass Labelling (TMT) for the relative multiple quantification of salivary proteins.

TMT	PROP taste Phenotype Group	Saliva Sample	TMT Label Reagents
TMT-1	Non-Taster	Basal -t0	126
		t1-CRW	127
		t2-CRW	128
	Taster	Saliva-t0	129
		t1-CRW	130
		t2-CRW	131
TMT-2	Non-Taster	Basal -t0	126
		t1-TRW	127
		t2-TRW	128
	Taster	Saliva-t0	129
		t1-TRW	130
		t2-TRW	131

TMT-1: Saliva t1 and t2: saliva collected after the oral exposure to the control red wine (CRW) considering Taster (T) and Non-Taster (NT) individuals; TMT2: saliva t1 and t2: saliva collected after the oral exposure to the tannin spiked red wine (TRW) considering T and NT individuals; Basal saliva t0 is the same sample in TMT-1 and TMT-2.

2.5.3. Fractionation

The samples were then fractionated using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, MA, USA) as described with minor modifications. The sample was re-swollen in 0.1%TFA and then, loaded onto an equilibrated, high-pH, reversed-phase fractionation spin column. A step gradient of increasing acetonitrile concentrations (5-50%) in a volatile high-pH (Triethylamine (0.1%) was then applied to the columns to elute bound peptides into nine different fractions collected by centrifugation. The fractions obtained from high-pH, reversed-phase 6plex-labeled mixture were dried and stored until analysis by mass spectrometry for quantification.

2.5.4. Analysis by Reverse Phase-Liquid Chromatography-Tandem Mass Spectrometry (RP-LC-MS/MS)

The fractions were resuspended in 10 µl of 0.1% formic acid and analysed by RP-LC-MS/MS in an Easy-nLC 1200 system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Scientific). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1mm × 20 mm C18 RP precolumn (Thermo Scientific), and then separated using a 0.075mm x 250 mm bioZen C18 RP column (Phenomenex) operating at 0.25 µl/min. Peptides were eluted using a 90-min dual gradient. The gradient profile was set as follows: 5−25% solvent B for 68 min, 25−40% solvent B for 22min, 40−100% solvent B for 2 min and 100% solvent B for 18 min (Solvent A: 0,1% formic acid in water, solvent B: 0,1% formic acid, 80% acetonitrile in water). ESI ionization was done using a Nano-bore emitters Stainless Steel ID 30 µm (Proxeon) interface at 2.1 kV spray voltage with S-Lens of 60%.

The instrument method consisted of a data-dependent top-20 experiment with an Orbitrap MS1 scan at a resolution ($m/\Delta m$) of 30,000 followed by either twenty high energy collision dissociation (HCD) MS/MS mass-analyzed in the Orbitrap at 7,500 ($\Delta m/m$) resolution. MS2 experiments were performed using HCD to generate high resolution and high mass accuracy MS2 spectra.

The minimum MS signal for triggering MS/MS was set to 500. The lock mass option was enabled for both MS and MS/MS mode and the polydimethylcyclosiloxane ions (protonated (Si (CH3)2O))6; m/z 445.120025) were used for internal recalibration of the mass spectra.

Peptides were detected in survey scans from 400 to 1600 amu (1 µscan) using an isolation width of 1.3 u (in mass-to-charge ratio units), normalized collision energy of 40% for HCD fragmentation, and dynamic exclusion applied during 60 seconds periods. Charge-state screening was enabled to reject unassigned and singly charged protonated ions.

2.5.5. Identification and Quantitative Data Analysis

Peptide identification from raw data (a single search was performed with all nine raws from the fractionation) was carried out using PEAKS Studio XPro search engine (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada). Database search was performed against uniprot-homo sapiens.fasta (80581 entries; UniProt release 12/2022) (decoy-fusion database). The following constraints were used for the searches: tryptic cleavage after Arg and Lys (semispecific), up to two missed cleavage sites, and tolerances of 20 ppm for precursor ions and 0.05 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation and fixed TMT 6 plex reagent labeling at the N-terminus and lysine residues[32,34,36]. False discovery rates (FDR) for peptide spectrum matches (PSM) and for proteins was limited to 0.01. Only those proteins with at least two unique peptides being discovered from LC/MS/MS analyses were considered reliably identified and sent to be quantified.

Quantitation of TMT labeled peptides was performed with PEAKS Studio XPro search engine, selected “Reporter Ion Quantification TMT” under the “Quantifications” options. We use Auto normalization mode that calculate a global ratio from the total intensity of all labels in all quantifiable peptides. The -10LgP, Quality and Reporter Ion Intensity, were used for Spectrum filter and Significance (PEAKSQ or ANOVA method) was used for peptide and protein abundance calculation. For the Protein quantification we consider protein groups for peptide uniqueness; we only use unique peptides for protein quantification and the modified peptides were excluded.

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3. Results

RP-LC-MS/MS analysis identified 1330 proteins in the basal (t0) saliva of Ts and NTs. Following wine oral exposure to CRW, 1329 and 1790 proteins were identified in Ts and NTs respectively, while a similar number of proteins, 1232 and 1231, for Ts and NTs were identified in the saliva collected after the oral exposure to TRW. Figure 2S provides a visual representation of these differences.

3.1. Differences in the saliva proteome before wine stimulation (t0) depending on PTS

Table 3 shows the proteins present in the saliva (t0) (before the exposure to the wines) that showed significant differences ($p<0.01$) on their relative abundance depending on the PROP taster status (PTS). The magnitude of these changes is represented by the value of the ratio t0 T/t0 NT. As shown in the table, a few number of proteins exhibited significant differences between both groups. For instance, T individuals showed higher abundance (t0 T/t0 NT >1) for the BPI fold-containing family A member 1 protein and for protein S100-A7. On the contrary, the ratio between T and NTs individuals, showed values <1 in the case of annexin A3, neutrophil elastase, haemoglobin subunits alpha and beta, and protein S100-A12, meaning that NTs had a higher presence of these saliva proteins. Figure 3S also shows the heat map graphic representation of these differences.

3.2. Time-dependent changes in the salivary proteome 30 and 60 seconds after the oral exposure to the control wine (CRW) depending on PTS

The abundance of salivary proteins 30 (t1) and 60 seconds (t2) after the oral exposure to the wine is shown in table 2S. This table also shows the relative changes compared to the abundance in basal saliva (t1/t0 and t2/t0) for both types of individuals (Ts and NTs).

In the case of Taster individuals, Figure 2a shows the representation of the most significant changes ($p<0.01$) in the salivary proteome (SP) after the oral intervention with CRW. As can be seen, 30s after the oral intervention with the wine, seventeen proteins experienced significant changes ($p<0.01$) in their abundance compared to basal saliva. Most of them (fourteen) significantly increased compared to basal saliva (t1/t0 >1). Considering from the largest to the smallest differences (the extent of this changes is shown in brackets), these proteins were, cystatin-D (4.2), cystatin-S (3.69), nucleobindin-2 (3.09), BPI fold-containing family A member 2 (2.78), cystatin-C (2.5), lactoperoxidase (2. 33), cystatin-SA (2.31), cystatin-SN (2.26), protein inducible protein (PIP) (2.09), carbonic-anhydrase-VI (CA-VI) (1.95), ribonuclease 4 (1.64) and dermicidin (1.62). In contrast, some proteins such as desmoplakin (0.99), protein S100-A7 (0.77) and ras-related protein-RAB 25 (0.57) significantly decreased 30s after the oral intervention with CRW compared to basal saliva (t1/t0 <1) (Figure 2a). Sixty seconds (t2) after the oral intervention with this wine, a very similar behaviour was observed and most proteins exhibited higher abundance at t2 compared to t0 (t2/t0 >1). These ratios were slightly lower than those reported for t1 (**Figure 2a**), indicating that most proteins seem come back to basal concentration. Only some proteins, such as dermcidin, desmoplakin, dermokine and desmocollin-1, exhibited a higher intensity at t2 than at t1. CA-VI was the only protein, from those that experienced significant changes that remained constant 30 and 60 seconds after the oral exposure to CRW in T individuals.

For non-taster (NT) individuals (**Figure 2b**), the relative changes observed in the salivary proteome 30 seconds (t1) after the oral intervention with CRW (t1/t0) were quite similar to those observed in T individuals. However, a lower number of proteins (twelve) experienced significant changes ($p<0.01$) compared to T individuals (seventeen) (Figure 2b). Eight proteins (cystatin-SN, CA-VI, lactoperoxidase, desmocollin-1, ribonuclease-4, protein S100-A1, ras-related-protein Rab-25 and dermcidin) that experienced changes in T individuals did not show significant changes ($p<0.01$) in NTs after the oral intervention with the wine. On the contrary, there were

Table 3. Significant differences ($p<0.01$) in the relative abundance of salivary proteins in basal saliva (t0) between T and NT individuals (t0 T/t0 NT).

Protein reference	Ratio t0 Taster/t0 Non-Taster	Coverage (%)	Peptides	Unique	Avg. Mass	Protein Name
P12429	0,55	51	22	20	36375	Annexin A3
P08246	0,58	50	17	17	28518	Neutrophil elastase
P68871	0,11	53	16	7	15998	Hemoglobin subunit beta
P69905	0,14	46	7	7	15258	Hemoglobin subunit alpha
Q9NP55	3,56	27	5	5	26713	BPI fold-containing family A member 1
P31151	2,68	35	4	4	11471	Protein S100-A7
P80511	0,5	47	5	5	10575	Protein S100-A12

Protein reference: Identified protein using the Uniprot accession; Coverage (%): minimum sequence coverage; #Peptides: Number of peptides in the protein; #Unique: Number of unique peptides in the protein; Avg. mass: molecular weight (kDa). (For proteins with lower level of significance, check the list of Table 1S)

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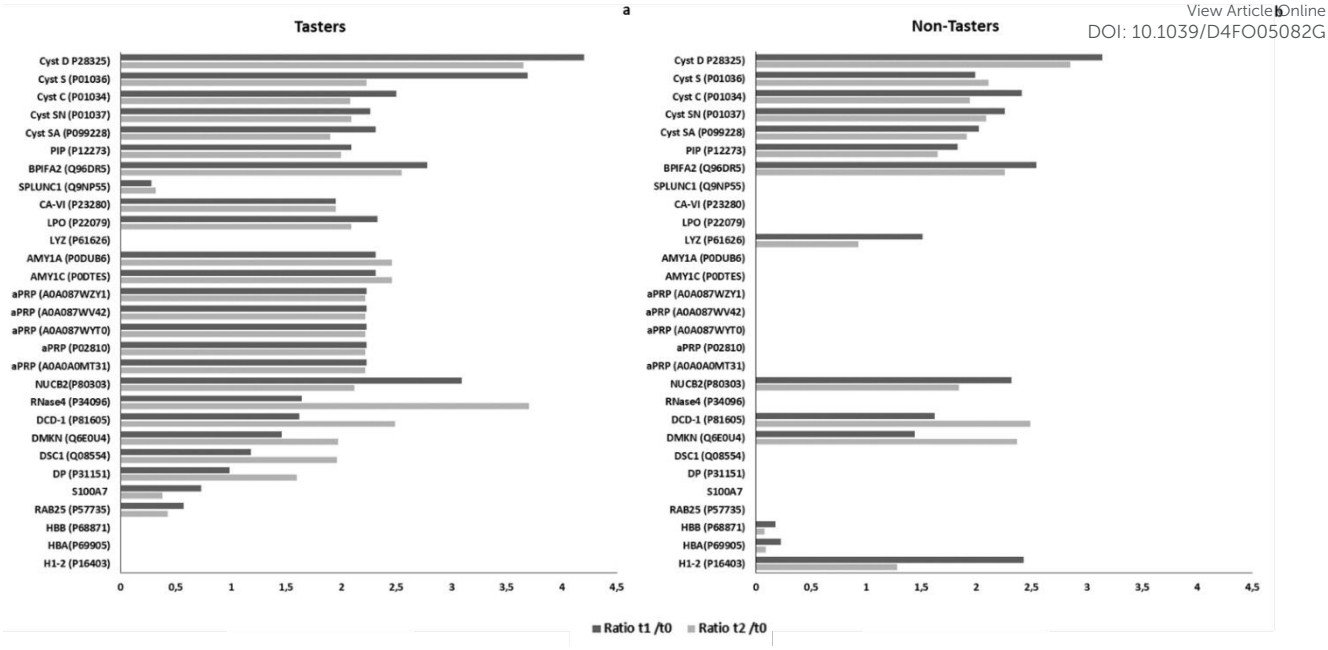

Figure 2. Significant changes (p<0.01) (compared to basal saliva) in the abundance of salivary proteins 30 and 60 seconds (t1/t0 and t2/t0) after the oral exposure to the wine (CRW) in saliva from a) Taster and b) Non-Taster individuals. The complete list of proteins that changed over time (p>0.01) is shown in Table 2S. Protein identification code is shown in brackets. Abbreviations: Cystatin D (Cyst D); cystatin S (Cyst S); cystatin C (Cyst C); cystatin SN (Cyst SN); cystatin SA (Cyst SA); protein-inducible protein (PIP); BPI fold-containing family A member 2 (BPIFA2); BPI fold-containing family A member 1 (SPLUNC1); carbonic anhydrase-VI (CA-VI); Lactoperoxidase (LPO); lysozyme C (LYZ); alpha amylase 1A (AMY1A); alpha amylase 1C (AMY1C); Salivary acidic proline-rich phosphoprotein ½ (PRP H1); Proline-rich protein 4 (PRP 4); Proline-rich proteoglycan 2-like (aPRP); nucleobindin2 (NUCB2); dermcidin (DCD-1): desmoplakin (DP); protein-S100A7 (S100A7); ras-related protein Rab-25 (RAB25); hemoglobin subunit beta (HBB); hemoglobin subunit alpha (HBA); histone H1.2 (H1-2)

some proteins (lysozyme C, haemoglobin subunit alpha and beta and histone H1.2) that did not change in Ts, but that did it in NTs (Figure 2b). Similar to what happen in T individuals, in the firsts 30 s after the oral intervention with the CRW, most proteins increased compared to basal saliva (t1/t0 <1). Interestingly, two proteins, histone H1.2 and lysozyme-C that did not experience significant changes in Taster individuals, significantly increased in NTs after the oral exposure to CRW showing t1/t0 ratios >1 (2,34 and 1.51 respectively). Additionally, two other proteins (haemoglobin alpha and beta) that neither changed in T individuals, significantly decreased in the case of NT individuals (t1/t2 ratios <1). Similarly, to what happened in Ts, sixty seconds after the oral exposure to CRW, most of these proteins were still present in higher abundance compared to basal saliva (t2/t0>1), although showing lower t2/t0 values, which as previously commented, might indicate that they start to come back to basal concentration.

3.3. Comparison of the changes in the salivary proteome after the oral exposure to the Control (CRW) and the tannin-spiked red wine (TRW) depending on PTS

The oral intervention with the tannin-spiked wine (TRW) also produced significant changes in the salivary proteome, affecting a higher number of salivary proteins (24 and 17 in Ts and Nts respectively) compared to the CRW. Similar to what happened with the control wine, an increase in protein abundance 30 and 60 s after the oral intervention with the wine was noticed (Table 3S). For better compare the effect of the intervention with both wines, Figure 3 only show the significant changes (p<0.01) in salivary proteins previously suggested to play a role in flavor perception. [37–47]. Additionally, Figure 3 only shows the changes in the first fifteen seconds after the intervention with the wines since results were very similar in the second sampling point (60 s) (Figure 4S).

As can be seen in this figure, (Figure 3), in both PROP phenotypes (T and NT), the oral exposure to TRW induces more changes compared to the exposure to the CRW. In addition, a clear effect of PTS was noticed. In fact, these changes were of higher magnitude and affected a higher number of proteins in the case of Ts (Figure 3a) compared to NTs (Figure 3b). In T individuals (Figure 4a), the oral exposure to TRW, significantly increased the abundance of most cystatins (cystatin-D, cystatin-S, cystatin-C, cystatin-SN, cystatin-SA), BPI-FA2, lactoperoxidase, PIP, zinc-α-2-glycoprotein, carboxypeptidase E, CA-VI and lysozyme C, compared to the exposure of CRW. The increase in abundance is roughly estimated between 37.5% for cystatin D and 1.5% for CA-VI. In fact, there were

some proteins (zinc-α-2-glycoprotein, carboxypeptidase E and lysozyme C) that increased compared to basal saliva after the oral intervention with TRW but that did not change with the CRW (figure 3a). As can be seen, the rise in acid PRPs was very similar independently on the wine type. Only some isoforms of PRP-4 did not change after the oral exposure to TRW in Taster individuals.

In NTs (Figure 3b), the intervention with TRW produced in general, less changes that did it in Ts. Compared to the CRW, the exposure to TRW slightly increased the abundance of cystatin-D (about 6,8%) and mainly cystatin SN, which did not change in this group of individuals after the exposure CRW. Additionally, figure 3b also shows that in NTs, there are some proteins such Zinc-α-2-glycoprotein, Carboxypeptidase E, CA-VI that did not changed compared to basal saliva with none of the tasted wines. Interestingly, lysozyme C experienced changes in their abundance after the oral exposure to CRW but not with TRW. It is worth to notice that most (acid) PRPs only increased after the intervention with TRW, but they did not increase after the exposure to CRW in these individuals.

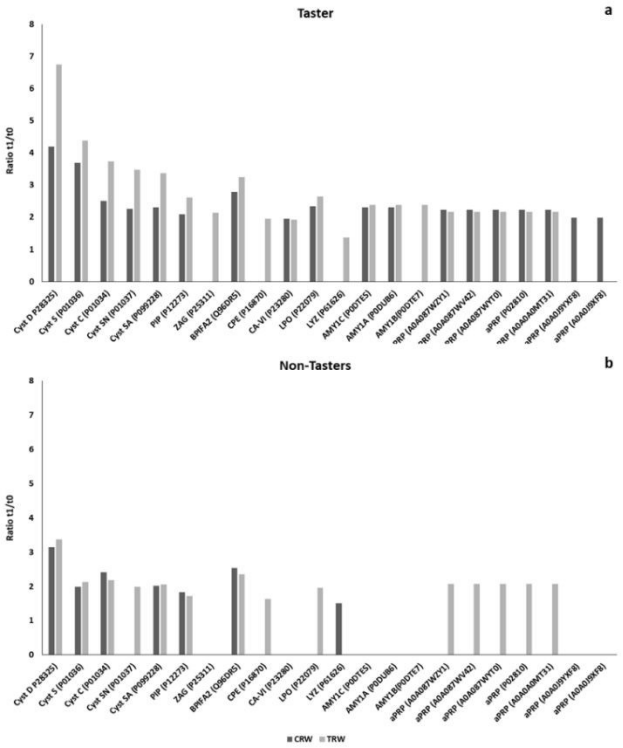

Figure 3. Significant changes (p<0.01) (compared to basal saliva) in the abundance of salivary proteins 30 s after the oral exposure to the CRW and TRW wines in saliva from a) Taster and b) Non-Taster individuals. The complete list of proteins that changed over time (p>0.01) is shown in Table 2S (CRW) and Table 3S (TRW). Protein identification code is shown in brackets. Abbreviations: Cystatin D (Cyst D); cystatin S (Cyst S); cystatin C (Cyst C); cystatin SN (Cyst SN); cystatin SA (Cyst SA); protein-inducible protein (PIP); zin-alpha-2-glycoprotein (ZAC); BPI fold-containing family A member 2 (BPIFA2); carboxypeptidase E (CPE); carbonic anhydrase-VI (CA-VI); Lactoperoxidase (LPO); lysozyme C (LYZ); alpha amylase 1C (AMY1C); alpha amylase 1A (AMY1A);); alpha amylase 1B (AMY1B); salivary acidic proline-rich phosphoprotein ½ (PRP H1); proline-rich protein 4 (PRP 4); proline-rich proteoglycan 2-like (aPRP).

4. Discussion

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The main objective of this study was to evaluate the time-dependent changes in the salivary proteome (SP) after the oral exposure to wine considering the individual PROP taster status (PTS), which might contribute to explain recent findings, related to differences in the time-course perception of astringency depending on PROP responsiveness [26]. To do so, we followed an untargeted proteomic approach with the saliva collected from twelve individuals (young women, 23 y.o. average age), six of them with positive PROP responsiveness (taster group, T) and another six with no responsiveness (no-taster, NT), that were classified in previous studies [22]. Saliva collection was performed 30 and 60 seconds after the oral exposure to the wines. Although astringency perception following oral stimulation could be very variable to recede (100 to 300 seconds or even longer), [24,30,48–50], the selected period catches the early protein response, which might be more related with the time-curse evolution of astringency during wine tasting [20,21,26]. Additionally, by only recruiting young women, we tried to reduce the impact of other potential sources of variation in the salivary proteome, such as sex and age [29,51–53]. For the experimental test, individuals rinsed their mouths with two different wine types, a red wine (CRW) and the same wine spiked with a commercial tannin (gallotanin) (TRW), which might also affect the long-lasting astringency perception following wine tasting. In fact, previous studies have shown that tannin concentration and type can be an important factor that affect the specificity of different types of PRPs to complex and precipitate [80]. To avoid the carry-over effect of polyphenols, individuals firstly tried the CRW and 15 minutes later the spiked-tannin wine (TRW). Other preventive measurements already suggested for this type of assays [24] such as the use of a pectin: water solution for mouth rising were also used. Besides of this, previous studies [22] confirmed the absence of total polyphenols in the saliva collected 15 minutes after the oral intervention with different types of wines.

All the collected saliva (basal saliva, and saliva 30 and 60 s after the oral intervention with the two wines) from individuals of the same PTS were pooled in order to have two groups of saliva, from T and NT individuals. This minimises interindividual differences and it was necessary due to the restrictions imposed by the proteomic procedure using Tandem Mass Labelling (TMT) in which only six different types of samples can be done and compared at the same time. Nonetheless, as previously explained in material and methods (section 2.5.2), since basal saliva (t0) was the same in the two TMTs, it was possible to compare 12 different samples. The limitation of this MS procedure might be neglected compared to the novelty of using a non-targeted approach to check the changes in the whole SP, as opposite of targeting specific salivary proteins [27,30]. As far as authors known, this is the first time using this approach for checking the time-course changes in the SP in a realistic food (beverage) consumption situation.

Considering both PROP taste phenotypes, results from proteomic analysis allowed us to identify above 1300 proteins in whole saliva before the oral intervention with the wines (basal saliva-t0). Although more than 3000 proteoforms have been described in

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human saliva [54], the number of proteins identified in the present study, is within the range of values previously described using other MS-approaches (LC-MS/MS), which might vary from 1050 to about 2340 proteins [55–57]. The high variability of salivary proteins identified in the different studies could be associated to a great number of factors, such as salivary flow, epithelial leakage, gingival and periodontal inflammation, or enzymatic degradation [58] but also on the use of different methodologies and analytical approaches.

The effect of PTS on the saliva proteome before the intervention with wine, was rather limited since there were not many significant differences ($p<0.01$) between both groups of individuals (Table 3). From the proteins that exhibited significant differences between PROP taste phenotypes, most of them (Annexin A3, Neutrophil elastase, Haemoglobin subunit α and β and protein S100-A12), were more abundant is NTs, while only two proteins (BPI fold-containing family A member 1 and Protein S100-A7) were significantly more abundant in Ts. Some of these proteins have some relevance for clinic diagnosis [59–61] and others (neutrophil elastase) can have antimicrobial and bactericidal activity [62], but, in the best of our knowledge, they do not seem to have any function on flavour perception. The lack of clear effect of PTS on the proteome of basal saliva agrees with previous results of Rodrigues and co-authors in which using in gel-proteomic approach, they only evidenced some significant differences on the salivary proteome of women supertasters (STs) compared to STs men and NTs (men and women) [53]. Specifically, they showed lower abundance of some proteins such as IgK and PIP, but a higher abundance of CA-VI (gustin protein). A higher prevalence of the genotype AA responsible for the expression of CA-VI in individuals with higher PROP responsiveness was also observed[12]. Different studies have also confirmed the relationship between CA-VI and bitter taste perception [11,18,29,37,40,42,44,63,64]. It has been suggested that this could be due to a single nucleotide polymorphism of the CA-VI gene (rs2274333), located in exon 3, which alters bitter taste perception, especially in response to 6-n-propylthiuracil (PROP), contributing to this taster condition, highly expressed in Ts [11]. Nonetheless, the relationship of CA-VI protein with bitter taste perception is still controversial as shown in a recent study involving a large cohort of individuals (n=1117), in which authors found that polymorphism in CA-VI gen was not related to any taste or somatosensory sensation, such as astringency [14].

Besides CA-VI, some basic PRPs (such as PS-1 and II-2-peptides) have been related to a higher PROP responsiveness [18]. However, as shown in Table 1, in the present study, we did not find significant differences between T and NTs in t0 saliva in this type of proteins, even when decreasing the statistical significance level (Table 1S). Reasons related to methodological differences, such as the use of different types of saliva (stimulated *vs* non-stimulated), analytical approaches (gel-based, MS-based, targeted, untargeted, etc.), or other aspects related to the characteristics of the saliva donors (sex, age) might be some of the reasons.

In spite of the practically lack of differences in the basal SP depending on PTS, the exposure to wine produced significant changes in some proteins that were different when comparing both taste phenotypes (Figure 2, Table 1S). A significant rise ($p<0.01$) in the abundance of most identified proteins was noticed 30s after the oral exposure to the wine in both PROP taste phenotypes (Figure 2). Interestingly, the significant changes affected a higher number of proteins in T than in NT individuals. In Ts, from seventeen proteins that experienced changes after oral wine stimulation, fourteen of them, significantly increased. Among them, different type of cystatins (Cys-D, Cys-S, Cys-C, Cys-SA, Cys-SN) and other proteins previously related to flavour perception such as BPI, lactoperoxidase, PIP and CA-VI.

Salivary cystatins have often been associated to bitterness perception [65–67]. For instance, an increase in Cys-S after the early and long-term exposure to polyphenol-rich foods have been observed and related to differences in food preferences [39,68]. Cys-SN is also involved in blocking bitterness perception [69]. Besides its role in bitterness, [42] also observed higher expression of CysD and Cys SN in individuals very sensitivity to oleic acid, thus, these cystatins can also be involved in fatty perception. Additionally, PIP has been identified as a predictor of bitter taste acceptance [70], while BPI fold-containing family A member 2 (BPIFA2) protein is expressed in the oral mucosa [71] increasing after the oral exposure to pungent 6-gingerol [72], also suggesting its potential role on bitter perception.

Interestingly, different types of acid proline rich proteins (aPRPs) also increased after the oral stimulation with the wine (Figure 2), but only in T individuals. In fact, in the case of NTs, the number of proteins that changed their abundances after wine stimulation was lower (thirteen) and eight proteins that positively changed in T individuals, did not experienced any change in NTs; among them, Cys-SN, CA-VI and lactoperoxidase. Sixty seconds after the oral stimulation with the wine (t2), we observed a slight decrease in most of these proteins, showing practically the same differences between T and NT than in t1 (Figure 2).

As previously suggested, wine should have induced the stimulation of parotid gland and the secretion of protein storage granules containing different types of low molecular weight proteins with high phenol binding capacity (PRPs, cystatins, etc.) [28]. Results from the present work agrees with those reported by Melis and co-authors, that also observed an increase of different types of low molecular weight proteins, specifically two sub-types of aPRPs (PRP-1 and PRP-3) and one cystatin sub-type (Cyst-SN) one minute after the oral rinsing with cranberry juice, mainly in Ts individuals [29]. In a further work, Yousaf and co-authors studied the time-course changes of specific salivary proteins after the oral stimulation with cranberry juice and a cranberry polyphenol extract, also finding a significant increase in aPRP five minutes after the oral exposure to the stimuli that remained elevated 10 min later independently of the stimuli type [30].

Compared to the above-mentioned previous studies, in the current one, we observed a higher number of proteins that changed after wine stimulation. This could be due to differences in composition of the stimuli (wine compared to cranberry juice/extract) but also on the use of an untargeted proteomic approach instead of focusing in looking for specific salivary proteins. In spite of this, results from the present and previous proteomic studies seem to confirm that the

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stimulation with (complex) polyphenol-type beverages favoured the early release of cystatins and acidic PRPs.

Remarkably, we did not find significant changes in basic PRPs, at least, in the first minute after wine stimulation in spite that in this period astringency perception reaches its maximum intensity values [26]. This agrees with other previous proteomic studies [29,30], but contrast with the outstanding role of this group of proteins as the origin of astringency [73–76]. This discrepancy could be explained by the different feedback provided by *in vitro* models to study protein-polyphenol interactions in astringency perception compared to studies performed under physiological and more realistic food consumption conditions in which many different types of proteins are considered at the same time. Anyhow, it is important to recognise that astringency is a very complex phenomenon in which many other factors besides SPs seem to be involved (genetic, psychological aspects, etc.) [77,78].

Interestingly, as shown in Figure 3, compared to the CRW, the exposure to the tannin-spiked wine (TRW) produced different changes in the SP of both types of individuals. These changes were also of higher magnitude in Ts compared to NTs (Figure 3a *vs* Figure 3b). In Ts (Figure 3a), we observed an increase in the abundance of many types of Cystatins, PIP, and the presence of some proteins that were not previously identified after the stimulation with CRW, such as Zinc-α-2-glycoportein, carboxypeptidase E or α-amylase. The two first proteins have been previously related to bitter perception [29,79,80]. Mounayar and colleagues also reported an overexpression of zinc-α-2-glycoprotein (Zn-α-2-GP) together with CA-VI and SN and D cystatins in individuals who are more sensitive to oleic acid (C18:1) [42]. Moreover, α-amylase has also found at higher levels in STs than NTs in response to cranberry juice [30], which agrees with our results.

In the case of NTs, the exposure to TRW produced a significant rise in aPRPs and Cys-SN that did not change during the oral exposure to CRW. The presence of this type of tannin (gallotain) or the increase in tannin concentration might have promote the stimulation of salivary glands and in turn, the release of these proteins. Supporting this, previous studies have shown that wine tannin concentration is an important factor that affect the specificity of different types of PRPs to complex and precipitate salivary proteins [80].

Results regarding the effect of gallotanin show that small variation in stimuli type (presence of different polyphenols or different concentrations) seems to provoke a different early salivary profile. The SPs released after wine stimulation can react with some of the polyphenols that in spite of swallowing, can be still present as residual polyphenols in saliva [28]. In a first step, they can form persistent soluble aggregate with salivary proteins, which do not coalesce and precipitate, while in a second step, the complexes reach a size at which it is no longer soluble and precipitates from saliva, which is the origin of the astringency sensation [4]. However, it is possible that residual polyphenols could not only be present in circulating saliva but also adhered into oral mucosa [81]. All of these polyphenols might keep stimulating the release of low molecular binding proteins (aPRPs, cystatines, α-amylase, etc.) as long as the stimuli is still present in the oral cavity. This might explain the little differences in proteins that we observed 30 and 60 seconds after wine stimulation. The extent of this stimulation might determine the time-course changes observed in the SP and might depend on how quickly these polyphenols could be washed out from the oral cavity. Thus, aspects such as the stimuli concentration, stickiness or binding capacity of polyphenols to oral surfaces, and composition of the food/beverage matrix (e.g. presence of ethanol, polysaccharides, etc.) might also affect. In this case, it could be expected that a higher salivary flow rate might also induce a higher wash out of polyphenols from the oral cavity, which agrees with the inverse relationship between astringency and flow rate found in previous studies [82].

Additionally, tannins can be adhered to mucins in the oral mucosa [83–85], likely inducing a higher stimulation of low salivary protein release, explaining the higher SP changes observed with the tannin-enriched wine (TRW) compared to the CRW in both group of individuals. Interestingly, recent studies, have suggested that astringency depends not only on the thermodynamic tendency to form the complex between tannins and salivary proteins but also on the time required to dissociate the complex [86].

Results from the present study also support an effect of PTS on the changes induced in the SP following wine stimulation, which agrees with previous studies that also reported similar changes one minute after the oral stimulation with a different type of stimuli [29]. In contrast, Yousaf and colleagues, did not find clear PROP-related effects 5 and 10 minutes after the stimulation with the same cranberry juice used in previous studies [29,30]. This disagreement is explained by the authors because of the differences in the protein sampling points (1min *vs* 5 min), which gives a different "snapshot" of SP after oral stimulation. This seems a plausible explanation considering results from the present study, in which we show how shorter sampling times (30s and 60 s) seems to capture an early SP protein profile in which differences between PROP taste phenotypes are more evident.

Our findings also agree with pioneers works of Dinnella and colleagues, who found that high responsive subjects (those rating highest the astringency of a tannin solution), accumulate a higher concentration of proteins with high phenol-sequestering ability (PRPs, cystatins, histatins) and a greater amount of other proteins with lubricating properties (amylases, glycosylated PRPs), compared to the low responsiveness group after stimulation with tannic acid [27].

These evidences seem to support that the higher differences in the early SP after wine stimulation in Ts compared to NTs, agrees with the higher astringency perception over time previously observed in these individuals [26]. Additionally, the enhancing effect of TRW on SPs, mainly in T individuals, agrees with the significant reduction in the tmax parameter (time to reach the maximum astringency intensity) previously observed in Ts and NTs individuals. The effect of this additive was much higher in Ts individuals, meaning that this additive produced a quicker perception of the astringency, which agrees with the higher number and intensity of salivary proteins release observed in the present study.

Something to be noticed as well is the lack of consensus in the scientific literature on the effect of PROP-phenotype on wine

astringency perception. Some studies show a positive correlation [5,22,26] while others do not [87–89]. In this regard, besides differences in methodology and the complexity and multimodality of wine astringency [22], this current study has proven that the early SP changes over time are important to reveal differences in this sensory modality by PROP taste phenotype. In view of this, and as previously suggested [88], dynamic sensory methods, rather that rating the astringency intensity at one single point, seems to be better suited for revealing individual differences in this oral sensation.

Intriguingly, we still do not know why individuals with a genetically determined high capacity to bind PROP, also might have a higher salivary protein response after red wine stimulation and presumably, a higher sensitivity to astringent compounds. SPs have been considered as the "first line of defence" to protect the animals against the negative effects of tannins, which might have anti nutritional or harmful consequences [41]. In presence of these type of chemical stimuli, there is a physiological response consisting in the release of high-binding phenol proteins, with PROP Taster individuals showing an enhance response compared to NTs, as shown in the present study. Additionally, we noticed the release of other types of salivary proteins (Immunoglobulin A; lactoperoxidase, lactotransferrin) involved on protective mechanisms for defending the oral cavity against physical or chemical damage [90]. These proteins also experienced more changes in T than in NTs after wine stimulation (table 2S). Therefore, PROP taste phenotype seems to act such as "defence phenotype", with Taster individuals exhibiting higher capacity to combat potential harmful [42]chemicals, though different mechanisms (higher release of SPs with higher polyphenol binding capacity, antimicrobial and other defence SPs, enhancing capacity to bind bitter compounds, etc.) but sharing the same physiological objective. In line with this, some authors have also speculated that the NTs salivary microbiome seems to be less stable to environmental perturbations (oral exposure to cranberry polyphenol extract), while the ST microbiome may be more stable to this intervention, conferring beneficial oral health status to STs [30]. In any case, new studies will be necessary to support this and to provide new insight in this appealing topic.

Finally, it is important to highlight some limitations of the present study. Firstly, results related to the changes on the salivary proteome refers to young women and might not be the same when considering other cohorts (elderly, men, etc.). Additionally, although we did not notice appreciable differences in the weight and height of the participants, we did not perform a strict control of potential cofounded variables such as body mass index (BMI) [12,53,91,92]. Moreover, in spite of the powerful of the proteomic analysis, this technique implies some constraints related to the number of samples to work with and compare in the same analysis and the necessity to use pooled saliva. Besides, we used a rather small sample size (12 individuals, six of each phenotype), which was due to the restrictions imposed to the participants (different PTS, similar age, minimum concentration of total salivary proteins, adequate volume of saliva, etc.). Additionally, it could have also been interesting to check the effect of other type of polyphenol addition (besides a commercial gallotanin), or other concentrations, since we used a very low amount. Moreover, we still do not know at what time SPs recovers after wine stimulation. However, collecting repeated samples of saliva in the same experiment was very challenging, due to the low saliva volumes and the discomfort of this practice for the participants. Finally, the sensory meaning of the changes in the SP observed in the present work have been compared and discussed considering the sensory feed-back of a trained panel formed by T and NTs individuals, which were not the same that the participants of the present study [26]. In any case, the present work provides the first insights on the changes in the salivary proteome of T and NTs individuals in real wine consumption conditions, which will complement previous *in vitro* studies, likely contributing to our understanding in the individual differences on wine astringency perception.

5. Conclusion

The application of an untargeted proteomic approach allowed us to evidence the time-course changes on the overall salivary proteome after wine stimulation in individuals (young female women) from different PROP taster status (Ts and NTs). The lack of significant differences in the salivary proteome between both phenotypes in basal (resting) saliva, contrast with the larger differences observed after wine stimulation in different types of salivary proteins already related to flavour perception. Fifteen seconds after the oral exposure to wine, Ts exhibited higher changes in the number and abundance of these proteins compared to NTs. Among them, a higher abundance of different types of Cystatins, CA- VI and different sub types of acid PRPs was noticed. The exposure to the spiked tannin wine (TRW) promoted the release of more SPs and in higher abundance compared to CRW, and this effect was greater in Ts than in NTs. In the former, an increase in the abundance of some proteins such as Zinc-α-2-glycoportein, carboxypeptidase E or α-amylase that were not observed with the CRW, were found. Sixty seconds after wine stimulation, a slight and similar decrease in the abundance of most proteins was observed in both phenotypes, but differences between Ts and NTs were still evident, independently of the wine type. Overall, these results show that Ts and NTs individuals seem to have a different salivary pattern release upon wine consumption that involve many types of polyphenol-binding salivary proteins, which are tightly related with astringency perception. These results agree with previous findings that showed differences in astringency perception over time in individuals from different PTS, supporting the importance of the early salivary profile after wine stimulation for explaining individual differences in this oral sensation.

Author contributions

R.I.V.-M.: Investigation, Data curation, Formal analysis, Methodology, Writing—original draft; C.M-G.: Investigation, Supervision, Writing—review and editing, Project administration, Funding acquisition; A.M-R.: Data curation, Formal analysis, Software, Writing-review & editing; M.A.P.-B.: Conceptualization, Supervision; Investigation, Writing-review & editing, Project administration, Funding acquisition.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the Supplementary Information.

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DATA AVAILABILITY

The data supporting this article have been included as part of the Supplementary Information