ROLE OF PROP TASTER STATUS AS A MARKER FOR INDIVIDUAL DIFFERENCES IN ASTRINGENCY, SALIVARY PROTEINS AND ORAL HEALTH

By

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ABSTRACT OF THE DISSERTATION

Role of PROP Taster Status as a marker for individual differences in astringency perception, salivary proteins, and oral health

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Astringency is a sensation commonly described as drying, roughing, and puckering of the oral surfaces, which is experienced upon consumption of tannin-rich foods. Although there is more than one mechanism at play, it is widely accepted that astringency is mainly caused when salivary proteins are precipitated by polyphenols in the mouth. Individual differences in the perception of astringency are well known but not well-understood. Sensitivity to the bitter compound, 6-n-propylthiouracil (PROP), controlled by polymorphisms in the *TAS2R38* gene, has been observed to associate with differences in perception and liking for astringency. Additionally, previous research from our lab (Melis 2017) has shown PROP sensitivity may also be related to variation in salivary proteins released after stimulation with polyphenols. As these salivary proteins are key players in

maintaining a healthy oral environment, PROP sensitivity may then also be a marker for oral health.

The aims of this dissertation were to investigate if PROP sensitivity characterized by PROP Taster Status is a marker for astringency perception and liking as well as variation in salivary proteins. An exploratory aim of this dissertation was to understand if taste perception, salivary proteins and the oral microbiome are related to one another and if PROP Taster Status can help us understand these relationships. We selected cranberry polyphenols, native to the cranberry fruit, *Vaccinium marcoporan* as an ecologically relevant model to study these research questions.

In the first study, subjects evaluated cranberry juice cocktail supplemented with varying levels of cranberry polyphenols for key flavor attributes and liking. PROP-classified groups did not perceive astringency differently from these samples but instead perceived differences in other flavor attributes. The results showed that other individual factors, such as ethnicity or gender, may have a larger influence than PROP taster status in the perception of astringency from cranberry juice.

In the second study, we studied the time course of salivary proteins released after stimulation with cranberry-derived stimuli to understand if differences in astringency perception by the PROP groups may be explained by variation in salivary proteins. We observed both an effect of PROP taster status and gender on this phenomenon. Specifically, PROP super-tasters (ST) were observed to have higher levels of alpha amylase after stimulation with cranberry juice ($p=0.014$-0.000) in comparison to PROP non-tasters (NT); an additional interaction with

gender was observed, whereby male STs had higher levels of bPRPs after stimulation with cranberry-derived polyphenols. This study showed that there is variation in the salivary protein response with respect to PROP taster status after exposure to an astringent stimulus.

The final study explored the influence of PROP taster status on the interrelationship between taste perception, salivary proteins, and the oral microbiome. PROP non-tasters and super-tasters used a cranberry-polyphenol oral rinse for 11 days. Results showed evidence for differences in the oral microbiome at baseline but not at the end of the intervention. Further analyses revealed that the oral rinse altered the microbiome in NTs but not in STs. Using correlation networks, we observed that the oral environment of STs may have more complex relationships with salivary proteins and oral microbiome than NTs.

Taken together, these studies suggest that the presence of polyphenols in the mouth initiates astringency and other oral sensations that are mediated by salivary proteins which play a crucial role in protecting the oral cavity against insult and injury. Even brief exposure to cranberry polyphenols was sufficient to modify one indicator of oral well-being, the oral microbiome. PROP status appears to be an integral part of this dynamic system and deserves more attention as a marker for individual differences in oral responses to polyphenols.

DEDICATION

To my parents,

Shagufta Butt and Muhammad Yousaf

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Chapter 3: PROP Taster Status and the Oral Microbiome has been published in *Nutrients* in the Special Issue: Taste and Olfaction in Health:

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LIST OF ABBREVIATIONS

CJ: Cranberry Juice, unsweetened

CJC: Cranberry Juice Cocktail

CPE: Cranberry Polyphenol Extract

MT: Medium-taster

NT: Non-taster

ST: Super-taster

PROP: 6-n-propylthiouracil

1. INTRODUCTION

1.1. Astringency, mechanisms, and salivary proteins

Astringency is an oral sensation typically experienced upon consumption of foods rich in polyphenols. Such foods include but are not limited to red wine, tea, fava beans, coffee, grapes, cranberries, apples, persimmons, and red currants as well as unripe fruits. Astringency is an umbrella term, which is generally marked by specific subqualities: drying, roughening, or puckering of the oral surfaces. Drying signifies a loss of lubrication in the oral cavity, roughening implies an experience of friction while puckering involves shrinking of the oral surfaces (Gawel 2001, Hamada 2019, Kershaw 2019). These astringent subqualities can vary depending on the type of polyphenolic stimulus (Lee 1991) being consumed. For instance, while flavonols (a type of phenolic stimulus) can impart a 'rough and puckering' astringency, flavonon glycosides give rise to a much smoother and even 'velvety' astringent sensation (Hufnagel 2008). In addition to polyphenols, other components or factors of an astringent food can also affect this experience e.g. sourness, viscosity and temperature in a cranberry juice model have been shown to affect the intensity of perceived astringency (Peleg 1999). Thus, any investigation of astringent sensations is highly dependent on the food context and matrix.

The exact cause and mechanism of astringency is unclear. The most widely accepted mechanism is the protein-polyphenol complex formation. According to this mechanism, once dietary polyphenols enter the oral cavity, the process of mastication mixes the food with saliva and these polyphenols are exposed to salivary proteins, which can be present in the mobile phase of saliva or in the layer of saliva coating the oral mucosa. First, the salivary proteins in the mobile phase of

saliva are complexed with the polyphenols. Initially soluble, these complexes quickly turn insoluble in the continued presence of tannins. Next, as the salivary proteins from the mobile phase of saliva are depleted, the polyphenols start forming aggregates with proteins adsorbed onto the oral pellicle. At this point, the salivary pellicle layer coating the oral tissues is eroded and dryness in the mouth is experienced. This is not surprising as some of the salivary proteins particularly gPRPs and mucins provide saliva with its characteristic lubricant properties so upon complexation, they are unable to maintain their function (Ma 2016). It is unclear whether the perceived dryness is due to the insoluble aggregates eroding the lubricating salivary pellicle or due to direct interaction between free astringent stimuli and oral tissue exposed once the salivary lubrication is lost. It is likely to be a sum of all mechanisms combined. Figure 1.1 (Gibbins and Carpenter 2013, Ma 2016) illustrates this multi-step process.

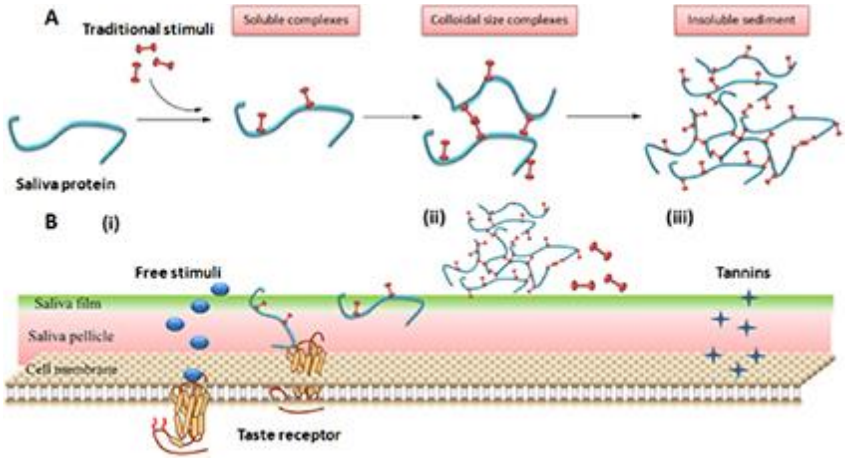

Figure 1.1: Various hypotheses that explain the development of astringency (Ma 2016)

In terms of protein-polyphenol combinations, there are several major classes of proteins that have been demonstrated to participate in these interactions and are outlined below:

Proline-rich proteins

The human salivary proteome is largely comprised of a multigene family of proline-rich proteins (PRPs) (Bennick 2002). PRPs are also found in several other mammals and their structure is typically characterized by a signal peptide, a transition region, a repeat region and a carboxyl terminal region (Ann 1985, Ann 1987). They are so-called due to a large number of proline residues, which give them a high affinity for binding tannins (Wroblewski 2001). PRPs can be further divided into three classes: (1) acidic PRPs (aPRPs), (2) basic PRPs (bPRPs) and (3) glycosylated PRPs (gPRPs). In humans, 6 genes are responsible for encoding the PRP family. Two genes, PRH1 and PRH2 encode aPRPs while the remaining four, PRB1, PRB2, PRB3 and PRB4 encode bPRPs and gPRPs (Kim 1993). Together these genes give rise to more than 20 PRPs across the three classes, which start out as similar pro-proteins and then undergo modifications and/or cleavage to turn into acidic, basic or glycosylated forms (Stubbs 1998, Bennick 2002). For instance, acidic PRPs contain a highly acidic N-terminal end and include PRP1 and PRP3, which are the most widely studied proteins in terms of tannin-binding properties. Basic and glycosylated PRPs have pro-proteins that are structurally similar to aPRPs but then undergo cleavage prior to secretion.

PRPs are known antimicrobial peptides. PRPs are secreted from the parotid glands and after post-translation modifications can be found both in the mobile and

adsorbed layer of saliva. While their functions range from maintaining the oral pellicle, dental enamel, calcium homeostasis, their role in taste and astringency perception has been studied only to a limited context in human models. Specific PRPs have been found to influence bitter taste perception (Melis 2013, Melis 2017).

Histatins

Histatins are a family of 12 histidine-rich proteins that are only found in the saliva of humans and some monkeys (Minaguchi 1989). The most prominent proteins in this family are HRP1, HRP3 and HRP5 (Sabatini 1989). Only HRP1 and HRP3 are unique and encoded by different genes; the remaining ten members of this family are cleavage products of these major proteins. They have also been shown to precipitate in the presence of tannins.

Histatins are secreted mostly from the parotid glands and submandibular glands. However, recently they have also been shown to be secreted from the minor intrinsic salivary glands called Von Ebner's glands, located around taste papillae on the tongue. Together with PRPs, histatins have been demonstrated to have antimicrobial (Du 2017) and antifungal properties (Han 2016). Their role in taste and astringency perception has been limited in investigation to a few studies only.

Cystatins

Cystatins are a family of proteins that are not only associated with oral health but have been demonstrated to play an important role in immunomodulation in other areas of the human body (Ochieng 2010). Cystatin proteins are protease inhibitors. In humans, there are three main salivary cystatins: S, SA and SN and all

have been suggested to play a primary role in the re-mineralization of the teeth as well as formation of the salivary pellicle and protection of oral tissue against proteolytic destruction (Baron 1999, Heller 2017).

Cystatins are secreted by the submandibular and parotid glands although the former secretes approximately five times as much cystatins as the latter. Thus, submandibular glands are thought to mostly contribute to production of salivary cystatins. Cystatins have been demonstrated to influence bitter taste perception (Mozel) as well as related to measures of astringency perception (Melis 2017).

Statherins

Statherin is a protein encoded by the gene STATH (Amado 2010). It is a low molecular weight protein that keeps ionic calcium and phosphate stable in the saliva and prevents their spontaneous precipitation (Vitorino 2005). In doing so, it is an essential part of the dental pellicle as it prevents the formation of hydroxyapatite crystals on the surface of the teeth (Yin 2006, McDonald 2011, Vukosavljevic 2012). Additionally, statherins plays a role in mitigating or preventing oral disorders such as periodontal diseases, dental caries, and oral mucosal infections. Statherins have been shown to have a relatively high affinity for condensed tannins, nearly at the same level as that of PRPs.

Statherin is produced by both parotid and submandibular glands. It is a relatively less-studied salivary protein in taste and astringency perception research.

Alpha-amylase

Alpha amylase is a hydrolase enzyme that catalyzes the conversion of starch into oligosaccharides (Amado 2010). It is produced in two areas of the human body:

salivary amylase in the oral cavity and pancreatic amylase in the pancreas. The salivary amylase jump-starts the digestion of polysaccharides at the moment of food ingestion and has an essential role in taste perception, oral digestion and prevention of oral and dental diseases.

Tannins from various sources such as black tea and pine nut coat extract have been shown to inhibit salivary amylase and reduce its hydrolytic activity. This results in a decreased conversion of starch into oligosaccharides and not only disrupts oral digestion but can also alter taste perception. It has also been shown that carbohydrates can inhibit protein-polyphenol interactions (Soares 2012) as they compete with polyphenols for aggregation with proteins. Although this effect was demonstrated in pectin and polygalacturonic acid (Soares 2012), it is possible that unhydrolyzed starch resulting from the inhibited activity of alpha-amylase can act in the same way. Additionally, a 2010 study (Mandel 2012) determined the relationship between AMY1 gene copy number and salivary amylase levels. It was found that there are genetic differences in the expression of salivary amylase. As a result, this can affect the amount of starch that an individual is able to digest in the oral cavity. Therefore, the amount of active alpha amylase present in saliva and consequently the amount of starch that is orally digested is likely to affect astringency perception via interfering with protein-polyphenol interactions.

Alpha-amylase is secreted by the parotid glands. Recently more evidence was found for interindividual variation in the alpha amylase and its relationship to taste perception, where amylase levels were found to be negatively correlated with sweetness and saltiness perception (Lamy 2020).

Mucins

At least 20 already identified mucins line wet epithelial surfaces throughout the human body such as the gut, respiratory tract, reproductive tract and eyes. In the context of saliva, there are four mucins that are secreted: MUC1, MUC4, MUC5B and MUC7 (Frenkel 2015). However, MUC5B is mainly responsible for the 'wetness' of saliva as it is a gel-forming protein. MUC7 typically exists as a monomer or dimer and does not contribute to gel formation. The remaining proteins, MUC1 and MUC4 are membrane-associated and localized as they line the ducts of the salivary glands and are involved in signal transduction (Liu 2002).

Studies have shown that MUC5B and MUC7 play a part in protection of the oral cavity by binding to antibacterial salivary proteins. For instance, MUC7 and MUC5B have been shown to bind aPRPs, bPRPs, statherin and Histatin 1 at their N-terminal domains (Takehara 2013). This also suggests that they may act as carrier for other salivary proteins and transport them from secretion sites to other areas in the oral cavity (Frenkel 2015, Frenkel and Ribbeck 2015).

Mucins are secreted by the sublingual glands. Their role in taste and astringency perception has not been well-studied.

1.2. TAS2R38, eating behavior and salivary proteins

Genetic taste blindness to PROP is a recessive trait controlled by the bitter-taste gene *TAS2R38*. Polymorphisms in this gene produce changes in amino acid residues at three locations giving rise to two common forms: AVI, the insensitive form and PAV, the sensitive one. Individuals who carry two recessive alleles

(AVI/AVI) are considered phenotypic non-tasters (NT); heterozygotes (AVI/ PAV) are considered medium-tasters (MTs) and carriers of two dominant alleles (PAV/PAV) are considered super-tasters (ST) (Guo 2001, Bufe 2005). This variation in amino acid residues causes a structural change in the bitter-taste receptor TAS2R38 and affects the binding affinity for PROP. For instance, super-tasters (STs) can bind PROP strongly and therefore experience the strongest bitterness, while non-taster (NTs) individuals do not bind PROP and experience none to very little bitterness. The intermediate PAV/AVI haplotype individuals are medium-tasters (MTs) and have a moderate affinity for binding PROP. The percentage of phenotypic groups in the population corresponds to 30% non-tasters and 25% super-tasters respectively, with the remaining 45% as medium-tasters.

The use of PROP impregnated filter-paper disks is a valid method for phenotyping subjects into the three taster groups that strongly correlate with TAS2R38 genotypes (Tomassini Barbarossa 2015). Typically, super- and medium-tasters gravitate towards foods that are less bitter in comparison to non-taster individuals as they may be more sensitive to oral stimuli and irritants. Numerous studies have shown that PROP status is a general marker for oral sensations such as bitterness, sweetness, fattiness and astringency. As bitterness and astringency can be negative attributes affecting consumer choice, this can have significant impact on food selection

((Drewnowski 2000, Lesschaeve 2005, Jaeger 2009). PROP non-tasters perceive less intensity from these sensations and prefer higher concentrations of these sensory qualities in their foods, while super-tasters show the opposite responses (Tepper 2008, Tepper 2017). For instance, Figure 1.2 shows selected figures from Melis et al (2017) in which non-taster males perceived less astringency and bitterness from cranberry juice cocktail supplemented with tannic acid and gave it higher liking ratings in comparison to super-taster males. Although some studies have inconsistent findings with this phenomenon (Sandell 2015, Duffy 2016), there is similar evidence in the literature that shows that bitter-sensitive individuals tend to have a lower intake of fruits that can be bitter e.g. lingonberries (Sandell 2015) and are also predicted to have lower liking of similar fruits (such as bilberries and crowberries), which are characterized by bitterness, sourness and astringency (Laaksonen 2013, Laaksonen 2013).

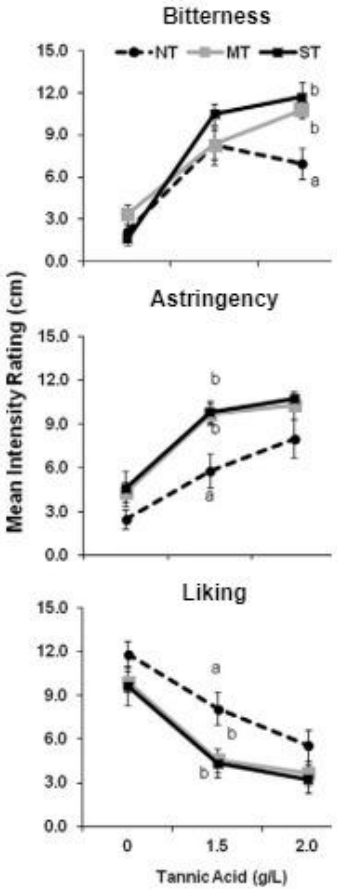

Figure 1.2: Sensory Data Excerpt from

In the last two decades, our lab has shown that non-tasters select diets that are higher in fats, sweets, and energy than super-tasters and this difference impacts body weight (Ullrich 2004, Goldstein 2005, Goldstein 2007, Tepper 2008, Shafaie

2013, Tepper 2014). Importantly, the consumption of sugar and sweet desserts plays a critical role in the development of oral disease (Mishra 2011).

Most recently TAS2R38 has been linked to differential release of selected salivary proteins:

In our recent work (Melis 2017), a taster-specific response in release of salivary proteins in response to stimulation with cranberry juice was observed. Specific subtypes of aPRPs and Cystatins (specifically PRP1 and PRP3; Cyst SN) were released in higher concentrations in tasters in comparison to non-tasters after stimulation with cranberry juice. It should be noted that these proteins are heavily involved in immune response against oral pathogens. Figure 1.3 shows the data. The idea that PROP taster status may be connected to variation in salivary protein expression, particularly those involved in bitter taste perception has already been corroborated (Cabras 2012, Melis 2013).

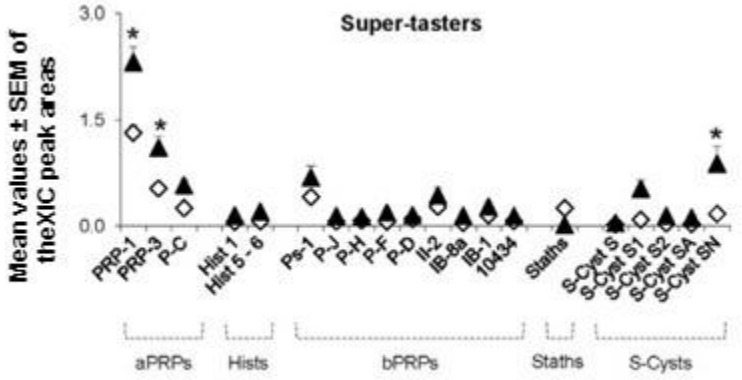

Figure 1.3: Mean values ± SEM of the XIC peak areas of salivary proteins levels in resting (✧) and saliva stimulated with cranberry juice (✦) in super-tasters (n=25). Note levels of PRP-1 and PRP-3 as well as S-Cyst SN rise significantly in super-tasters following stimulation. This effect is not significant in non-tasters (data not shown) (Melis 2017)

These proteins have known anti-pathogenic activity (Hemadi 2017) and the polymorphisms in these proteins have been linked to higher risk of developing oral disease (Hong 2014, Stromberg and Marell 2017, Wang 2018). Since we have observed a difference in the levels of these proteins with respect to PROP taster status, it is possible that individuals have compromised status of oral health due to an interaction of these factors.

1.3. TAS2R38 and immunity

TAS2R38 has been previously linked in host defense responses in the upper respiratory tract and evidence suggests that this gene may have a similar role in oral microbial defenses. The TAS2R38 receptor resides in non-oral tissues such as the upper airways, where it functions as a microbial sensor against pathogens. In this role, TAS2R38 receptors detect bacterial quorum-sensing molecules via ciliated cells (Lee 2015). For example, TAS2R38 is activated to release the antimicrobial, nitric oxide, in the presence of quorum-sensing molecules expressed by *Psuedomonas aeruginosa*, a pathogen involved in sinus disease (Lee 2015). Individuals who express the nonfunctional TAS2R38 receptor (i.e. NTs) show lower detection of these molecules leading to greater biofilm formation (Hamilos 2015) in the sinus mucosa and are more susceptible to *P. aeruginosa* infection with higher risk of developing chronic rhinosinusitis (CRS). Indeed, individuals with the non-taster phenotype are highly overrepresented in CRS patients requiring surgery (Lee 2012, Adappa 2014).

Recent research suggests that TAS2R38 performs similar functions in the oral cavity. An *in vitro* study showed that oral pathogens could induce a taster-status

specific expression of TAS2R38 in gingival epithelial cells (GECs) (Gil 2015). GECs derived from PROP-classified individuals were exposed to the oral pathogens *S. mutans* and *P. gingivalis*. Cells from super-tasters showed a 4.3-fold increase in the induction of TAS2R38 mRNA vs. a negligible change in cells from non-tasters (Gil 2015). Human data also show that non-tasters may be more susceptible to dental caries in children (Pidamale 2012, Pidamale 2012) as well as adults (Wendell 2010, Yildiz 2016). However, the mechanisms that may orchestrate this individual variation in oral health due to PROP taster status are not well-studied. One possible mechanism could be via modulation of the oral microbiome. Most recently, a study demonstrated a potential mechanism whereby bitter taste receptors detect quorum-sensing molecules in the oral cavity, they induce a cascade of events that release of antimicrobial peptides (Zheng 2019). Since antimicrobial peptides can influence the microbiota, this relationship merits further investigation.

A healthy oral environment reflects a delicate balance of beneficial and pathogenic microbes. In healthy humans 96% of the cultivable oral bacteria fall into six phyla in order of abundance: *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria*, *Spirochaetes and Fusobacteria* (Dewhirst 2010, Verma 2018). Most oral diseases are caused by a disruption in the proportions of the members of these phyla (dysbiosis). Given its interaction with microbes in the airways as discussed above, it is possible that TAS2R38 receptors also modulate the oral microbiome. The study of individual variation in oral microbiome has mostly been conducted in disease-affected populations e.g., in people affected by dental caries. Recently it was demonstrated that the oral microbiota PROP genotypes can also vary (Sandell 2018,

Cattaneo 2019). However, we do not know much about individual variation in oral microbiome in healthy populations with respect to the PROP taster status phenotype as well as the associated levels of salivary proteins expressed.

1.4. Cranberries and oral Health

Cranberries are unique among high-acid, astringent fruits in that they contain the greatest abundance of Type-A proanthocyanidins (Foo 2000), which inhibit bacterial adhesion. This mechanism is the basis of how cranberry polyphenols mitigate urinary tract infections (Foo 2000) and also work against oral pathogens (Bodet and Ofek 2008). This role has been confirmed in *in vitro* experiments against key oral pathogens such as *S. mutans* in dental caries and *P. gingivalis* in periodontitis. Despite this evidence, only two limited clinical studies have evaluated the use of cranberry polyphenol extract (CPE) in reducing oral pathogens. The first study confirmed the effectiveness of cranberry-derived polyphenols in lowering *S. mutans* (Weiss 2004), while the second study established that these polyphenols were just as effective in lowering *S. mutans* count as Chlorhexhidine, the active ingredient in commercial mouthwash used to treat periodontitis (Khairnar MR 2015). Other than these studies cranberries have not been evaluated in how they would affect the overall microbial profile and whether they can be used in treating cases of oral dysbiosis.

1.5. Specific aims and hypotheses

The specific aims of this project are as follows:

AIM I: PROP and astringency: Determine if genetic variation in the ability to taste the bitter compound PROP, a marker for taste perception and food preferences, plays a role in astringency perception and liking of cranberry juice.

We hypothesize that:

1. PROP non-tasters will perceive less astringency from cranberry juice and
2. give it higher liking ratings in comparison to PROP tasters.

Rationale:

Much of past research on astringency and liking of astringent foods has used tannic acid as an astringent stimulus or to modulate the levels of astringency in model foods. As tannic acid is neither palatable nor part of normal human diets, the results obtained by these studies do not necessarily reflect 'real-world' responses to high-polyphenol diets. Secondly, no other team has used cranberry juice as an astringent model while measuring consumer taste perception and liking. Other work uses model foods that are not commonly consumed in America e.g. bilberry (European crop), aronia berry (American crop, but not commonly consumed), wine (widely consumed but has high variation due to grape variety and origin). On the other hand, cranberries are an important American crop. Cranberry-derived juices and polyphenol extracts, are readily accessible and widely consumed in various foods e.g. as cranberry sauce, craisins, cranberry juice cocktail etc. Given their economic and geographic importance as well as high polyphenol content, we will use cranberry juice as a model astringent. To modulate levels of astringency in our samples, we will use cranberry polyphenol extract (CPE) in different concentrations. CPE is GRAS and as it is already used in commercially available beverages, its use

will pave way for results that are translatable to real-world dietary behavior. These data will help determine whether PROP taster status is an effective tool in understanding and predicting individual variation in the perception of astringency.

AIM 2: PROP, astringency, and salivary proteins: Examine the time course of salivary protein response after stimulation with cranberry-derived astringent stimuli and determine interindividual differences with respect to PROP taster status and. We hypothesized that:

- PROP super-tasters will have elevated levels of salivary proteins following stimulation, in line with previous observations [52] and that
- Salivary protein levels will return to baseline earlier in STs than in non-tasters after stimulation.

Rationale:

Current understanding of salivary protein variation and its influence on astringency among PROP phenotype groups is limited. Additionally, nearly all of the literature studying oral recovery from astringency following exposure to a stimulus varies in methodology. Studies assume that it takes anywhere from about 5 minutes to nearly 30 minutes for the oral surfaces to completely reset following exposure and as such there is no consensus on how much time is needed for full recovery. Most commonly research uses a 5- or 10-minute break between introducing astringent stimuli. Our proposed methods will not only give a snapshot of salivary proteins profile at 5- and 10-minutes post-stimulation but will also link it to PROP groups. The results of this

experiment will provide novel insight into how quickly and in what ways PROP groups are able to recover from a polyphenolic stimulus.

AIM 3: PROP, salivary proteins, and oral health: Determine if there is variation in the oral microbiome with respect to the PROP phenotype, if daily rinsing with cranberry-polyphenol extract (CPE) oral rinse would alter the oral microbiome and if there would be changes associated with salivary protein profile or alteration of taste perception as an outcome of this intervention.

We hypothesized that:

- the oral microbiome structure would vary with PROP taster phenotype,
- the CPE oral rinse would alter the composition and diversity of the oral microbiome, and that
- these changes would be associated with changes in salivary proteins and taste perception.

Rationale:

The status of oral health is a result of a combination of various factors: genetic (e.g., polymorphisms in certain genes), lifestyle (e.g., consumption and frequency of certain foods) and oral hygiene habits. It has been well-established that irregular or poor hygiene habits lead to poor health and so most of the personal care industry intervenes by developing oral care products and devices such as toothpastes, toothbrushes, oral rinses, dental floss etc. It is also common knowledge that sugary and acidic foods can lead to dental decay and in combination with poor oral hygiene

can aggravate these issues. However, there is not enough research and public knowledge on how genetic factors can predispose individuals to lowered oral health status, whether it is through the foods they gravitate towards or a differential expression of protective salivary proteins. In our case we will be evaluating how the PROP phenotype may be related to lower or higher risk of developing oral disease and if salivary protein profiles can provide an explanation for these differences. Since, PROP non-tasters and super-tasters make up approximately 55% of the population, this issue deserves attention.

Secondly, this research will be the first human study to link PROP, oral health, salivary proteins and test the potential of CPE in this context. The data will pave way for personalized functional food or the development of an oral rinse that could lower pathogenic bacteria without the risk of developing resistance such as that observed with antibiotics use.

2. PROP TASTER STATUS & ASTRINGENCY PERCEPTION

Neeta Y. Yousaf and Beverly J. Tepper

2.1. Abstract

The objective of this study was to investigate if PROP taster status and other individual factors such as age, gender, ethnicity, and BMI are markers of variation in perception of astringency and other flavor attributes of cranberry juice. Participants (n=125) evaluated cranberry juice cocktail samples (CJC) supplemented with cranberry-derived polyphenol extract, CPE (added in 0, 0.3, 0.5 and 0.75 g/L) as well as control samples (unsweetened cranberry juice, CJ; aqueous solution of 0.75g/L CPE). Subjects evaluated the samples for sweetness, sourness, thickness, bitterness, astringency, cranberry flavor, overall flavor and liking using a 15-cm end-anchored line scale. The data was analyzed using an ANCOVA and machine learning tools (specifically regression trees and random forest modeling) to examine if the latter approach would extract more meaningful insights about role of personal factors on sensory perception of cranberry-derived stimuli. The ANCOVA revealed robust stimulus effects but no effect of PROP taster status on astringency perception was observed; instead, PROP taster status was observed to affect cranberry flavor perception and liking, where ST subjects perceived more flavor and liked them less. An effect of ethnicity was also observed, where Caucasian subjects generally perceived more bitterness and astringency from the samples and liked them less in comparison to Asian subjects. Regression trees generally agreed with ANCOVA results, but their visualized framework showed that each sensory attribute was influenced by a different set of independent variables. Random forest modeling confirmed that each independent variable had a different explanatory power for each sensory attribute. Together, these data show that PROP taster status has a

limited role in astringency perception of cranberry-juice when cranberry polyphenols are used as an astringent stimulus and disagrees with previous reports, which utilize lab models. The findings underscore the need to study human sensory perception using ecologically relevant food models and emphasize that personal factors, particularly ethnicity, must be an essential consideration while designing experiments to study sensory perception of complex foods. Finally, this study validates the capabilities of machine learning tools in the study of complex sensory datasets.

2.2. Introduction

Astringency is a well-known sensation marked by drying, puckering, and roughing of the oral surfaces (Lee 1991) upon consumption of polyphenol-rich foods. Although astringency is an expected and often desirable trait in certain foods such as coffee, tea, or dry red wine, it is often thought of as an unpleasant sensation. Indeed, numerous studies show that astringency can be a negative driver of acceptance of many foods and beverages including tea (Xu 2018), fruits (de Beer 2012), juices (Koppel 2010, Lawless 2013, Mayuoni-Kirshenbaum 2013), whey beverages (Childs 2010), cheese (Torri 2016), nuts (Lipan 2020), chocolate (Virgens 2020) and wine (Niimi 2017). Astringency has also been associated with consumer perceptions of lower quality (Koppel 2010, Sáenz-Navajas 2013). Since polyphenol-rich foods have substantial health benefits (Cory 2018, Durazzo 2019), it is important to understand how astringency is perceived and to develop strategies to mitigate their negative sensory attributes to enhance acceptability of such foods.

There is considerable individual variation in the experience of astringency which is well-known. This variability is shaped by differences in familiarity, diet, gender, and ethnicity, as well as personal traits such as food neophobia, nutrition attitudes, sensitivity to disgust, and sensitivity to reward and punishment among others (Dinnella 2011, Laaksonen 2013, Robino 2016, Corsi 2017, De Toffoli 2019, Cravero 2020).

Studies have also examined the role of genetic variation in the *TAS2R38* gene as a general marker for a range of oral sensations including alcohol burn, pungency, oral tactile perception as well as astringency. PROP taster status is the phenotypical manifestation of polymorphisms in the *TAS2R38* gene, which codes for the bitter taste receptor TAS2R38. Individuals with homozygous dominant alleles for this gene (PAV/PAV) experience greater bitterness from common foods and are called super-tasters (STs) (Kim 2003, Bufe 2005). Individuals who are recessive for this gene (AVI/AVI, phenotypic non-tasters, NTs) experience little PROP bitterness in comparison to STs; heterozygotes (medium tasters, MTs) perceive moderate bitterness from PROP. Generally speaking, PROP STs perceive greater intensities of these oral sensations in comparison to NTs which reduces their preferences for, and selection of foods with these sensory qualities (Tepper 2008). Studies specifically investigating the influence of PROP status on the acceptance and consumption of astringent foods have produced mixed results. Pickering (2004; 2006) reported that PROP STs experienced greater astringency from red wines in comparison to NTs (Pickering 2004, Pickering 2006). Contrary to what was expected, Laaksonen et al (2013) reported that PAV homozygotes (phenotypical PROP STs) gave lower

astringency ratings to berry juice fractions supplemented with polyphenols (Laaksonen 2013) than AVI homozygotes (phenotypic PROP NTs). Nevertheless, the PAV homozygotes disliked the samples the most. More recently, Melis and colleagues (2017) showed that PROP taster status played a role in the perception and liking of cranberry juice cocktail modified to enhance astringency, but only when examined separately by gender. Specifically, they reported that PROP ST males perceived greater bitterness and astringency from cranberry juice cocktail supplemented with tannic acid and gave lower liking ratings to it than did non-taster males (Melis 2017). Other researchers have been unable to find robust evidence of such relationships (Thorngate 1995, Smith 1996, Duffy 2016, Carrai, Campa et al. 2017).

There are numerous reasons for the discrepancies across studies. First, differences may lie in the type of astringent stimulus used. In the early studies, alum, tannic acid, or grape seed tannin were used as model stimuli (Ishikawa 1995, Smith 1996, Robinson 2004), which are not consumed on their own in typical diets. Since the intensity and quality of astringency is highly dependent on the type of astringent stimulus (Fleming 2015), extrapolating the findings from laboratory stimuli to real-world complex foods, such as fruit juices may not be valid. Secondly, study populations tested in these studies have varied widely. Many studies utilized homogeneous populations (typically majority White Caucasian populations from North America and Western Europe) (Törnwall 2011, Duffy 2016); some examined more ethnically-diverse consumer groups (Yang 2020, Yousaf 2020), and other did not report on the ethnic mix of their participants (Laaksonen 2013, Torri 2016,

Kershaw 2019, Griffin 2020). The lack of ethnic diversity and balance in taste studies is not unique to studies on PROP status and astringency. When Burgess et al (2018) examined the role of *CD36* polymorphisms on fatty acid taste sensitivity they tested a well-described cohort of Caucasian and East Asian participants (Burgess 2018). By doing so, they were able to demonstrate different gene effects in the two ethnic groups that were unobservable when all subjects were examined as a single group. Thus, the investigation of astringency with more weight placed on personal characteristics using ecologically representative food models deserves more attention. Yet another difference may lie in the data treatment or type of statistical tests used. Studying more than a few individual factors of variation with traditional statistical testing (e.g., using analysis of variance) often yields complex datasets, with the presence of two or three-way interactions, challenging a meaningful interpretation of the data. Recent work (Yang 2020) has used machine learning tools, specifically regression trees, to distill a complex sensory datasets into a visualized framework. Thus, there is an opportunity to use machine learning to uncover important relationships between taste perception and individual factors of variation.

The first objective of the present study was to investigate if PROP taster status and other individual factors such as age, gender, ethnicity, and BMI are markers of variation in perception of astringency and other flavor attributes of cranberry juice. Similar to our recent work on polyphenols and salivary protein release (Yousaf 2020), we used cranberry juice and cranberry cocktail which we supplemented with cranberry-derived polyphenols as an ecologically relevant

model stimulus. We hypothesized that PROP STs would perceive greater bitterness, astringency, and intensity of other flavor attributes in comparison to NTs and this would negatively affect their liking of cranberry juice.

The second objective of the study was to utilize a machine-learning based modeling approach to study these research questions. Traditional analysis of covariance (ANCOVA) was used alongside machine learning models, to determine if the latter methods extracted more meaningful information from the data, particularly with respect to parsing out the role of individual variability.

2.3. Methods

2.3.1. Subject Recruitment

A total of 125 healthy adults (male and female), 18-45 years of age were recruited from the Rutgers University community through an email distribution list. Subjects were screened for PROP taste sensitivity; only PROP non-tasters and super-tasters were admitted into the study into groups balanced for gender. Subjects were also screened for general suitability (e.g., demographics, health information) and familiarity with cranberry juice and products and consumption within the last 2 years.

Exclusion criteria included major metabolic diseases (diabetes, kidney disease, etc.), pregnancy, lactation, food allergies, and the use of medications that interfere with taste or smell functions (e.g., steroids, antihistamines, or anti-depressants). Participants who were determined to be medium-tasters for the PROP phenotype were also excluded.

The study was approved by the Rutgers University Arts and Sciences Institutional Review Board (Approval#13-309M). All subjects provided informed consent and were compensated monetarily for their participation.

Table 2.1 shows subject characteristics. The subject pool was mostly Caucasians (n=77), and the rest were Asians (n=48). Mean participant age and BMI were 21.8 ± 0.39 years and 24.9 ± 0.4 kg/m^2, respectively. Taster by gender subgroups were approximately balanced.

Gender	PROP Classification	n (of 125)	Ethnicity (n)		Age	BMI
			Caucasian	Asian	(years)	(kg/m^2)
Female	**NT**	30	22	8	22.1 ± 1.0	25.2 ± 0.9
	ST	33	23	10	22.2 ± 0.9	24.5 ± 1.0
Male	**NT**	30	13	17	21.1 ± 0.4	24.8 ± 0.8
	ST	32	19	13	21.7 ± 0.7	25.0 ± 0.6

Table 2.1: Subject characteristics.

2.3.2. PROP Taster Status:

The participants were screened and classified according to PROP taster status via the paper disk method (Zhao 2003), which has previously been tested for validity and reliability (Zhao 2003, Carta 2017, Melis 2017) and strongly correlates with tongue electrophysiological recordings (Pani 2017, Sollai 2017).

Briefly, subjects place a filter paper disk impregnated with 1.0 mol/L NaCl on the tip of the tongue until it is thoroughly wet. They rate the taste intensity of the disk using the labeled magnitude scale (LMS), a 100-mm scale anchored with the phrases “barely detectable” to “strongest imaginable”. This procedure is repeated with a second paper disk impregnated with 50 mmol/L PROP (6-n-propyl–2-thiouracil, P3755, Sigma-Aldrich). Subjects are instructed to rinse with spring water

at room temperature before and in between tasting each paper disk. Subjects are categorized as non-tasters (NT) if they rate the PROP disk < 15 mm on the LMS; they are categorized as super-tasters if they rate the PROP disk > 67 on the LMS. All others are classified as medium tasters. NaCl ratings do not vary with PROP status in this method. Therefore, NaCl ratings are used as a reference standard to clarify the taster status of subjects who give borderline ratings to PROP. This strategy is based on the rationale that non-tasters give higher ratings to NaCl than to PROP, medium tasters give equivalent ratings to both stimuli and super-tasters give higher ratings to PROP than NaCl.

2.3.3. Test Stimuli:

Cranberry juice with various levels of astringency, as modulated by addition of cranberry polyphenol extract, (CPE), was used as a test stimulus. There were a total of 6 samples in this study: CJ, CPE, CJC, Low, Med and High. Table 2.2 shows the details for each of these samples.

	Sample	Details
1	CJ	Cranberry Juice, unsweetened
2	CPE	0.75 g/L Cranberry Polyphenol Extract in spring water
3	CJC	Cranberry Juice Cocktail, CJ with 8.75% w/v sugar
4	Low	0.30 g/L CPE in CJC
5	Med	0.50 g/L CPE in CJC
6	High	0.75 g/L CPE in CJC

Table 2.2: Sample formulations.

Cranberry juice (CJ) was made from frozen cranberries, donated by Ocean Spray (Chatsworth, NJ). The cranberries were frozen at -20 C until use. Cranberry juice was made in small batches to maintain freshness using a standard recipe. Briefly, 300 g of berries were defrosted, washed and cooked on a stovetop with 648 mL of

spring water. The mixture was filtered through cheesecloth, mixed with 52.5 g of sucrose (CJC) and allowed to cool. This sample was cranberry juice cocktail (CJC). The final concentration of sucrose in the CJC was 87.5 g/L (8.75% w/v). Batches of cocktail were prepared as needed, the day before subject testing, and refrigerated at 4C until 30 min before use.

The cranberry-derived polyphenol extract solution (CPE) was made using a carrier-free powdered extract (Ocean Spray, MA) with the final solution with 0.75 g/L. 0.75 g of powdered CPE was added to pre-warmed 100 ml of spring water, while stirring on a hot plate to facilitate dissolution. This solution was prepared as needed, the day before subject testing, and refrigerated at 4C until 30 min before use.

Cranberry juice cocktail was supplemented with CPE in three concentrations: at 0.3 g/L (Low), 0.5 g/L (Med) and 0.75 g/L (High). These concentrations were selected after extensive benchtop screening for palatability to select a range that demonstrates the lower and higher acceptable limits. For these samples, CPE powder in the respective concentration was mixed directly into freshly prepared CJC. These samples were prepared as needed, the day before subject testing, and refrigerated at 4C until 30 min before use.

All samples were served at room temperature. Order of presentation was randomized and there was a forced 5-min break in between sample evaluation.

2.3.4. Taste Ratings:

Intensity ratings for key sensory attributes of the cranberry juice cocktail was collected using standard, 15-cm line scales anchored with the phrases “very weak” to “very strong.” Attributes of interest included sweetness, bitterness, sourness, astringency, thickness, cranberry flavor and overall flavor. Overall liking of the samples was collected with a similar scale anchored with “dislike extremely” to “like extremely.” Sensory ratings were collected electronically using RedJade data collection software, where the ballot is presented on a computer screen and subjects make their assessments electronically.

2.3.5. Experimental Procedures:

Subjects were instructed to refrain from consuming astringent foods for approximately 8 hours prior to each session. A list of such foods was provided to them. They were also prohibited from eating, drinking (except plain water), chewing gum, using breath mints, mouthwash or brushing their teeth for 2 hours prior to the test sessions.

Subjects participated in three test sessions scheduled at the same time on three different days. All testing took place in individual testing booths. The entire testing sequence took approximately 3 weeks to complete. Figure 2.1 shows the experimental procedure.

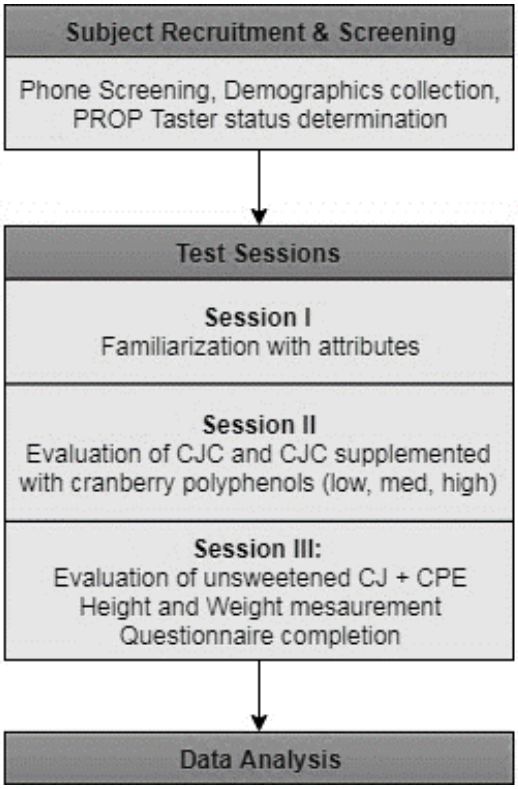

Figure 2.1: Experimental Procedure.

Session I: Familiarization Task:

During session 1, Subjects were presented with prototypical reference solutions for sourness (1.9 g/L citric acid), bitterness (0.6 g/L caffeine) and astringency (1.38 g/L alum) to familiarize subjects with these attributes. Subjects then participated in a matching exercise where they were asked to identify the above-mentioned reference solutions with the correct sensory attribute. If a subject answered incorrectly, they were re-directed to practicing sampling the solutions again until they matched the samples correctly. Only participants who answered correctly could progress in the experiment.

Finally, subjects then practiced evaluating these attributes by rating the intensity of sourness, bitterness, and astringency of a plain cranberry juice cocktail (CJC) sample.

Session II: Sample Evaluations

Subjects were presented with CJC with 0, 0.3, 0.5 and 0.75 g/L of added CPE with 5 min breaks in between sample evaluations. These levels were determined by extensive benchtop screening and were chosen to represent acceptability and palatability. The lowest concentration (i.e., 0.3 g/L) could be discriminated from the standard CJC in pilot testing. Beyond the highest concentration of CPE (i.e., 0.75 g/L), the CJC was not palatable any longer.

Session III: Sample Evaluations & Questionnaire Completion

Subjects evaluated unsweetened cranberry juice (CJ) as well as CPE in water using the same procedure as in session 2.

Subjects completed the 3-Factor Eating Questionnaire by Stunkard & Messick (Stunkard 1985) for assessment of general eating attitudes in addition to food preference questionnaire for astringent foods (Melis 2017). Demographic information collected in this study included gender, age, ethnicity, country of birth, and length of time living in the U.S. Height and weight were also measured for BMI calculations.

2.3.6. Data Analysis:

XLSTAT Software (Addinsoft New York USA) was used to perform all statistical analyses. Normality testing was conducted using the Shapiro-Wilk test, while homogeneity of variances was tested using Levene's test.

ANCOVA:

An ANCOVA model was applied to determine effects of independent variables on the intensity of the following attributes: sweetness, sourness, bitterness, astringency, thickness, cranberry flavor, overall flavor and liking ratings. Independent variables were a combination of grouping factors and covariates. PROP taster status, gender, ethnicity, and sample type were used as the main factors, while Age and BMI were the covariates. The results output is shown in Table 2.3. Post-hoc test was conducted for Sample Type using Duncan's test ($p<0.05$). Other significant effects are also shown in the table, but no post-hoc tests were conducted for them.

Regression trees:

Given the complex and multifactorial nature of the dataset, regression trees were used as a secondary approach to examine the data. CHAID regression trees were computed, in which an F test is conducted for each explanatory variable. If a significant difference is found ($p<0.05$), a node is created. Each node was set to 5% difference. Next, validation was performed for each tree, where a subset of data was randomly selected, and the explanatory power of the tree was tested. The fits of the trees were compared using the Mean Square Error (MSE) value and the models were optimized until the MSE value was minimized as much as possible.

The trees were computed for each of the attributes: sweetness, sourness, bitterness, astringency, thickness, cranberry flavor, overall flavor and liking using the same factors as in the ANCOVA model above. Additionally, age was converted to a discrete factor with two levels: Under 21 years or Over 21 years of age. BMI was converted to a discrete variable with two categories: Normal (≤ 25 kg/m^2) or Overweight (> 25 kg/m^2).

Random Forest Modeling:

A random forest model was used to evaluate feature importance i.e., the relative impact of the independent variables (PROP taster status, gender, ethnicity, sample type, age, and BMI) on each of the following attributes: sweetness, sourness, bitterness, astringency, thickness, cranberry flavor, overall flavor and liking ratings. Random forest modeling is a machine-learning approach, in which multiple regression trees are computed (similar to the above description), each using a subset of the dataset. By computing a large number of regression trees, the data is sampled repeatedly. The output is then averaged across these trees allowing for a comparison of variable importance.

A random forest model was used to evaluate the effect of the independent variables on each of the attributes. Hyper-parameters were set at 1000 trees. A

score was assigned to each factor in how important it was in explaining variance in those ratings. A heatmap was then computed for each of these factors across the various taste attributes.

2.4. Results

2.4.1. ANCOVA results

ANCOVA results revealed several significant effects of individual factors of variation (Table 2.3).

	Sweetness	Sourness	Bitterness	Astringency	Thickness	Cranberry Flavor	Overall Flavor	Liking
R^2	0.448	0.463	0.134	0.140	0.138	0.486	0.363	0.550
F	**22.402**	**23.778**	**4.274**	**4.494**	**4.419**	**26.048**	**15.699**	**33.643**
Pr > F	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Age	0.116	0.445	1.886	0.769	0.010	0.594	**13.208**	**4.359**
	0.733	0.505	0.170	0.381	0.920	0.441	**0.000**	**0.037**
BMI	3.180	1.217	1.750	0.464	0.391	0.033	**4.316**	2.990
	0.075	0.270	0.186	0.496	0.532	0.856	**0.038**	0.084
PROP Taster Status	0.926	2.947	1.055	0.392	0.001	**5.640**	1.117	**4.546**
	0.336	0.086	0.305	0.531	0.974	**0.018**	0.291	**0.033**
Gender	0.145	2.925	1.593	0.472	2.966	0.118	2.536	1.794
	0.703	0.088	0.207	0.492	0.085	0.731	0.112	0.181
Sample Type	**113.424**	**112.270**	**12.617**	**18.750**	**20.823**	**128.417**	**74.343**	**167.699**
	< 0.0001	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Ethnicity	1.118	1.795	**23.297**	**9.239**	0.344	1.189	0.073	**14.136**
	0.291	0.181	**< 0.0001**	**0.002**	0.558	0.276	0.787	**0.000**
Taster*Gender	0.413	**12.183**	0.191	1.955	0.463	**14.980**	3.147	0.335
	0.520	**0.001**	0.662	0.162	0.496	**0.000**	0.076	0.563
Gender*Sample	0.486	**2.841**	1.123	0.464	0.228	0.492	0.327	0.728
	0.787	**0.015**	0.346	0.803	0.950	0.782	0.897	0.602

Table 2.3: ANCOVA model results. Effects of main factors and covariates (Age, BMI) on sensory attribute intensity and liking ratings of cranberry juice cocktail samples. For each independent variable, first row shows the F value, while the second row shows the associated p-value. Values in bold show statistically significant differences ($p<0.05$).

First, the most prominent effect was observed in sample type, which significantly affected ratings of all sensory attributes as well as liking (Fig. 2.2).

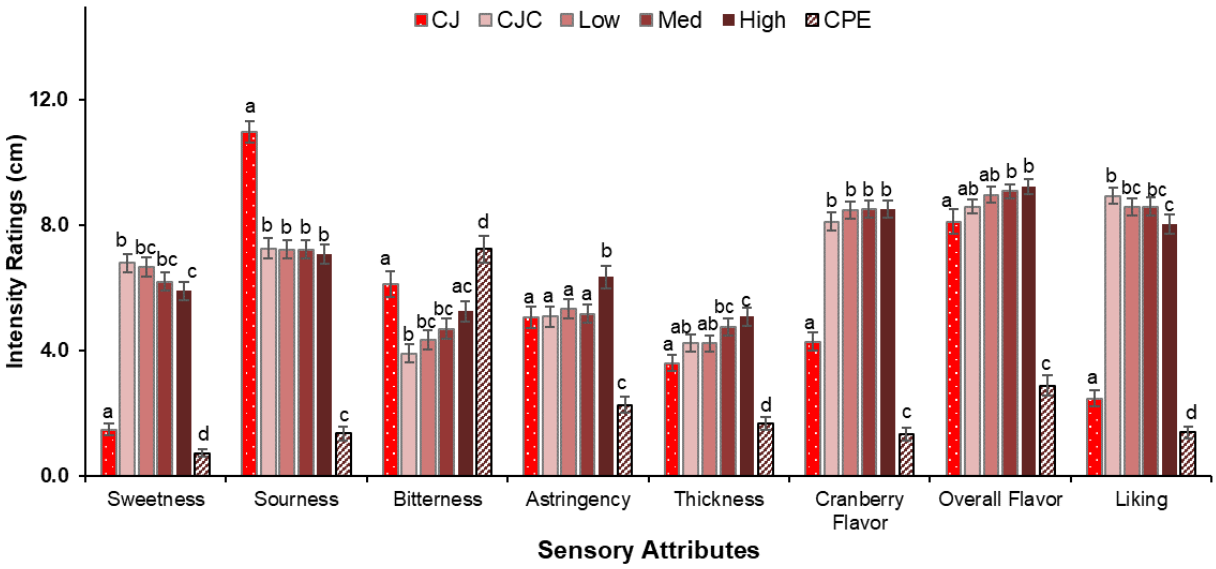

Figure 2.2: Effect of sample type on attribute intensity ratings (Mean ± SEM cm) of the CJ, CJC, Low, Med, High and CPE samples. Different superscripts (a, b, etc.) denote statistically significant differences based on post-hoc analyses ($p<0.05$).

The two controls, CJ and CPE were generally perceived to be distinct from the four CJC samples, which were clustered more closely. CPE was given the lowest intensity ratings for all sensory attributes except bitterness and was liked the least. Among the four CJC samples, CJC was the sweetest, while High was rated as most bitter, thickest, had overall flavor, and liked the least. Low and Med samples were given intermediate ratings.

Several other significant effects were also revealed by the ANCOVA (Table 2.3). An overall main effect of ethnicity was found for three attributes: bitterness ($p <0.0001$), astringency ($p=0.002$), and liking ($p=0.000$). Overall, Caucasian subjects

gave higher intensity ratings to both bitterness and astringency in comparison to Asians and liked them less than did Caucasian subjects.

There were also two significant interactions. First, a gender*sample ($p=0.015$) interaction affected sourness ratings. This was mainly due to male subjects giving higher sourness ratings to CJ in comparison to females (11.8 ± 0.4 vs. 10.3 ± 0.5 respectively). Secondly, a taster*gender interaction was observed in sourness ($p=0.001$) and cranberry flavor ($p=0.000$) attributes. Specifically, female STs gave higher ratings to these attributes than female NTs. No difference was observed in male subjects with respect to PROP groups.

2.4.2. Regression Trees

Figures 2.3 (a-h) show regression trees computed for each of the attributes.

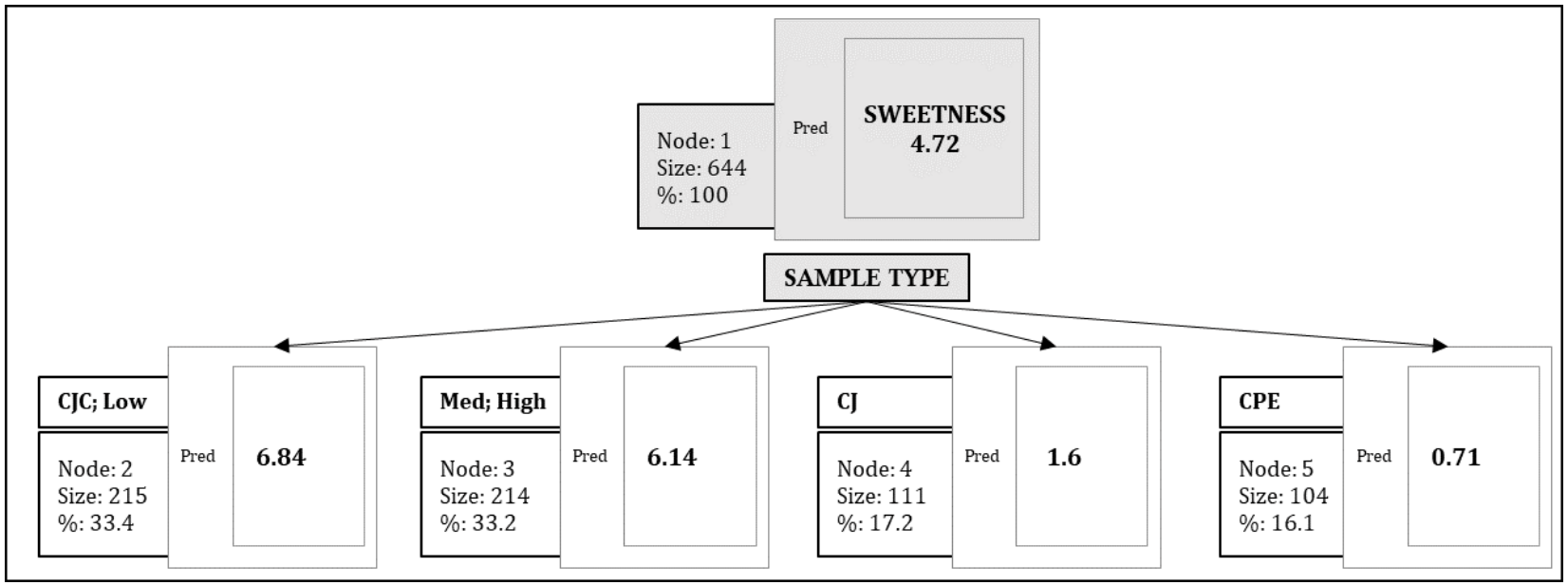

Figure 2.3a: Regression tree for sweetness intensity ratings. Node refers to a split based on a significant test ($p<0.05$). Size refers to number of subjects in a given split while % refers to the corresponding percentage of the total subjects in a given split. 'Pred' shows predicted values of intensity ratings on the 15-cm line scale.

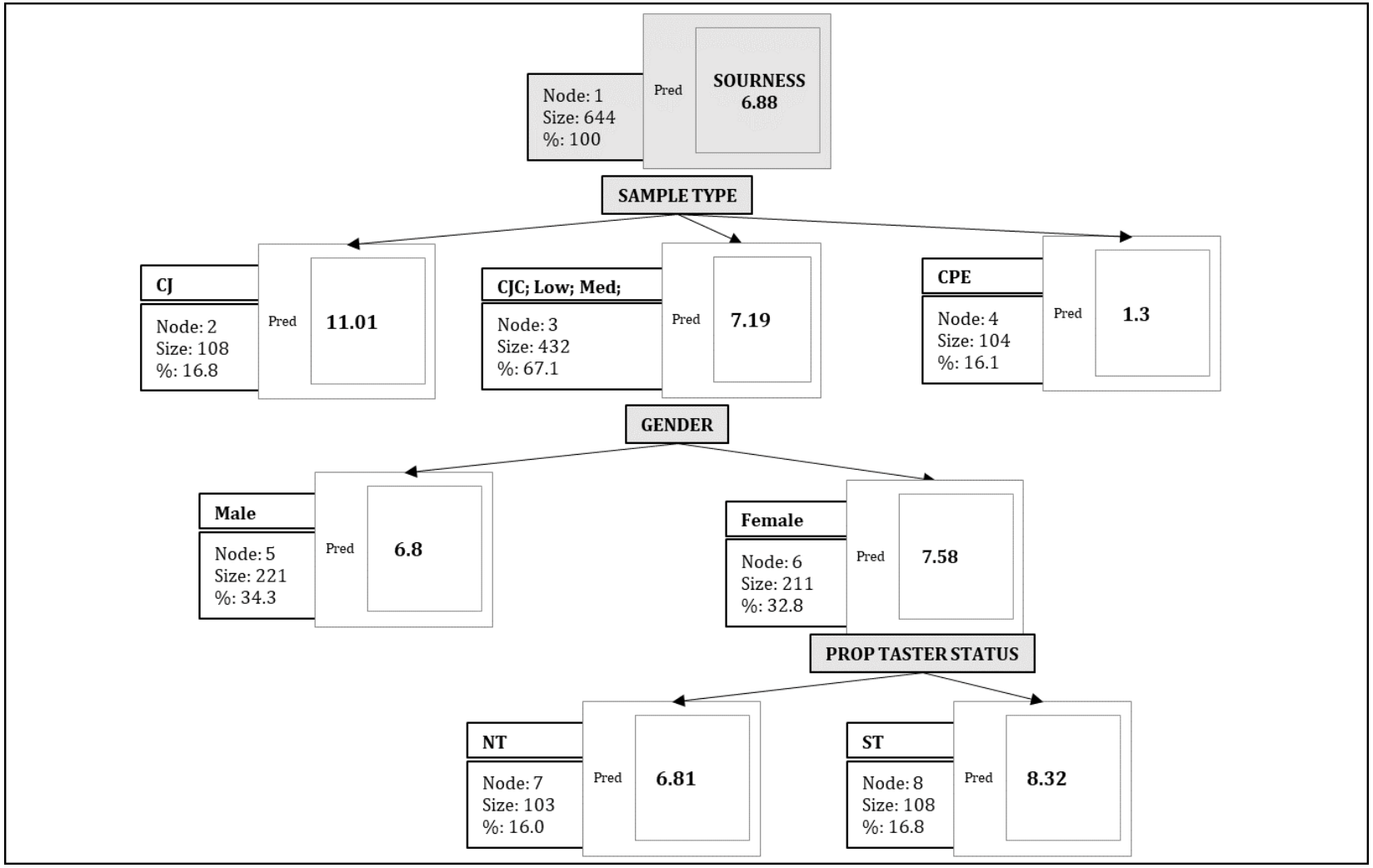

Figure 2.3b: Regression tree for sourness intensity ratings

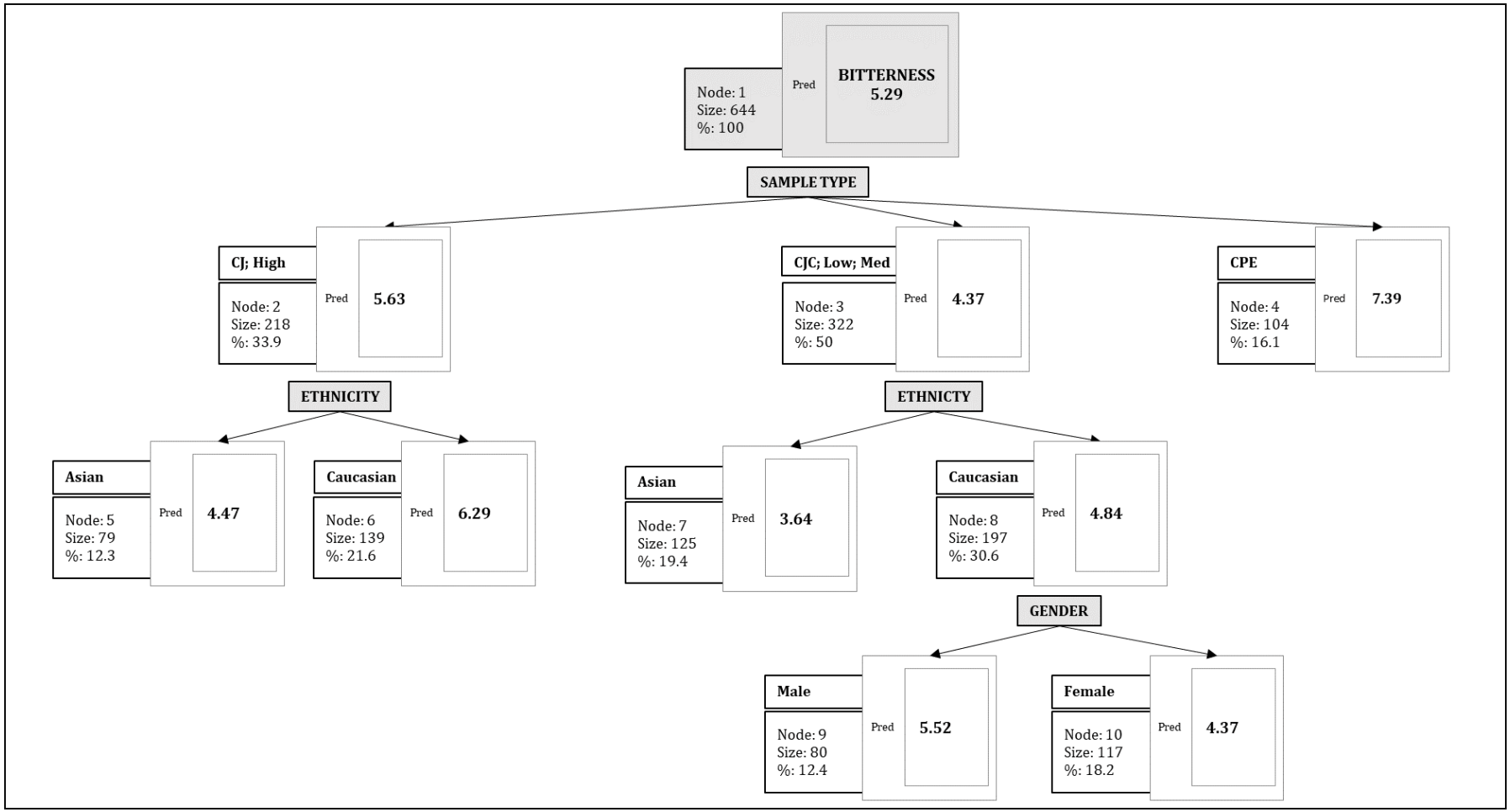

Figure 2.3c: Regression tree for bitterness intensity ratings

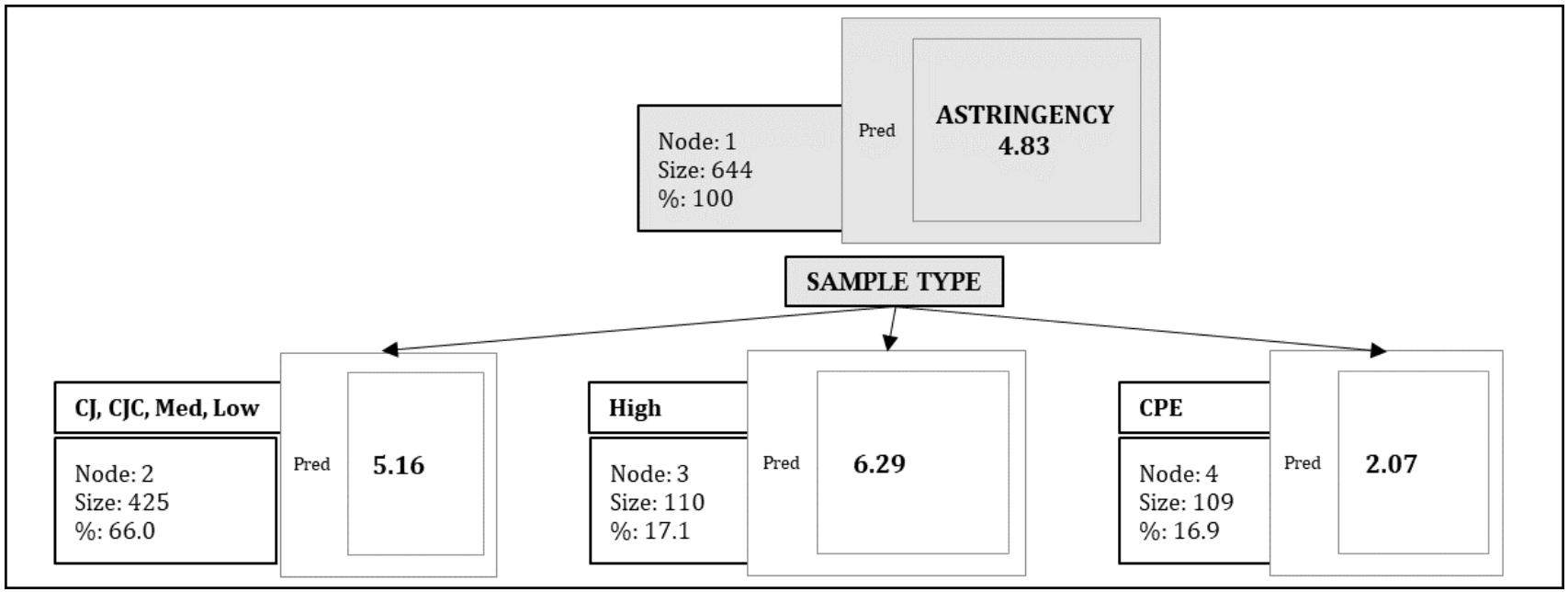

Figure 2.3d: Regression tree for astringency intensity ratings

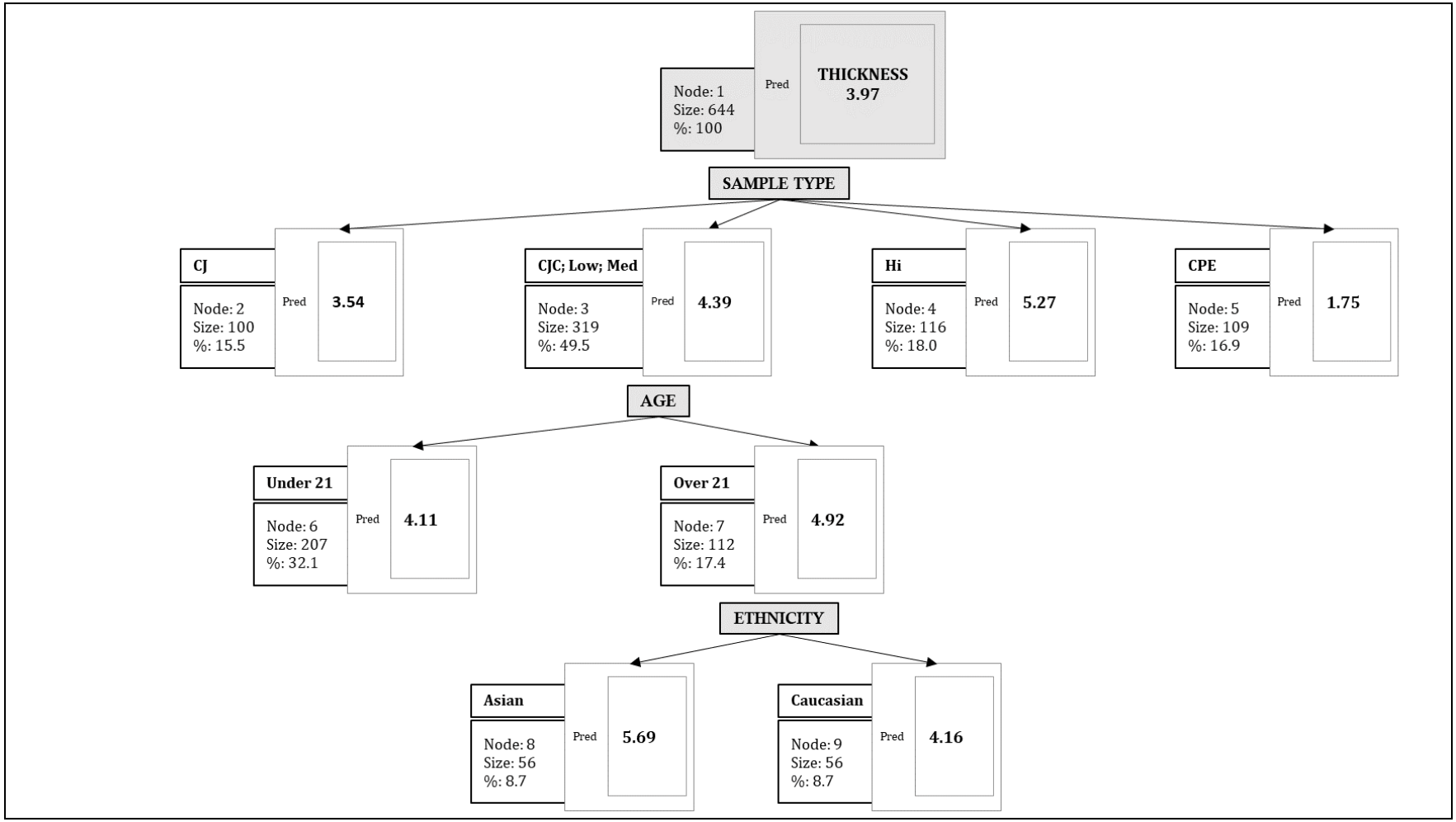

Figure 2.3e: Regression tree for thickness intensity ratings

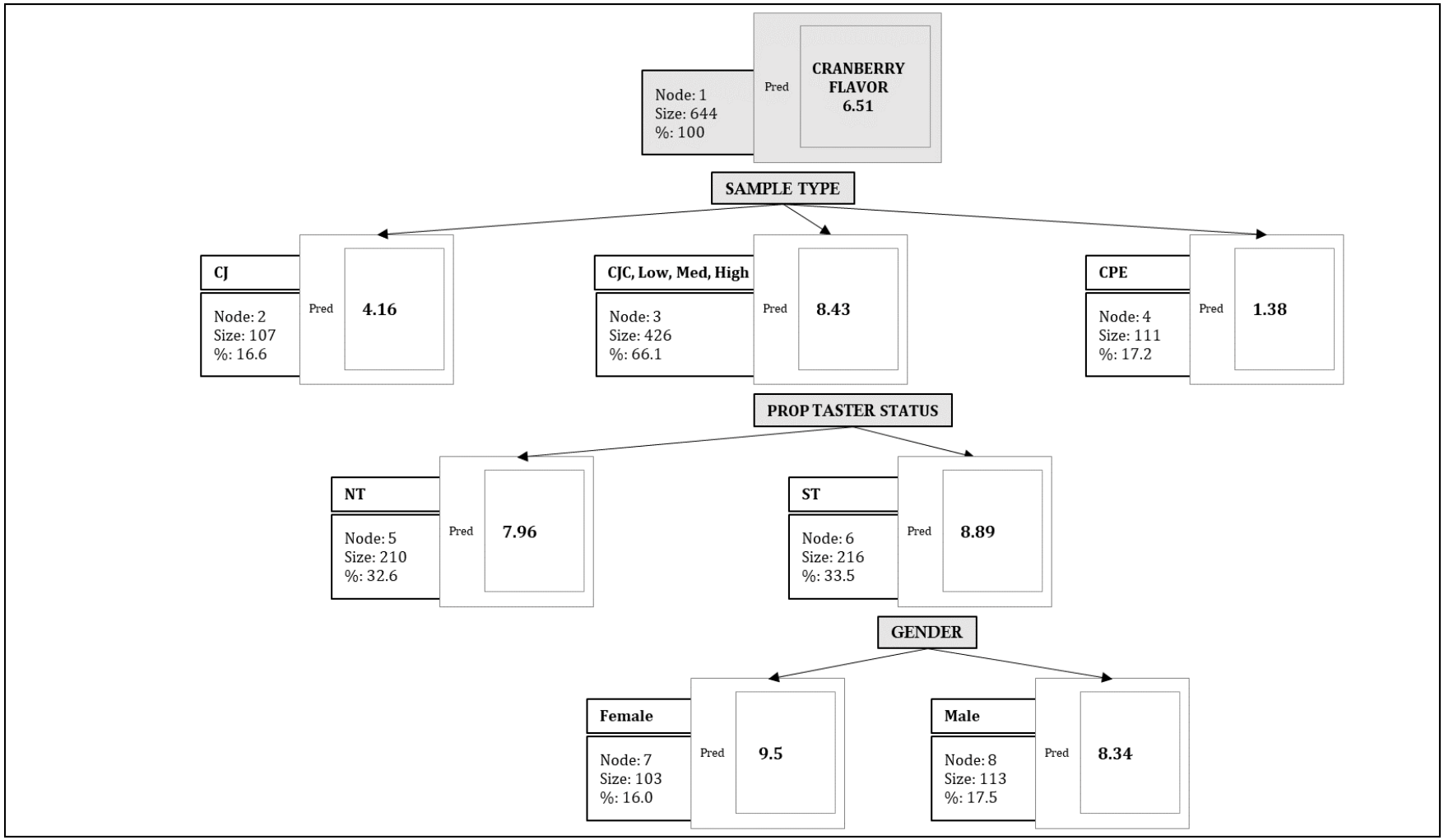

Figure 2.3f: Regression tree for cranberry flavor intensity ratings

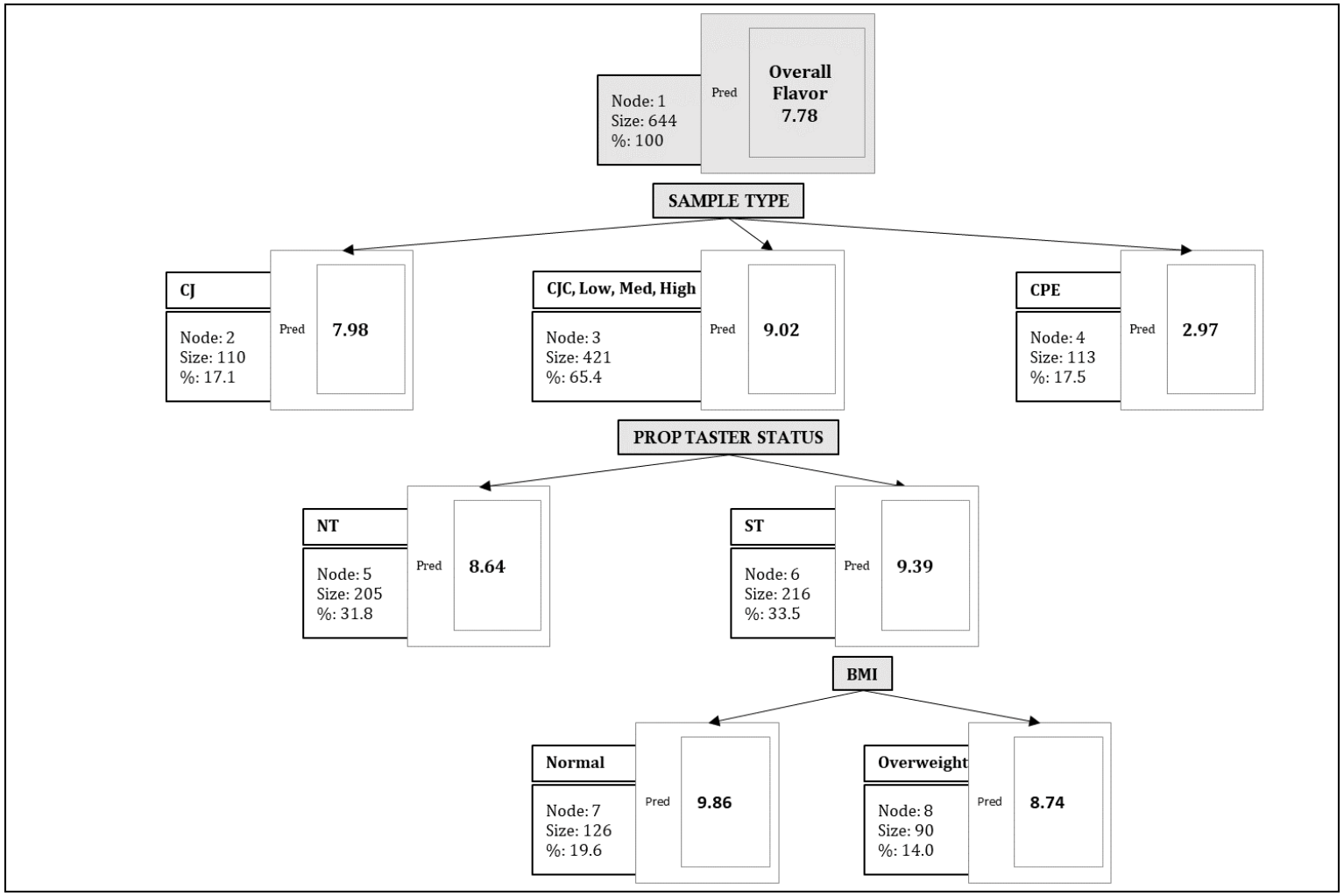

Figure 2.3g: Regression tree for overall flavor intensity ratings

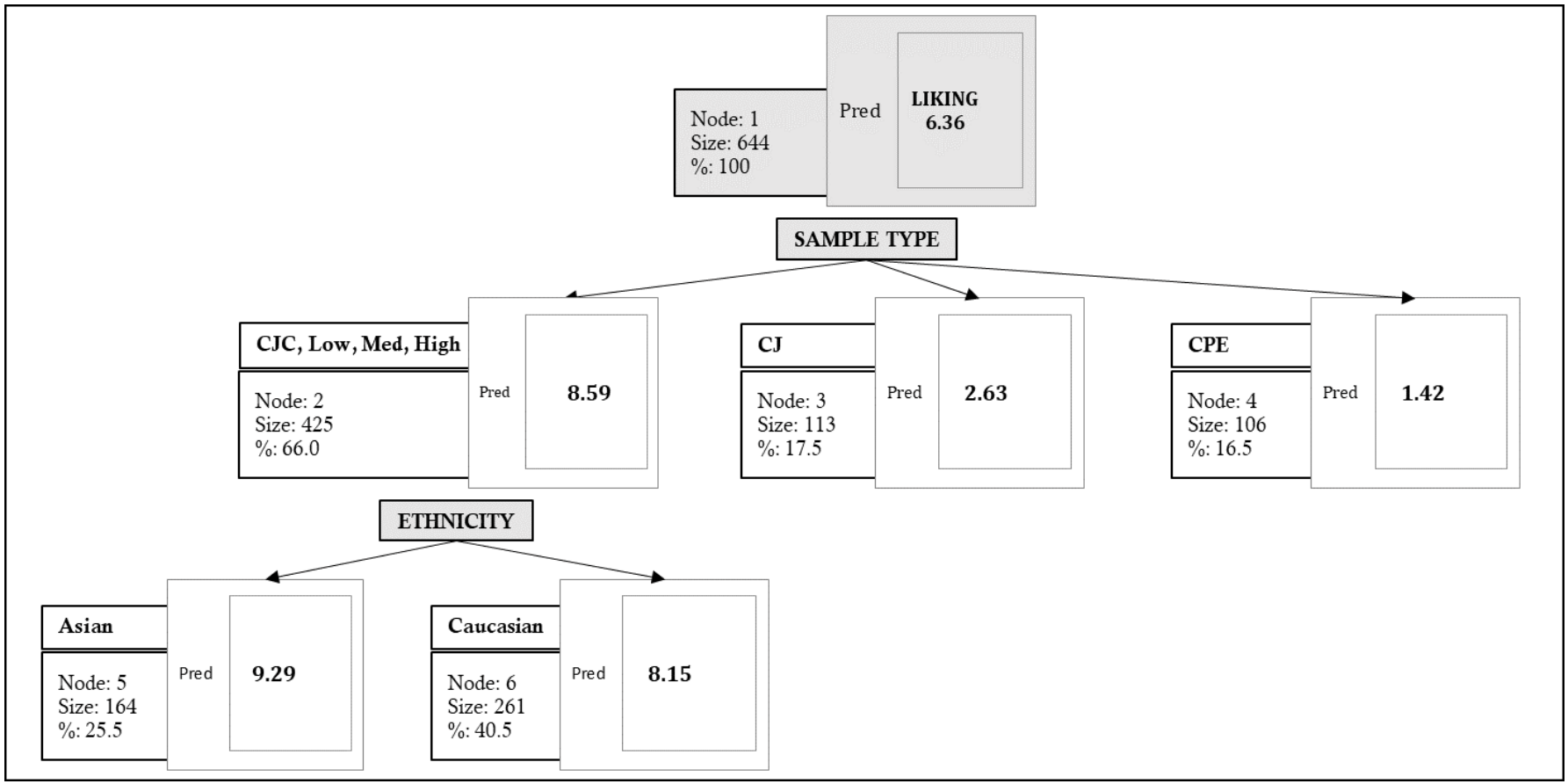

Figure 2.3h: Regression tree for overall liking ratings

For all the trees, sample type was always the first node in the tree, which indicated that the intensity for each attribute including liking was most dependent on sample type. Generally, branches from the 'Sample' node, led to further nodes, for CJ, CPE and another that grouped the CJC samples together. For sweetness (Fig. 2.3a) and astringency (Fig. 2.3d) attributes, the trees terminated at sample type and no other independent variables were found to have significant explanatory power.

For the remaining attributes, explanatory variables ranked differently in predictive power.

For sourness (Fig. 2.3b), 8 terminal nodes were generated. The first variable was sample type, which separated CJ, CPE and the CJC samples (grouped together). A further partition was observed for the CJC samples for the variable gender. Females gave higher sourness ratings in comparison to males. Within female subjects, PROP taster status was a significant explanatory variable of sourness ratings, where female ST subjects rated sourness as higher than female NTs.

For bitterness (Fig. 2.3c), after sample type, ethnicity accounted for the most variance. Caucasian subjects rated bitterness as higher in comparison to Asian subjects. Within the CJC samples, there was a split on ethnicity, wherein Caucasian male subjects rated the samples higher (12.4% of total) than female Caucasian subjects.

For thickness (Fig. 2.3e), sample type had the most explanatory power. Within the CJC samples, age was a significant predictor. Within the node Over 21

years of age, ethnicity was significant, wherein Asian subjects rated thickness as higher.

For cranberry flavor (Fig. 2.3f), within the CJC sample types, there was a further split on PROP taster status. Within STs (33.5% of the subjects), there was a split based on gender, whereby female STs gave higher ratings to cranberry flavor than male STs.

For overall flavor (Fig. 2.3g), PROP taster status was a significant variable for the CJC sample types, and within the ST subjects, BMI was a significant predictor. Subjects with a healthy BMI (19.6% of total) gave higher ratings to overall flavor.

Finally, for liking (Fig. 2.3h), there was a split on ethnicity for the CJC samples, with Asian subjects (25% of total), giving higher liking ratings to these samples.

2.4.3. Random Forest Modeling

Random forest modeling was conducted for each of the sensory attributes as well as liking. Results are summarized in a heatmap using feature importance based on mean square error values (Table 2.4).

Features	Sweetness	Sourness	Bitterness	Astringency	Thickness	Cranberry Flavor	Overall Flavor	Liking
Sample	**154.39**	**143.60**	**28.73**	**45.69**	**52.99**	**145.55**	**145.55**	**169.70**
Taster	10.60	20.22	0.91	2.55	-0.01	12.26	12.26	3.05
Gender	3.19	19.54	3.11	0.76	-1.67	11.41	11.41	4.80
Ethnicity	4.11	-1.32	15.29	5.60	2.59	4.04	4.04	8.68
Age	3.10	4.07	4.18	-1.31	6.84	2.07	2.07	3.58
BMI	11.30	6.90	-1.13	1.67	0.19	6.97	6.97	2.74

Table 2.4: Feature importance heatmap (using mean error square values) based on random forest modeling.

The greener a box is the most variation it can account for within the data for a particular attribute; a lighter color explains less variation within the data.

Across all the attributes, sample type was the single most important factor that influenced attribute intensity and overall liking ratings. However, after sample type, each feature ranked differently in its explanatory power for each sensory attribute.

For sweetness, PROP taster status and BMI were the most important after sample type. For sourness, cranberry and overall flavor, rankings were similar: after sample type, PROP taster status and gender were the most important factors. For both bitterness and astringency, ethnicity was the most important factor after sample type and had a higher importance score than PROP taster status. Age was the most important variable for thickness after sample type. Finally, like bitterness and astringency, ethnicity had the most impact on overall liking after sample type.

2.5. Discussion

Our objective was to understand individual differences in the perception of astringency and other key sensory attributes in cranberry- based stimuli including cranberry juice (CJ), an aqueous solution of cranberry phenol extract (CPE), and cranberry juice cocktail (CJC) supplemented with different concentrations of CPE. A major focus of the study was to better define the role of PROP taster status in the perception and liking of polyphenols derived from cranberries. Previous studies have examined the involvement of PROP taster status in astringency perception from an assortment of stimuli (polyphenol based and non-polyphenol-based), but

they have reported conflicting outcomes (Pickering 2004, Pickering 2006, Laaksonen 2013, Duffy 2016, Melis 2017). This lack of agreement warranted further study, as we did here, using model systems and fruit drinks containing the same native polyphenols. We utilized three approaches to address these objectives: ANCOVA, regression trees and random forest modeling.

First, using a traditional ANOVA approach, we showed that the samples varied widely in their perceived sensory attributes. Consistent with its description as a high acid fruit (Fong 2021), CJ (without added sugar) was extremely sour and lacking sweetness. CJ also exhibited moderate-high bitterness, astringency, cranberry flavor, and overall flavor and was disliked by subjects as a whole. In contrast, CPE was characterized as high in bitterness and astringency, relative to all other samples, but was weak in intensity for all other attributes. Liking for CPE was also low. This was expected since CPE was used purely as a model system to assess the perception of native cranberry polyphenols without the presence of other fruit constituents or added ingredients.

Surprisingly, supplementing the CJC samples with CPE did not alter sourness or cranberry flavor and astringency perception was only increased in CJC with the highest concentration of CPE. However, addition of CPE progressively decreased sweetness, and increased bitterness and overall flavor. Further, overall liking was highest for un-supplemented CJC and lowest for CJC supplemented with the highest concentration of CPE. These data suggest that adding native polyphenols to a formulated cranberry beverage alters its sensory profile but does not have a specific

or singular effect on astringency. These observations may be important for designing fruit beverages with added polyphenols to enhance flavor or health benefits.

Finally, we found that a moderate level of thickness was perceived in all the fruit-derived samples (except the CPE solution) and is likely associated with the presence of pectin from the fruit (Laaksonen 2013). We can only speculate as to why the perception of thickness increased with increasing CPE content of the samples. It is possible that CPE increased the sub-qualities of astringency including roughing, drying, or puckering which was described by Lee & Lawless (Lee and Lawless 1991) and reported to occur in other studies on astringency (Fleming 2015, Hamada 2019, Kershaw 2019, Pittari 2020). Subjects in our study may not have associated these sub-qualities with the astringency attribute. Rather, they might have interpreted these mouthfeel cues as texture or simply 'dumped' them in the thickness category. These data suggest that future studies seeking to understand the complexities of astringency perception should conduct a more fine-grained evaluation of this attribute, including its various sub-qualities.

Our prior work (Melis 2017) showed an interaction between PROP taster status and gender on the perception of bitterness and astringency and overall liking of CJC with added tannic acid (to modify astringency). Based on these results, we expected to find similar robust effects here. However, in the present study we observed no differences in bitterness or astringency of these samples with respect to PROP taster status. ANCOVA did show PROP- specific effects for other attributes

such as cranberry flavor (higher intensities experienced by STs) and overall liking (lower in STs). A few taster by gender interactions were also observed for sourness and cranberry flavor (with female STs giving the highest ratings). However, in general, PROP taster status did not have systematic and wide-spread effects on the perception of our samples. We did find that ethnicity, which is rarely considered in studies on astringency, was related to the perception of sourness, astringency and liking of the CJC samples. The reasons for these differences are unknown but could reflect cultural differences (Laing 1993, Pages 2007, Ferdenzi 2012) in familiarity with and acceptance of bitter and astringent fruits and vegetables. Subjects in our study described themselves as either Caucasian or Asian (both East Asian and South Asian). In studying aroma perceptions of Caucasian and East Asian participants, Jin et al (2018) showed that the pungency of cinnamaldehyde, ubiquitous as sweet cinnamon spice in North America, was both less intense and less liked by East Asian subjects compared with Caucasian subjects (Jin 2018). Indeed, ethnic, and cultural differences in reactions to diverse aromas and food flavors have been reported in the literature.

One interesting observation from the ANCOVA results was that STs and specifically female STs perceived more cranberry flavor intensity from the CJC samples relative to their comparison groups.

Recent work (Cliceri 2021) has shown that astringency associated with polyphenols can be a main modulator of flavor. Thus, it is possible that the addition of CPE did not influence astringency perception, per se, in STs, but these additions

were interpreted instead as flavor attributes. These findings suggest that STs may be more sensitive to polyphenol-linked flavor changes, which could modify the overall flavor profile of a complex food or beverage. Thus, focusing solely on perceived differences in bitterness and astringency between NTs and STs may miss important differences in how these two groups appreciate polyphenol-rich foods. Future studies should emphasize testing realistic foods and beverages rather than simple model systems which have limited ecological validity.

We then used regression trees in to visualize the effects of various factors on each of the sensory attributes. There were some generalities between the trees and results of ANCOVA. First, sample type was always the first node to be generated among the trees and thus accounted for the most variation in the dataset. Second, for most of the attributes, CJ and CPE samples were categorized separately than the CJC samples which was consistent with the ANCOVA findings which showed that these two samples were perceived as outliers.

Interestingly, the trees demonstrated that each sensory attribute was influenced by a different set of factors. For some attributes, these factors overlapped with those extracted from the ANCOVA analysis (such as a PROP by gender interaction for sourness and cranberry flavor). For other attributes, such as overall flavor, a PROP effect was observed in the regression trees but not the ANCOVA. Sample type was the only factor contributing to sweetness and astringency perception in the regression trees. Sweetness was only influenced by sample type in both analyses. However, astringency perception was influenced by ethnicity in the

ANCOVA results, but not in the regression tree analysis. Despite the observed inconsistencies between methods, the regression trees unmasked effects of ethnicity related to bitterness, thickness, and especially overall liking. Indeed, ethnicity was the only factor contributing to overall liking of the CJC samples. Thus, in analyses where factors are considered simultaneously, only ethnicity explained the higher predicted liking of CJC samples by Asians as compared to Caucasians.

Similar to the regression tree analysis, random forest modeling (Table 2.4) also confirmed that ratings of each sensory attribute are driven and influenced by a different set of individual factors. The results are generally in agreement with the regression trees but provide an additional rigor of analysis. This is because regression trees are prone to overfitting to a dataset; thus even robust significant effects may actually be an overestimation and not generalizable to a larger dataset from a representative population. Regression trees, on the other hand perform many iterations of the analysis using a subset of the data and present 'averaged' results. Ranking the features using the importance scores in Table 2.4, we arrive at fairly same conclusions as the regression trees. For instance, for sourness, cranberry flavor and overall flavor, both PROP taster status and gender have nearly the same scores. This is consistent with the interactions we observed as well as the splits in the regression trees. Thus, this is a confirmation of our findings above, which also shows that different sensory attributes are influenced by different individual factors.

The present study has several strengths. First, we had a robust sample size with approximately balanced taster*gender subgroups. Secondly, we used a food stimulus that is already marketed and accepted by consumers in a commercial beverage (Ocean Spray Inc.) and we tested concentrations within the range of sensory palatability. Finally, we observed good general agreement between the regression tree results and results obtained via ANCOVA, although the regression tress revealed more complex relationships embodied in the explanatory variables. Indeed, our trees showed that different individual factors influence taste and flavor modalities differently and the variable importance is 'built into' the hierarchical nature of the trees. Admittedly, regression trees can be susceptible to 'overfitting,' which may be monitored by validating the model, as we did in this study. Nevertheless, using machine learning approaches such as regression trees or random forests are considered a useful approach in unmasking latent effects when multiple individual factors of variation are under study.

The findings of the present study must be interpreted in consideration of certain limitations. Our screening procedure only recruited subjects who were familiar with cranberry juice. We did not see major individual differences in liking, which could be a consequence of the nature of the study population i.e., only acceptors of cranberry juice were recruited into the study. In addition, we only examined a limited number of factors in this study, and a wide range of personal traits have been associated with astringency perception including alexithymia, variety seeking, sensitivity to reward and others (Robino 2016, De Toffoli 2019). Future work should incorporate these factors into studies using regression trees

and other data analysis approaches to develop more informed models of human responses to astringency.

2.6. Conclusion

Current understanding of how PROP taster status, and other individual factors, influence astringency perception and liking, is limited. While many studies clearly show a role for PROP taster status in development of astringency, others have conflicting results. Across these studies, there is great diversity in use of stimuli, underlying characteristics of the sample population and choice of methods of data treatment. Observations from the current study suggest that characterizing the study population and accounting for individual factors of variation is essential to understanding astringency perception.

2.7. Acknowledgments

Conceptualization- N.Y.Y., B.J.T.; Investigation, Formal Analysis, Data Curation, Visualization- N.Y.Y.; Writing- Original Draft-N.Y.Y., B.J.T.; Writing- Review & Editing- N.Y.Y., B.J.T.; Supervision, Funding Acquisition- B.J.T.

3. PROP TASTER STATUS & SALIVARY PROTEINS

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3.1. Abstract

Astringency is a complex oral sensation, commonly experienced when dietary polyphenols interact with salivary proteins. Most astringent stimuli alter protein levels, which then require time to be replenished. Although it is standard practice in astringency research to provide breaks in between stimuli, there is limited consensus over the amount of time needed to restore the oral environment to baseline levels. Here we examined salivary protein levels after exposure to 20 ml of a model stimulus (cranberry polyphenol extract, 0.75 g/L CPE) or unsweetened cranberry juice (CJ), over a 10 min period. Whole saliva from healthy subjects (n = 60) was collected at baseline and after 5 and 10 min following either stimulus. Five families of proteins: basic proline-rich proteins (bPRPs); acidic proline-rich proteins (aPRPs); histatins; statherin; and S-type cystatins, were analyzed in whole saliva via HPLC-low resolution-ESI-IT-MS, using the area of the extracted ion current (XIC) peaks. Amylase was quantified via immunoblotting. In comparison to baseline (resting), both stimuli led to a rise in levels of aPRPs (p < 0.000) at 5 min which remained elevated at 10 min after stimulation. Additionally, an interaction of PROP taster status and time was observed, wherein super-tasters had higher levels of amylase in comparison to non-tasters after stimulation with CJ at both timepoints (p = 0.014–0.000). Further, male super-tasters had higher levels of bPRPs at 5 min after stimulation with both CJ and CPE (p = 0.015–0.007) in comparison to baseline. These data provide novel findings of interindividual differences in the salivary proteome that may influence the development of astringency and that help inform the design of sensory experiments of astringency.

3.2. Introduction

Astringency is an everyday sensation that is experienced with consumption of polyphenol-rich foods. It is marked by drying, roughing, and puckering of the oral surfaces (Lee 1991) and is commonly associated with foods such as green tea, coffee, cocoa, berries, and red wine. The sensation of astringency lingers in the mouth with just a single exposure and intensifies with repeated exposure (Guinard 1986, Lyman 1990). Individual variation in the perception and liking of astringency is well known and could explain large differences in consumer acceptance of polyphenol-rich foods (Jaeger 2009, Dinnella 2011, Torri 2016). Dietary polyphenols can have both desirable and undesirable nutritional effects. Their excessive consumption has been linked with growth inhibition, decreased nutrient absorption and weight loss in animals (Mehansho 1987, Kondo 2014, Zhong 2018). However, due to their immunomodulatory and anti-inflammatory properties, polyphenols have also been shown to promote healthy gut bacteria and reduce risk for cardiovascular diseases, cancer, obesity, and metabolic syndrome (Saibandith 2017, Cory 2018, Durazzo 2019). Despite these health benefits, the astringent, and sometimes bitter, properties of these foods polarize consumers and make it challenging to incorporate polyphenols into everyday diets. Thus, developing a better understanding of the underlying mechanisms of astringency perception and the factors driving consumer hedonics for these foods is important from a public health perspective and could also guide the food industry in formulating new polyphenol-rich foods and beverages that are acceptable to a broad cross section of consumers.

Our understanding of the underlying mechanisms of astringency has evolved over the last 70 years but remains incomplete. Currently, the most widely accepted model for astringency is based on the interaction between polyphenols and salivary proteins. According to this model, when dietary polyphenols first enter the oral cavity, they form hydrogen bonds or hydrophobic interactions with proteins circulating in saliva to form aggregates which grow over time, becoming insoluble precipitates (Siebert 1996, Charlton 2002, Jobstl 2004). These proteins are mainly identified as acidic proline-rich proteins, histatins, cystatins and statherin. Other proteins also participate in this phenomenon, such as mucins, glycosylated and basic proline-rich proteins, which are mostly adsorbed onto oral surfaces and are essential in providing oral lubricity and protecting the salivary pellicle against damage and microbial insult (Frenkel 2015, Ployon 2018). Interaction of polyphenols with these proteins also forms large aggregates, eventually eroding the protective lubricating layers. Together, these actions generate the astringency sensation (Horne 2002, Nayak 2008), which, in evolutionary terms, might serve as a warning cue against toxicity from over-consumption of these plant materials. A great deal of our understanding of protein–polyphenol interactions in astringency perception has come from *in vitro* studies (Monteleone 2004, Pascal 2008, Soares 2011, Soares 2012, Brandao 2014, Brandao 2017, Silva 2017, Ramos-Pineda 2019, Soares 2019, Ramos-Pineda 2020). However, *in vitro* models cannot fully replicate the physiological conditions and dynamics of the human mouth.

The time course of astringency perception and oral recovery are poorly understood. It is known that astringency following oral stimulation takes anywhere

from 100 s to 300 s or even longer to recede (Lee 1991, Naish 1993, Valentova 2002, Bajec 2008), although the complementary work examining the salivary protein response over time is limited (Brandao 2014). The intensity of an astringent sensation, its quality (i.e., the predominance of different sub-qualities such as drying, roughing, puckering) and time course depends on the type and concentration of the stimulus used (Lee 1991, Peleg 1999, Hufnagel 2008). Different types of polyphenols (e.g., grape seed tannins, catechins) as well as metal salts and organic acids have been used in astringency studies, but they produce different profiles of astringency perceptions (Fleming 2015, Kershaw 2019). This diversity suggests that there may be more than one mechanism underlying the astringency response. This is underscored by findings that many astringent stimuli may not interact with proteins at all (Lee 2012). Astringency has also been described as a tactile or trigeminal response (Schobel 2014), which could involve chemosensation via activation of bitter receptors (Soares 2013, Soares 2018). The use of many different stimuli under varying conditions has led to no general agreement about the length of time necessary to reset the mouth after exposure to an astringent stimulus. Resolving this issue is particularly important for elucidating the mechanisms by which polyphenols interact with specific salivary proteins and for gaining insights into the broad array of other potential mechanisms involved in astringency perception. In the absence of clear consensus on when the oral cavity resets after an astringent exposure, sensory methodology has traditionally varied in the duration of rest breaks—lasting anywhere from a few minutes to even 30 min (Dinnella 2009, Xu 2018).

Superimposed on these uncertainties are individual differences in salivary responses mentioned previously. For instance, Dinnella and colleagues showed that subjects who can maintain relatively constant salivary protein levels after exposure to astringent stimuli experience less astringency (Dinnella 2009), whereas the inability to replenish these levels is associated with higher experience of astringency, especially upon repeated sampling (Dinnella 2010). Other work has shown that familiarity, consumption and liking of polyphenol-rich food can impact perception of astringency as well (Dinnella 2011). Genetic variations may be another source of interindividual differences in astringency perception. Variation in the bitterness perception of 6-n-propylthiouracil (PROP) is a general marker for differences in oral sensations (Tepper 2008) and has also been studied in the context of astringency perception (Pickering 2004, Pickering 2006). Variation in the bitter taste receptor gene, *TAS2R38,* leads to a structural change in the bitter-taste receptor TAS2R38, which affects the binding affinity and, as a result, bitterness perception of PROP. For instance, super-tasters can bind PROP strongly and therefore experience the strongest bitterness, while non-taster individuals do not bind PROP (Tan 2012) and experience none to very little bitterness. Typically, super- and medium-tasters select foods that are less bitter in comparison to non-taster individuals as the former may be more sensitive to oral sensations in wine, fruits and vegetables (Dinehart 2006, Pickering 2006, Sandell 2015). Indeed, studies using bilberry and crowberry juices supplemented with their native polyphenol extracts, have shown that super-tasters (*TAS2R38* homozygous dominant individuals) dislike the more astringent version of these juices (Laaksonen 2013).

Other work has shown that tasters may consume astringent fruit such as lingonberries less frequently (Sandell 2015).

Melis and colleagues (Melis 2017) were the first to study the role of PROP taster status in the perception of and salivary response to another astringent fruit, the North American cranberry (*Vaccinium marcoporan*). Cranberries are unique among astringent fruits with the greatest abundance of type-A proanthocyanidins (condensed tannins) (Blumberg 2013). Following oral stimulation with unsweetened cranberry juice, PROP tasters had higher levels of selected salivary proteins (specific subtypes of acidic proline-rich proteins and cystatins) in comparison to non-tasters. Accompanying sensory experiments used tannic acid-supplemented cranberry juice cocktail to modify astringency perception. Cranberry juice cocktail (cranberry juice with added sugar) was used instead of cranberry juice since the latter is routinely disliked by consumers. Results showed that non-taster male subjects perceived less bitterness and astringency from and gave higher liking ratings to tannic-acid supplemented cranberry juice cocktail compared to taster male subjects. These data suggest an important role for PROP taster status and gender in astringency perception and liking although this association has not been observed in all studies (Törnwall 2011, Feeney 2014, Duffy 2016, De Toffoli 2019).

The objectives of the present study were two-fold. First, the study examined the time course of salivary protein response after stimulation with unsweetened cranberry juice (CJ) or cranberry-derived polyphenol extract (CPE). The second objective was to determine interindividual differences in the context of PROP taster

status and gender after stimulation with CJ or CPE. We hypothesized that PROP super-tasters will have elevated levels of salivary proteins following stimulation, in line with previous observations (Melis 2017) and these levels will return to baseline earlier than in non-tasters.

3.3. Methods

3.3.1. Subject Recruitment

Healthy adults (n = 60), between 18–45 years of age were recruited from the Rutgers University community through an email distribution list. Subjects were screened for PROP taste responsiveness; only PROP non-tasters and super-tasters were admitted into the study into groups balanced for gender. Subjects were also screened for general suitability (e.g., demographics, health information) and familiarity with cranberry juice and cranberry products. They had to have consumed such products within the last 2 years. Exclusion criteria included major metabolic diseases (diabetes, kidney disease, etc.), pregnancy, lactation, food allergies, and the use of medications that interfere with taste or smell functions (e.g., steroids, antihistamines, or anti-depressants). Participants who were determined to be medium-tasters for the PROP phenotype were excluded.

The study was approved by the Rutgers University Arts and Sciences Institutional Review Board (Approval#13-309M). All subjects provided written informed consent and were compensated monetarily for their participation.

3.3.2. PROP Taster Status

The participants were screened and classified according to PROP taster status via the paper disk method (Zhao 2003), which has been previously tested for validity and reliability (Zhao 2003, Tomassini Barbarossa 2015, Carta 2017, Melis 2017, Melis 2017, Melis 2018), and strongly correlates with tongue electrophysiological recordings (Pani 2017, Sollai 2017). In this procedure, subjects place a filter paper disk impregnated with 1.0 mol/L NaCl (Sodium Chloride, S671-500, Fisher Scientific) on the tip of the tongue for 30 s. They rate the taste intensity of the disk using the labeled magnitude scale (LMS), a 100-mm scale anchored with the phrases "barely detectable" to "strongest imaginable." This procedure is repeated with a second paper disk impregnated with 50 mmol/L PROP (6-n-propyl–2-thiouracil, P3755, Sigma-Aldrich). Subjects rinse with room-temperature spring water before and in between tasting each paper disk. Subjects are classified as non-tasters (NT) if they rate the PROP disk <15 mm on the LMS; they are categorized as super-tasters (ST) if they rate the PROP disk >67 on the LMS. All others are classified as medium-tasters. NaCl ratings do not vary with PROP status in this method. Therefore, NaCl ratings are used as a reference standard to clarify the taster status of subjects who give borderline ratings to PROP. This strategy is based on the rationale that non-tasters give higher ratings to NaCl than to PROP, medium tasters give equivalent ratings to both stimuli and super-tasters give higher ratings to PROP than to NaCl.

3.3.3. Test Stimuli

Two oral stimuli were used: cranberry juice (CJ) and cranberry-derived polyphenol extract (CPE). CJ was made from fresh cranberries frozen at −20 °C until use and donated by Ocean Spray Cranberries Cooperative in Chatsworth, NJ. CJ was made in small batches using a standard recipe where 300× *g* of berries were defrosted, washed, and cooked on a stovetop under medium heat for 10 min with 648 mL of spring water. The mixture was filtered through cheesecloth and cooled to room temperature. CPE solution was made using a carrier-free powdered extract (Ocean Spray Cranberries, Inc, Lakeville, MA, USA) added to spring water at a concentration of 0.75 w/v g/L. The water was pre-warmed under low heat on a stirring hot plate to facilitate dissolution. Both stimuli were prepared as needed, the day before subject testing, and refrigerated at 4 °C until 30 min before use. The samples were served at room temperature.

3.3.4. Experimental Procedures

Overall Study Design

Subjects participated in two test sessions. They were instructed to refrain from consuming astringent foods for approximately 8 h prior to each session. A list of such foods was provided to them. They were also prohibited from eating, drinking (except plain water), chewing gum, using breath mints, mouthwash or brushing their teeth for 2 h prior to the test sessions. In addition, they were asked to refrain from consuming any alcoholic beverages for 24 h prior to each session.

During session 1, subjects were familiarized with the procedures and completed demographic questionnaires. During session 2, subjects provided saliva samples by spitting directly into a plastic polypropylene cup, had been refrigerated at 4 °C until ready to be used. First, resting saliva was collected over 1 min to enable the measurement of baseline salivary protein levels. After a 5 min rest period, subjects were given 20 mL of one of the two astringent stimuli (either CJ or CPE), asked to swish the sample in their mouth and then swallow it completely. Subjects then provided saliva at 5 and 10 min after swallowing.

After a 20 min break, the subject was provided with the second astringent stimulus and followed the same procedure for saliva collection as above. As a result of this procedure, there were five saliva collections, a resting sample, and saliva collected at 5 and 10 min after exposure to each stimulus (CJ and CPE). The order of presentation (CJ first or CPE first) was randomized across subjects (Figure 3.1).

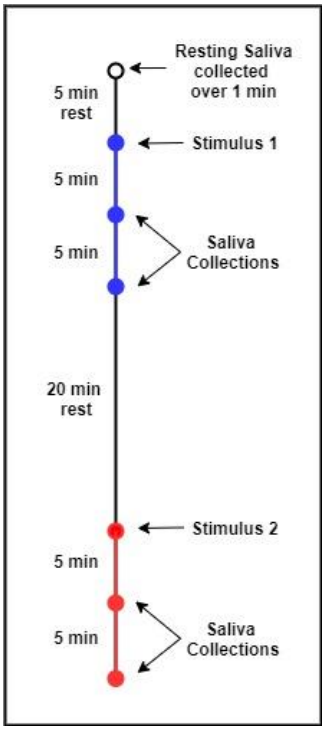

Figure 3.1. Saliva collection timeline.

Saliva Treatment

Each saliva sample was transferred into two microcentrifuge tubes, which had been maintained on ice (0.5 mL per tube); one tube was prepared for High Performance Liquid Chromatography-low resolution- Electrospray Ionization- Ion Trap- Mass Spectrometry (HPLC-ESI-MS) analysis while the other was prepared for immunoblot procedure.

For the HPLC-ESI-MS analysis, 0.2% trifluoroacetic acid (TFA, Sigma-Aldrich, St. Louis, MO, USA) was added to the saliva in a 1:1 v/v ratio. These samples were centrifuged at 8000× *g* at 4 °C for 15 min. The supernatant was separated from the pellet and stored at −80 °C until chromatographic analysis. For the immunoblot procedure, a protease inhibitor cocktail solution [mix of 1 tablet/1.4 mL of cOmplete® Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA)

and 175 mM NH_4HCO_3 (Ammonium Bicarbonate, Sigma-Aldrich, St. Louis, MO, USA)] was added to saliva in a 1:2 v/v ratio. These samples were stored at −80 °C until Dot-blot immunoblot analysis.

Total Protein Content Quantification

Bicinchoninic Acid (BCA) Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) was used to quantify total protein content of the saliva samples according to manufacturer's instructions. The total concentration was used to normalize protein levels for dot blot analysis.

Alpha Amylase Analysis

Alpha Amylase purification

To purify amylase protein, a volume of 6 mL of whole saliva was collected and treated with 0.2% TFA, as previously described. The sample was injected in an Ultimate 3000 Micro HPLC apparatus (Dionex, Sunnyvale, CA, USA) equipped with a photodiode detector (UV-VIS) and the chromatographic column was a Vydac-C8 with 5 µm particle diameter (column dimensions 250 × 10 mm) (Hesperia, CA, USA).

The following solutions were utilized for purification: 0.06% (v/v) aqueous TFA (eluent A) and 0.05% (v/v) TFA in acetonitrile-water 80/20 (eluent B) with flow rate at 2.8 mL/min. Salivary proteins were eluted using a linear gradient from 0% to 60% of B in 40 min, and from 60% to 100% of B in 5 min. Protein detection was carried out at a wavelength of 214 nm. The total injected saliva volume was 800 µL. Collected salivary fractions were analyzed in HPLC-ESI-MS and the fraction containing amylase was lyophilized and then dissolved in 0.1% v/v TFA for a total

volume of 500 µL. BCA assay was then performed, as previously described, to determine the concentration of amylase in the resulting solution.

Alpha Amylase Quantification

The concentration of alpha amylase in the salivary samples (individual samples, un-pooled) was estimated semi-quantitatively by using dot-blot technique, where the protein samples were spotted directly onto a PVDF membrane (0.2 µm pore size; Immun-Blot® PVDF Membrane, Bio-Rad Laboratories, Inc., Italy). To set up a dot blot assay, the saliva samples treated with cOmplete protease inhibitor cocktail were first diluted with Tris Buffered Saline (TBS: 20 mM Tris-HCl pH 7.6, 150 mM NaCl) so that each diluted sample would have the same amount of total protein content (adjusted to 0.38 µg/µL). Before transferring the samples on to the PVDF membrane, it was pre-wetted with methanol for 1 min, then transferred to TBS for 2 min.

The amylase fraction, purified from whole saliva, was used as a standard in 4 concentrations (0.01, 0.02, 0.05, 0.1 µg/µL). All samples and standards were spotted onto the wet PVDF membrane in triplicate. Specifically, each test sample was spotted in a volume of 1µL (0.38 µg/µL of total protein content). The membrane was blocked with blocking agent 5% of BSA (Bovine serum albumin, Sigma Aldrich) in TBS-T buffer (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. Subsequently, the membrane was incubated with primary antibody (dilution 1:1000; Amylase G-10: sc-46657-Santa Cruz Biotechnology, Inc.) in 5% of BSA in TBS-T buffer, for 1 h. Three washes for 5 min with TBS-T buffer were performed and the membrane was incubated for 1 h with secondary antibody

(dilution 1:5000; Rabbit anti-Mouse IgG, Secondary Antibody, HRP ThermoFisher Scientific). After three further washes with TBS-T, the membrane was incubated for 5 min with ECL substrate (Clarity Western ECL Substrate, Bio-Rad, Laboratories, Inc, Italy) for fluorescence signal development and captured on the Chemidoc MP Imaging System (Bio-Rad, Hercules, CA, USA). Analysis of images obtained were performed using Image Lab 6.0.1 software (Bio-Rad Laboratories Inc.). Signals of samples were determined and shown as intensity values which were transformed by the software with volume tools in value of concentration (μg/μL) for each sample by using the standards as references. Each sample was analyzed in triplicate with acceptable coefficient of variation (CV%) set as below 15%.

3.3.5. HPLC-Low Resolution-ESI-IT-MS Analysis

Table 3.1 shows the salivary proteins and peptides analyzed in each of the salivary samples (individual samples, un-pooled) collected using the HPLC-low resolution-ESI-IT-MS technique according to (Cabras 2012). 30 μL of the acidic soluble fraction corresponding to 15 μL of whole saliva was used (1:1 v/v dilution). Only proteins/peptides characterized in human saliva by applying the same analytical conditions in previous studies (Messana 2008, Castagnola 2012) were analyzed in the present investigation. Average mass values (Mav), obtained by deconvolution of averaged ESI-MS spectra automatically performed by using MagTran 1.0 software (Zhang 1998), and elution times of proteins/peptides were compared with those determined under the same experimental conditions in our previous studies (Messana 2008, Castagnola 2012).

Experimental Mav were also compared with the theoretical ones available at the UniProt-KB human data-bank (http://us.expasy.org/tools). The quantification of each protein and peptide was based on the area of the HPLC-ESI-IT-MS extracted ion current (XIC) peaks. The XIC analysis reveals the peak associated with the peptide of interest by searching, along the total ion current chromatographic profile, the specific multi-charged ions generated by the protein. The area of the ion current peak is proportional to concentration, and under constant conditions it may be used to perform relative quantification of the same analyte in different samples (Ong 2005).

Protein (Swiss-Prot Code)*	Experimental Average Mass (Da) ± SD (Theoretical)	Elution Time (min ± 0.5)
Acidic proline-rich phosphoproteins family (aPRPs):		
P-C peptide (P02810)	4370.9 ± 0.4 (4370.8)	13.6-14.5
PRP-1 type di-phosphorylated (P02810)	15515 ± 2 (15514-15515)	22.9-23.3
PRP-1 type mono-phosphorylated	15435 ± 2 (15434-15435)	23.9-24.3
PRP-1 type non-phosphorylated	15355 ± 2 (15354-15355)	24.2-24.7
PRP-1 type tri-phosphorylated	15595 ± 2 (15594-15595)	22.6-22.9
PRP-3 type di-phosphorylated (P02810)	11161 ± 1 (11161-11162)	23.3-23.8
PRP-3 type mono-phosphorylated	11081 ± 1 (11081-11082)	23.8-24.2
PRP-3 type non-phosphorylated	11001 ± 1 (11001-11002)	24.8-25.1
PRP-3 type di-phosphorylated Des-Arg_{106}	11004 ± 1 (11005-11006)	23.5-23.8
Histatin family (Hists):		
Histatin-1(P015515)	4928.2 ± 0.5 (4928.2)	23.3-23.8
Histatin-1 non-phosphorylated	4848.2 ± 0.5 (4848.2)	23.4-23.8
Histatin-6 (P15516)	3192.4 ± 0.3 (3192.5)	14.0-14.4

Histatin-5 (P15516)	3036.5 ± 0.3 (3036.3)	14.2-14.7
Basic proline-rich protein family (bPRPs):		
Ps-1	23460 ± 3 (23459.0)	17.0-18.0
P-J	5943.9 ± 0.5 (5943.6)	14.1-14.7
P-H (P02812/P04280)	5590.2 ± 0.5 (5590.1)	15.0-15.5
P-F (P02812)	5843.0 ± 0.5 (5842.5)	14.3-14.8
P-D (P010163)	6949.5 ± 0.7 (6949.7)	15.2-15.8
II-2 (Tot):		
- II-2 (P04280)	7609 ± 1 (7609.2)	18.7-19.1
- II-2 non-phosphorylated	7529 ± 1 (7529.2)	19.5-19.8
- II-2 Des-Arg$_{75}$	7453 ± 1 (7453.0)	18.8-19.2
IB-8a (Tot):		
- IB-8a (Con1+)	11888 ± 2 (11887.8)	17.1-17.8
- IB-8a (Con1-)	11898 ± 2 (11896.2)	17.1-17.8
IB-1 (Tot):		
- IB-1 (P04281)	9593 ± 1 (9593.4)	18.8-19.3
- IB-1 non-phosphorylated	9513 ± 1 (9513.4)	19.4-19.7
- IB-1 Des-Arg$_{96}$	9437 ± 1 (9437.2)	19.0-19.4
Statherin family (Staths)		
Statherin di-phosphorylated (P02808)	5380.0 ± 0.5 (5379.7)	28.9-29.5
Statherin mono-phosphorylated	5299.9 ± 0.5 (5299.7)	28.7-29.1
Statherin non-phosphorylated (P02808)	5220.5 ± 0.5 (5219.7)	28.4-28.8
Cystatin family (S-type Cysts)		
Cyst S non-phosphorylated (P01036)	14,186 ± 2 (14185)	36.5-37.1
Cyst S mono-phosphorylated (S1)	14,266 ± 2 (14265)	36.6-37.1
Cyst S di-phosphorylated (S2)	14,346 ± 2 (14345)	36.8-37.2
Cyst SN (P01037)	14,312 ± 2 (14313)	34.8-35.2
Cyst SA (P09228)	14,347 ± 2 (14346)	38.4-38.9

PB	5972.9 ± 0.5 (5792.7)	29.4-30.5

Table 3.1: List of salivary proteins and peptides quantified by HPLC-low resolution-ESI-MS. *Identification was based on the chromatographic behavior and comparison of the experimental mass values with the theoretical ones reported in the Swiss-Prot Data Bank (http://us.expasy.org/tools).

3.3.6. Statistical Analyses

Statistical analyses of the protein data were performed with XLSTAT Statistical and Data Analysis Solution (Addinsoft 2020, New York, NY, USA) and SAS 9.4 Analytical Software (Cary, N.C. USA). Normality testing was done using the Anderson–Darling test while homogeneity of variances was tested using Bartlett's test. For non-normal distributions, data were transformed using the Johnson transformation.

To determine potential effects of stimulus order presentation (CJ first or CPE first) on alpha-amylase as well as protein families (totals of basic proline-rich proteins (bPRPs), acidic proline-rich proteins (aPRPs), statherin, histatin and S-type cystatins), a repeated measures ANOVA (between subjects factor: order; within-subjects factor: treatment, order*treatment) was used.

Transformed data were then analyzed using a repeated measures ANOVA with PROP taster status and gender as between-subjects factors to determine inter-individual differences. In early analyses, we included the effects of ethnicity in our models, but finding no effets of this variable, it was dropped from subsequent analyses. Post-hoc comparisons were performed with Bonferroni's method.

None of the transformations improved normality for the individual proteins. In these cases, Friedman's ANOVAs (non-parametric test) were performed on the untransformed data to understand the effect of stimulation on the individual protein levels. These analyses were conducted on individual bPRPs (P-F, P-J, P-D, P-H, IB-8a Tot., II-2 Tot., IB-1 Tot. and Ps-1), aPRPs (PC, PRP-1, PRP-3), histatins (Hist 1 and Hist 5 and 6), S-type cystatins (Cyst S, Cyst S1, Cyst S2, Cyst SN and Cyst SA) and PB. Finally, post-hoc comparisons for the individual proteins (17 in all) were done following Bonferroni corrections (p = 0.05/17 = 0.002) to adjust for the large number of comparisons.

To understand variation in individual protein levels with respect to PROP taster status and gender, multiple Kolomogrov–Smirnov pairwise comparisons were performed at each time point. Specifically, cumulative distribution frequency curves of non-taster subjects were compared to those of super-taster subjects at resting, 5 and 10 min after CJ and CPE exposure. Similarly, comparisons were made for gender differences at each of the time points. However, this approach did not reveal significant effects of PROP taster status and gender at the individual protein level. Therefore, these data are not reported.

Data reported in the figures are means (±SEM) and show original, untransformed values.

3.4. Results

Table 3.1 shows subject characteristics. The subject pool (n = 60) was mostly Caucasians (n = 43) and the rest were Asians (n = 17). Taster x gender subgroups were approximately balanced with 25.0% female non-tasters (n = 15), 26.7% female

super-tasters (*n* = 16), 23.3% male non-tasters (*n* = 14) and 25.0% male super-tasters (*n* = 15). Mean participant age was 22.0 ± 0.6 years.

Gender	PROP Classification*	Ethnicity (*n*)			Age	BMI
		n	Caucasian	Asian	(years)	(kg/m²)
Female	NT	15	12	3	21.7 ± 1.5	26.0 ± 1.5
	ST	16	12	4	23.3 ± 1.5	24.2 ± 1.5
Male	NT	14	9	5	21.4 ± 0.6	24.2 ± 1.0
	ST	15	10	5	21.6 ± 1.2	25.5 ± 1.0

Table 3.2. Subject Characteristics.

3.4.1. Effect of Stimulation on Protein Response: Protein Families

Overall order effects:

There was no effect of stimulus presentation order on levels of total aPRPs, bPRPs, statherins, histatins and S-type cystatins over the time course (p = 0.976–0.211, not significant).

Overall treatment effects:

The effects of stimulation with CJ and CPE on protein families are shown in Figure 3.2. There was a general trend of higher levels after stimulation with both CJ and CPE. However, this effect was only statistically significant for aPRPs ($F_{[4,225]}$ = 24.96, p < 0.000). aPRP levels were higher relative to baseline at 5 min and remained elevated at 10 min after stimulation with CJ (p < 0.000). The same pattern was observed after stimulation with CPE; levels were higher in comparison to baseline at 5 min (p < 0.000) and remained elevated at 10 min after stimulation (p <

0.000). No significant main effects of stimulation were observed on other protein families (bPRPs, statherins, histatins and S-type cystatins).

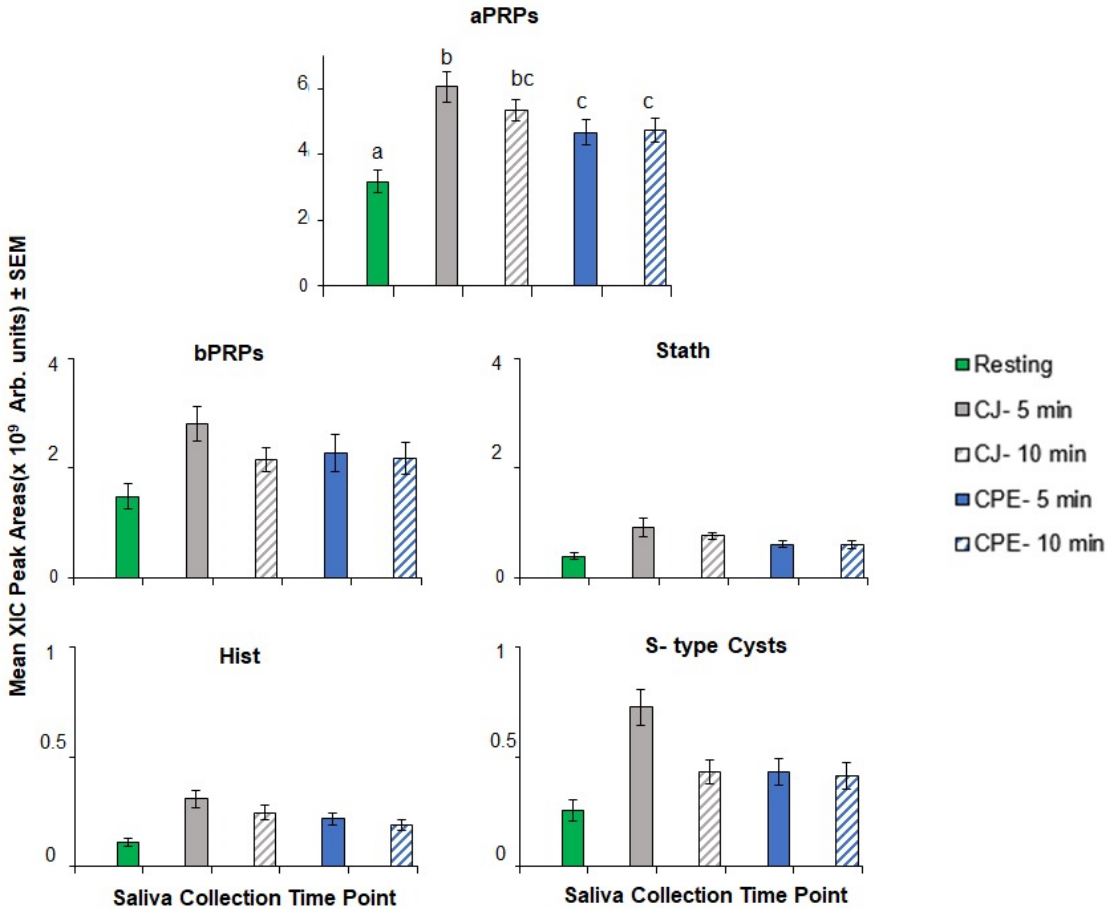

Figure 3.2. Extracted ion current (XIC) peak areas (mean values × 10^9 ± SEM) in arbitrary units for salivary protein families following stimulation with cranberry juice (CJ) and cranberry-derived polyphenol extract (CPE) (n = 60). Values with different superscript letters (a, b etc.) differ at $p < 0.05$.

Interaction effects:

There was no overall effect of stimulation on bPRP levels (with either CJ or CPE). However, there was a significant gender*taster*treatment effect ($F_{[4,224]}$ = 2.60, p = 0.037) on bPRPs (Figure 3.3.).

Separate analyses were conducted in non-taster and super-taster groups. There were significant differences in bPRP levels across the time course in super-taster subjects with CJ ($F_{[4,116]}$ = 3.20, p = 0.015) (Figure 3.3a). Super-taster males had higher levels at 5 min than they had at resting, and at 10 min these levels declined to intermediate values, which were not statistically different from levels at either resting or at 5 min. Female super-tasters showed a statistically significant, but small increase at 5 min, that was not considered to be physiologically meaningful. In comparisons by gender, male super-tasters had higher peak bPRP levels (at 5 min) in comparison to female super-tasters (p = 0.018). Male super-tasters showed the same pattern of response to CPE as they did for CJ; levels of bPRPs were higher at 5 min after stimulation (p = 0.007) compared to resting and declined to intermediate levels at 10 min. Female super-tasters did not show any effect of stimulation to CPE, and there were no differences between genders at any time point.

Within non-tasters (Figure 3.3b), females generally had higher levels of bPRPs at resting as compared to males (non-significant). No effect of PROP taster status was seen after stimulation with either CJ or CPE among non-tasters.

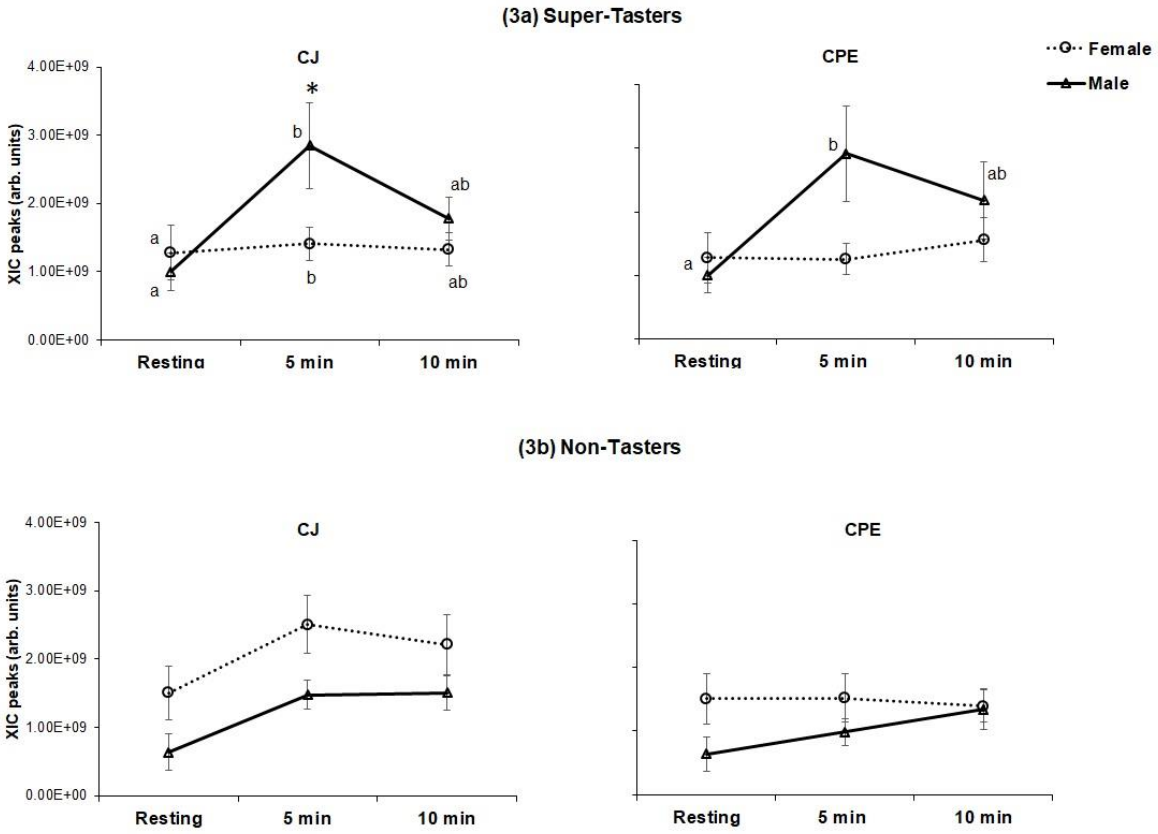

Figure 3.3 Effect of stimulation on total bPRP (basic proline-rich protein) levels in taster by gender subgroups. Mean extracted ion current (XIC) peak areas ± SEM (arbitrary units) are shown for super-taster (a) and non-taster subjects (b). Left panels show response to stimulation to CJ, while right panels show response to CPE. The same resting values were used for CJ and CPE. Superscripts (a, b) show significant differences in protein levels across the time course in each subgroup (male ST, female ST, male NT or female NT) and * shows differences between males and females at each time point. Non-taster females (n = 15), non-taster males (n = 14), super-taster females (n = 16) and super-taster males (n = 15).

3.4.2. Effect of Stimulation on Protein Response: Individual Proteins

The effects of CJ and CPE stimulation on the levels of individual proteins are shown in Figure 3.4 and Figure 3.5, respectively. In general, results showed that both stimuli led to increased levels of these proteins.

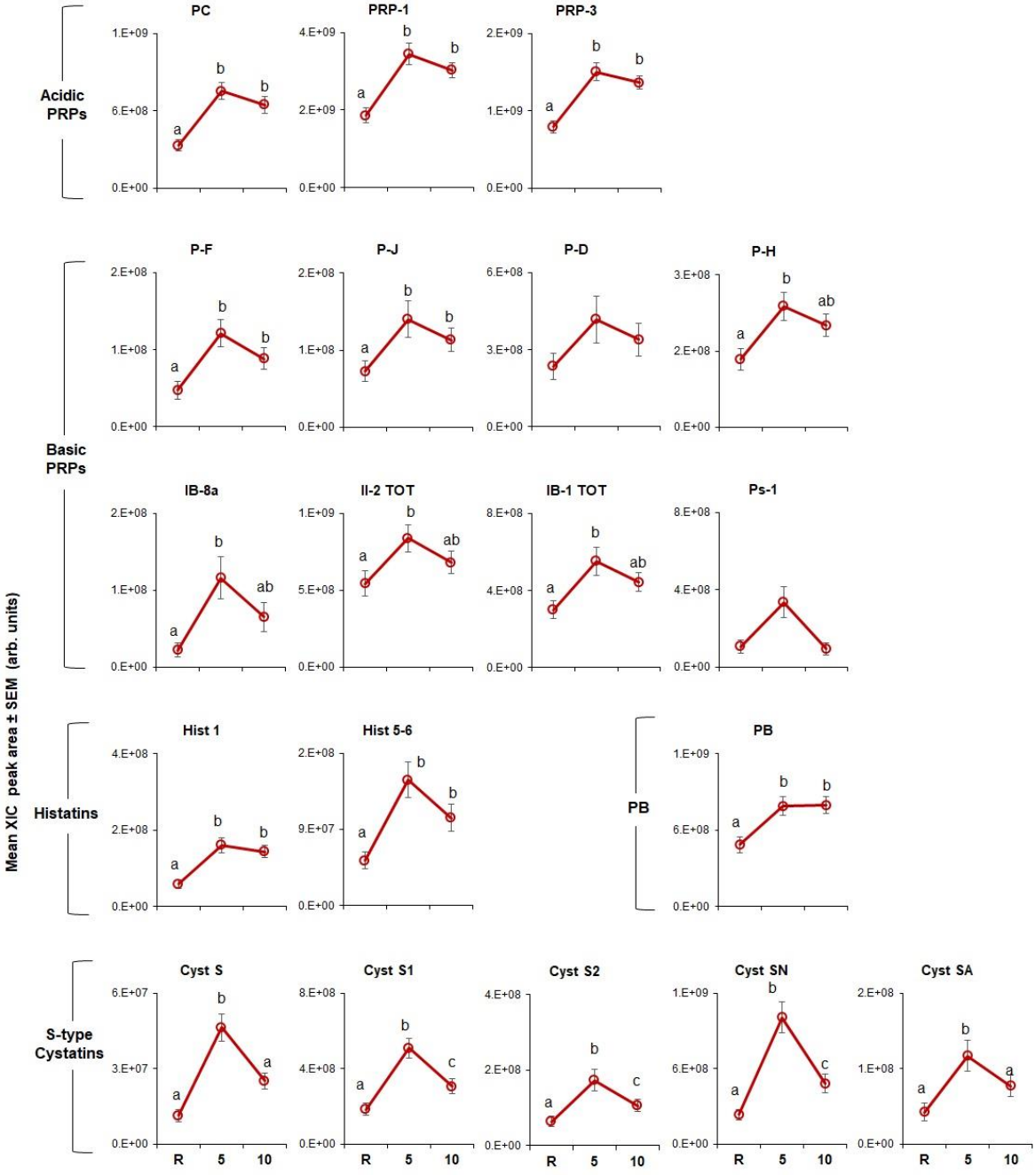

Figure 3.4. Effect of stimulation with CJ on levels of individual salivary proteins. Mean extracted ion current (XIC) peak areas ± SEM (arbitrary units) of individual

proteins measured at resting (R), 5 min (5) and 10 min (10) after stimulation (n = 60). Means within protein type with different superscript letters (a, b, etc.) are different at $p < 0.002$.

CJ stimulation (Figure 3.4) had a significant effect on all proteins (p = 0.002–0.0001) except for PD and Ps-1 (bPRP family). P-C, PRP-1, PRP-3 (aPRP family), PF and PJ (bPRP family) rose after stimulation with CJ at 5 min and remained elevated after 10 min. The same pattern was observed for Hist 1 and Hist 5, and Hist 6 (histatin family) and PB. Figure 3.4 also shows that, PH, IB-8a, II-2 and IB-1 (bPRP family), Cyst S and Cyst SA (S-type cystatins family) were also elevated at 5 min but fell to levels not statistically different from resting by 10 min. Three members of the S-type cystatins family, Cyst S1, S2, SN rose to peak levels at 5 min, trended downward, but remained elevated relative to baseline at 10 min.

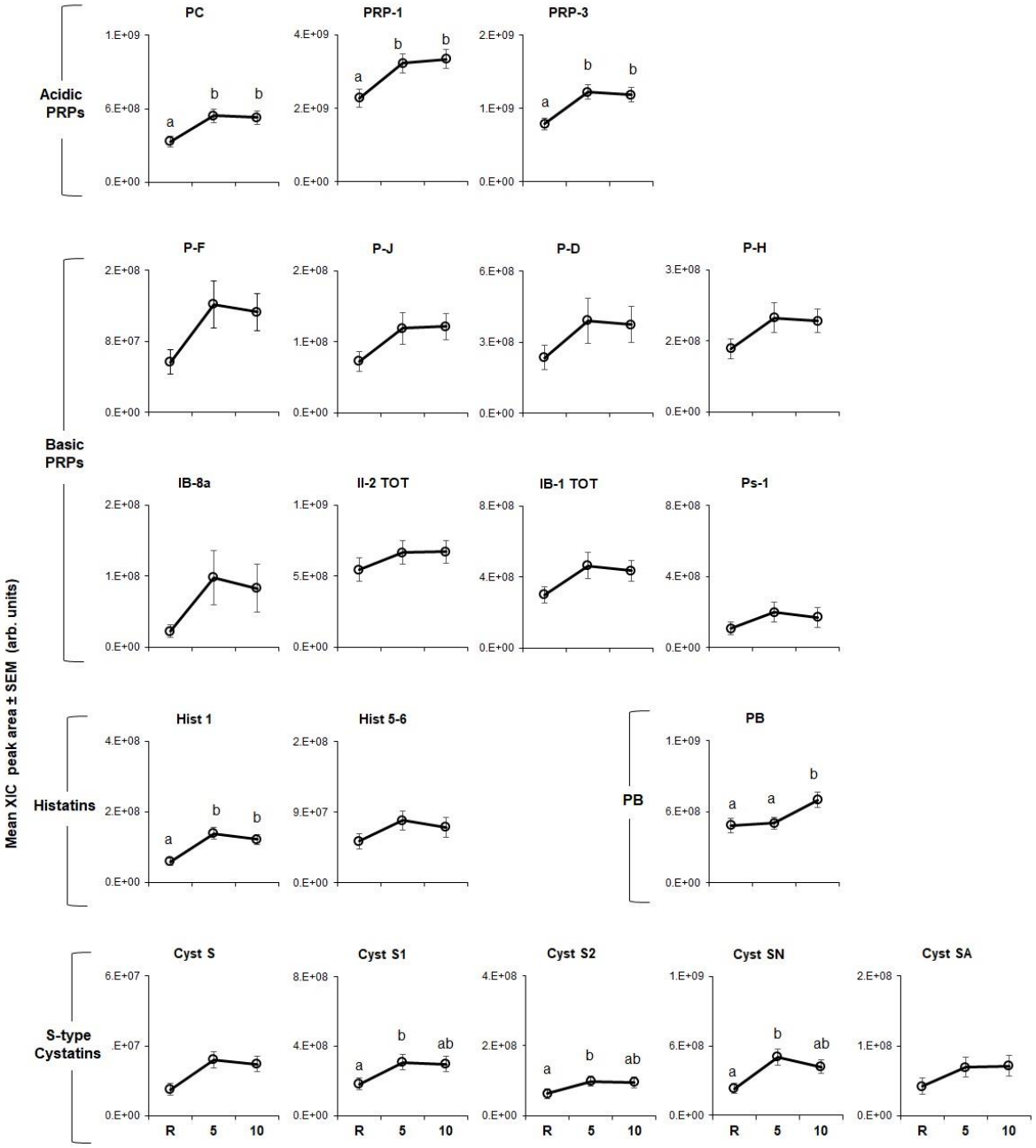

Figure 3.5. Effect of stimulation with CPE on levels of individual salivary proteins. Mean extracted ion current (XIC) peak area ± SEM (arbitrary units) of individual proteins measured at resting (R), 5 min (5) and 10 min (10) after stimulation (n = 60). Means within protein type with different superscript letters (a, b) are different at $p < 0.002$.

The effects of CPE (Figure 3.5) on individual proteins were less robust than they were for CJ ($p < 0.002$). Levels of all three aPRPs were higher at 5 min and remained elevated at 10 min after stimulation with CPE; the same effect was observed for Hist 1 (histatin family). Levels of Cyst S1, S2 and SN were also elevated at 5 min but fell to baseline by 10 min. In contrast, Hist 5, and Hist 6, Cyst S and Cyst SA and all bPRPs were unaffected by stimulation.

3.4.3. Effect of Stimulation on Protein Response: Alpha Amylase

Mean alpha-amylase levels in resting saliva were estimated at 0.029 μg/uL. Repeated measures ANOVA showed no overall main effect of stimulation by CJ or CPE on alpha-amylase levels. However, there was a significant taster*treatment interaction, in response to stimulation ($F_{[4,224]} = 5.95$; $p = 0.001$). As shown in Figure 3.6, PROP STs had higher levels of amylase than NTs at 5 ($p < 0.014$) and 10 min ($p < 0.000$) after exposure to CJ. No effect of stimulation was seen with CPE.

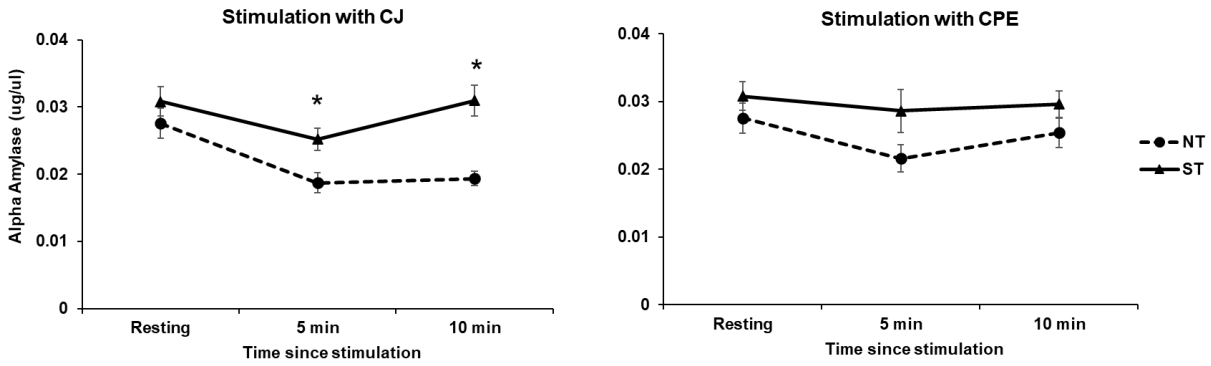

Figure 3.6. Effect of stimulation on alpha-amylase levels after stimulation with CJ (left) and CPE (right). * Indicates a difference between non-tasters and super-tasters ($p < 0.014–0.000$) at a given time point. Non-tasters ($n = 29$) and super-tasters ($n = 31$).

Finally, there was no effect of stimulus presentation order on levels of alpha-amylase over the time course (p = 0.762, not significant).

3.5. Discussion

The time course of astringency has been studied from a sensory perspective (Lee 1991, Naish 1993, Valentova 2002) but complementary work in salivary proteins has been limited (Brandao 2014) thus far. The first objective of this study was to examine salivary responses to CJ and CPE with respect to major protein families and individual protein sub-types within those families. First, at the family level (Figure 3.2), we found that oral stimulation with CJ and CPE led to a robust increase in aPRPs but not the other protein families, which only showed minor (non-significant) positive increases. This generally agrees with our previous findings (Melis 2017) with the same set of proteins showing that aPRPs rose robustly after oral stimulation with CJ. Another time-course study conducted by Brandao and co-workers (Brandao 2014) also reported that aPRPs were most responsive to stimulation with condensed tannins, while bPRP levels were relatively unaffected. Interestingly, we observed the same outcomes for aPRPs as did Brandao et al. (Brandao 2014) even though our study was fundamentally different than the earlier study, which had a smaller sample size (triplicates of n = 4), used pooled saliva samples and repeated oral stimulation prior to beginning the time course. Despite these methodological differences, all three studies support an important role for aPRPs in the salivary response to oral polyphenols. Together, these findings seem to conflict with the earlier literature suggesting that bPRPs were primarily responsible for oral astringency due to their highest binding affinity to tannins (Lu

1998, Charlton 2002). However, recent competitive *in vitro* assays showed that aPRPs and histatins, mostly found in the mobile phase of saliva, are first in line to interact with polyphenols (in comparison to other proteins such as mucins or gPRPs, which are mostly adsorbed onto mucosal or dental surfaces) particularly at lower phenolic concentrations (Soares 2012). Presumably, all the astringent stimulus may not be cleared from the mouth upon swallowing. It is possible that interaction of protein with residual polyphenols in saliva may occur (Dinnella 2009, Dinnella 2010), leading to persistent soluble aggregate formation, which do not coalesce and precipitate, initiating the so-called 'second step' of protein–polyphenol interaction (Charlton 2002, Jobstl 2004).

An important observation in our study was that CJ was a more potent stimulus for protein responses than CPE, both at the family level (Figure 3.2) as well as for individual protein sub-types (Figure 3.4; Figure 3.5). Virtually all protein sub-types were significantly elevated by CJ, except two bPRP sub-types, PD and Ps-1. In comparison, CPE elevated all three aPRPs, but its effects on other protein types were more muted and less consistent than what we observed for CJ. CPE did not increase any of the individual bPRPs and only affected some of the S-type cystatins and histatins. Finally, we noticed that individual S-type cystatins, (e.g., Cyst S1, S2 and SN) returned to baseline at the end of 10 min after CJ but not after CPE.

There could be several explanations for these differences. First, CPE is a carrier-free flavor ingredient that contains a mixture of various flavanoids. A preponderance of these polyphenols are anthocyanins, flavanols and proanthocyanidins, which have all been shown to interact with salivary proteins

(Garcia-Estevez, Cruz et al. 2017, Ramos-Pineda 2019, Soares 2019). Although CJ contains these same polyphenols, it is also highly acidic and contains pectin. Pectins have been shown to hinder the complex formation between salivary proteins and polyphenols (Soares 2012). Acidic stimuli elicit saliva release, mainly from the parotid gland, which leads to an elevated level of proteins, such as proline-rich proteins in the oral cavity (Neyraud 2006, Neyraud 2009). Notably, bPRPs are only released from the parotid gland, where they make up 23% of the total protein secreted (Kauffman 1979). This could be an explanation for why bPRP levels rose after CJ but not to CPE stimulation. Second, recent work studying mixtures of proteins *in vitro* has shown that depending on the size of protein–polyphenol aggregates formed by other salivary proteins such as aPRPs, interactions between phenolic stimuli and bPRPs may be impeded (Ramos-Pineda 2019). It is possible that CPE formed aggregates with other salivary proteins, which prevented the interaction between bPRPs and CPE. Finally, a large component of our understanding of protein–polyphenol interactions comes from purified salivary protein fractions, whereas the present study analyzed whole saliva, which is a mixture of many proteins. These factors alone or in combination could have influenced the higher levels of proteins following stimulation with CJ in contrast to CPE.

The present study also examined the role of PROP taster status in salivary protein responses to CJ with the goal of replicating the earlier findings of Melis and co-workers (Melis 2017) and potentially extending these findings to CPE. Melis et al. (Melis 2017) showed PROP-specific effects of CJ on two sub-types of aPRPs (PRP-1

and PRP-3) and one of the Cystatin sub-types (Cyst-SN). Specifically, levels of these proteins rose after CJ stimulation in medium-tasters and super-tasters, but no increases were observed in non-tasters. The present findings diverge from our earlier results in that here, we observed no PROP-related effects on either aPRP or Cystatin sub-types. However, we did observe a taster by gender interaction of stimulation on the bPRP protein family (Figure 3.5) only in STs. Generally, levels of bPRPs among male STs rose significantly higher than those of female STs at 5 min after stimulation. CPE stimulation also raised bPRPs for male STs but these levels were not significantly higher than those of female STs. The reasons for the discrepant findings between studies are presently unclear. Nevertheless, an important difference is that Melis et al. (Melis 2017) measured protein levels at 1 min after stimulation, whereas the current study examined protein levels at 5 and 10 min. The dominance of different salivary proteins at different time points (i.e., aPRPs and cystatins at 1 min and bPRPs at 5 min after stimulation) suggests the sequential involvement of proteins at different stages of astringency development and that PROP-taster status may be an important marker at each stage of this process. Although methodologically challenging, repeated saliva collections at short intervals directly following CJ exposure may reveal important dynamics of the early protein response that would clarify our understanding of PROP effects as well as other individual differences in salivary function. Future studies should address this question. Interestingly, Melis et al. (Melis 2017) also found a gender dichotomy in their astringency perception experiment. Together, these data suggest a gender-specific role for PROP status that may link oral astringency perception with protein

responses. Greater recognition of these potential differences may be important for interpreting the results of future sensory studies and proteomic analyses.

To our knowledge, salivary amylase has not been measured in studies investigating the involvement of salivary proteins in astringency responses over a time course. We found no main effect of either CJ or CPE on amylase levels. However, a novel finding of this study was that STs had higher levels of amylase in response to CJ exposure than did NTs (Figure 3.6). Salivary amylase plays a key role in the oral digestion of starches by hydrolyzing complex carbohydrates to smaller sugars (MacGregor and Svensson 2001) and its expression has been shown to be influenced by high-fat and high-tannin diets in animal models (da Costa 2008, Rodrigues 2015). Individual differences in salivary amylase activity are well known and may be related to *AMY1* gene copy number (Elder 2018) in humans. Whether salivary amylase activity also varies with PROP taster status should be investigated in future studies as amylase has important implications in digestion, nutrient absorption and most recently, texture perception (Engelen 2003, de Wijk 2004, Bridges 2017, Lamy 2020).

Seminal work by Dinnella and colleagues (Dinnella 2009, Dinnella 2010) on individual variation in the astringency response utilized total protein concentrations (*D* values) in correlation with sensory responses to classify individuals into low or high responders to astringency. In the current study, we targeted specific proteins and peptides, which enabled us to offer insights into a selected individual difference, i.e., interindividual variation in salivary proteins associated with PROP taster status. Conducting a time-course of astringent

sensations in PROP-classified subjects could supplement our understanding of the sensory relevance of these differences. Examples of this could include use of temporal dominance of sensations or other time-intensity measures to track differences in the experience of astringent sub-qualities, which may be important for understanding this complex sensation.

The present study has several limitations. First, we did not measure salivary flow rate which has been correlated with measures of astringency perception in previous studies (Ishikawa 1995, Noble 1995, Horne 2002, Dinnella 2009). This decision was based on previous observations (Melis et al., unpublished) showing no meaningful associations between flow rate and protein variations. Nevertheless, salivary flow has also been shown to be associated with differences in gender and age (Srivastava 2008), particularly in the context of stimulation with sour stimuli (Li-Hui 2016). Other work has shown that there can be considerable inter-individual variation in salivary flow rate and other characteristics related to diet and physiological factors (Tukia-Kulmala 1993, Prodan 2015) which could have impacted the variables measured in this study. Second, we did not assess mucins, which are technically challenging to measure (Harrop 2012). Mucins play a key role in oral lubricity and including them in future studies will provide a more complete picture of astringency mechanisms. Third, in designing this study we weighed the potential impacts of presenting the two stimuli in different sessions on different days, each with its own baseline (potentially increasing session-to-session variability) or presenting them sequentially in a single session with a long rest period in between samples. We ultimately decided on the second scenario in which

sample presentation was randomized to minimize order effects, and a 20 min rest period in between samples. In exploratory analyses we probed the data for order effects (i.e., the effects of subjects receiving CJ first vs. CPE first) but were unable to detect these differences. However, it is not known if the 20 min rest period was sufficient to reset protein levels to baseline. Finally, we did not collect saliva samples in replicates, i.e., participants were exposed to each stimulus only once. It is possible that there could be variation in the findings such as between-day variability if replications were performed. Nevertheless, whether small, underlying differences influenced our data is not known and should be considered in interpreting our findings.

3.6. Conclusion

These data show that proteomic changes may linger even at 10 min after an astringent high-polyphenol food enters the oral cavity. Therefore, researchers are cautioned against utilizing shorter breaks when administering astringent stimuli in sensory studies to allow the mouth sufficient time to recover. The present study used a time-course approach to study interindividual differences in salivary protein response to astringent stimulation and has provided novel insights into how PROP taster status may influence variation in astringency perception, i.e., via differential involvement of specific proteins, which may ultimately guide selection of polyphenol-rich foods.

3.7. Acknowledgments

Conceptualization, N.Y.Y., Me.M., B.J.T. and I.T.B.; methodology, formal analysis, investigation, N.Y.Y., Me.M., Ma.M. and C.C.; data curation, N.Y.Y., Me.M. and Ma.M.;

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4. PROP TASTER STATUS & THE ORAL MICROBIOME

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4.1. Abstract

Diet and salivary proteins are known to influence the composition of the oral microbiome, and recent data suggest that TAS2R38 bitter taste genetics may also play a role. This study investigated the effects of daily exposure to cranberry polyphenols as an oral rinse on taste perception, salivary proteins, and oral microbiota. PROP super-tasters (ST, n=10) and non-tasters (NT, n=10) used 30 mL of an oral rinse (0.75 g/L cranberry extract in spring water, CPE) twice daily for 11 days while consuming their habitual diets. 16S rRNA sequencing showed that the NT oral microbiome composition was significantly different than that in STs at baseline ($p=0.012$) but not after the intervention ($p= 0.525$). Stratifying for inter-individual variation, principle coordinates analysis using unweighted UniFrac distance showed that CPE significantly modified microbiome composition in NTs ($p=0.023$) but not in STs ($p=0.096$). The intervention also altered specific salivary protein levels (α-amylase, MUC-5B and selected S-type Cystatins) but no changes in sensory perception were observed. Correlation networks between oral microbiota, salivary proteins and sensory ratings showed that the ST microbiome had a more complex relationship with salivary proteins, particularly proline-rich proteins, than that in NTs. The differences in oral microbiota composition in NTs and STs and their distinct response to polyphenols may have implications for oral health.

4.2. Introduction

Diverse microorganisms inhabit the oral cavity and oral microbiota forms the second largest microbial community in humans, after the gut (Caselli 2020). The interactions between oral microbiota, host and environmental factors influence microbial homeostasis and ultimately human health (Lamont 2018). The study of taste perception and its relationship with oral microbiome is an emerging area of research, which has thus far not included analysis of salivary proteins as an important co-variate.

Salivary proteins play an essential role in maintaining a healthy oral environment. Notably, salivary proteins such as basic proline-rich proteins (bPRPs), acidic proline-rich proteins (aPRPs), histatins, cystatins, mucins, statherins and amylase exhibit antimicrobial and/or antiviral properties and maintain oral lubricity and mineral composition of dental surfaces that collectively form a key cornerstone of oral immunity (Pateel 2021). Many of these proteins directly or indirectly inhibit bacterial growth via disruption of their enzymes or manipulate bacterial adhesion mechanisms, therefore, affecting biofilm formation (Lynge Pedersen 2019, Benahmed 2021). In addition to oral immunity, many salivary proteins also influence taste and mouthfeel perception of polyphenol-rich foods. Salivary proteins may bind dietary polyphenols to form complexes; these complexes may be partially responsible for the sensation of astringency. In addition, this binding can elicit a cascade of protective mechanisms that defend the oral cavity against physical or chemical damage and may regulate the amount of polyphenols

available downstream for digestion (Ho 1992, Kondo 2014). This is important as over-consumption of polyphenol-rich foods can be aversive to animals and humans (Lesschaeve 2005). Nevertheless, feeding a polyphenol-rich diet to mice upregulates key salivary proteins, while reducing aversions to such foods (Torregrossa 2014). In a short-term intervention with human subjects, Crawford et al (Crawford 2020) showed that daily consumption of a polyphenol-rich chocolate-flavored cow's milk, rich in polyphenols, increased levels of salivary PRPs and Cystatins, and these effects were related to minor changes in taste perception (Crawford 2020). Therefore, evidence suggests that, in addition to their protective role in the oral cavity, salivary proteins may influence taste perception as well.

Genetic variability in taste sensitivity to the bitterness of 6-n-propylthiouracil (PROP) has been used as an oral marker for the perception and acceptance of bitter and strong-tasting foods with nutritional implications. This genetic variability is regulated by polymorphisms in the *TAS2R38* gene, which codes for the bitter taste receptor TAS2R38. Single-nucleotide polymorphisms in this gene result in structural variation in the TAS2R38 taste receptor, which affects the binding affinity of the bitter taste molecule 6-n-propylthiouracil (PROP). Individuals who are homozygous dominant for this gene, phenotypic PROP super-tasters (STs), have a strong binding affinity for PROP and experience intense bitterness from it, while homozygous recessive individuals are non-tasters (NTs), do not bind PROP very well and experience little to no bitterness from it; heterozygous individuals experience intermediate bitterness and are called medium-tasters (MTs).

Perception and liking of astringent, polyphenol-rich fruits has been studied in relation to PROP taste responsiveness (Laaksonen 2013, Sandell 2015, Duffy 2016). Using cranberry-derived stimuli, Melis and colleagues (Melis 2017) showed that PROP STs' saliva had higher levels of acidic PRPs and Cystatins in comparison to NTs after tasting unsweetened cranberry juice. In the same study, male NTs reported lower bitterness and astringency from cranberry juice cocktail supplemented with tannic acid (to modify astringency) and gave it higher liking ratings in comparison to PROP STs (Melis 2017). More recently, we showed that PROP STs had higher levels of salivary α-amylase in comparison to NTs following oral exposure to cranberry-derived stimuli (Yousaf 2020). We also found that male STs had higher levels of the proteins after oral stimulation when compared to other subgroups. These data suggest that the TAS2R38 phenotype may play a critical role in salivary protein release to cranberry polyphenols. Considering cranberries are abundant in type-A proanthocyanadins (PACs) with known strong oral antimicrobial properties (Foo 2000, Foo 2000, Weiss 2004, Bodet and Ofek 2008, Khairnar MR and 6:35-9 2015), these findings have potential implications for immunity and oral health.

Numerous studies in the dental literature have shown that NT adults and children have more dental caries than taster individuals (Wendell 2010, Pidamale 2012, Pidamale 2012, Yildiz 2016). Although a range of genetic and environmental factors are involved in caries development (diet, oral hygiene, other genes) (Isola 2020), these observations suggest there may be innate differences in caries risk between NTs and STs. In light of our findings outlined above showing that oral

exposure to cranberry polyphenols elicited higher protein responses in STs compared to NTs, we propose that STs may be better protected against dental caries by virtue of their greater ability to mount a protein response. There is strong evidence that the TAS2R38 receptor plays a sentinel role in protection from upper airway disease and the NT phenotype is associated with greater risk and severity of disease (Lee 2012, Adappa 2014, Lee 2015). Thus, our suggestion that TAS2R38 may serve a role in supporting oral health including maintaining healthy microflora is well supported by parallel studies in upper airway health. . Only two previous studies have reported associations between TAS2R38 genotypes and oral microbiota (Sandell 2018, Cattaneo 2019), but the focus of these experiments was on taste rather than oral health. Thus, any associations between the PROP taster phenotype in conjunction with measurement of salivary proteins and oral health remains unexplored.

Thus, the present pilot study had three research objectives: 1) to determine if there is variation in the oral microbiome with respect to the PROP phenotype, 2) if daily rinsing with cranberry-polyphenol extract (CPE) oral rinse would alter the oral microbiome and 3) if there would be changes associated with salivary protein profile or alteration of taste perception as an outcome of this intervention. We hypothesized that the oral microbiome structure would vary with PROP taster phenotype, that the CPE oral rinse would alter the composition and diversity of the oral microbiome, and that these changes would affect salivary proteins and taste perception. Whether these potential changes in the oral microbiome, salivary

proteins and/or taste perception would be differential with the PROP phenotype was an additional exploratory aim of this study.

4.3. Methods

4.3.1. Subject Recruitment

Healthy adults (*n* = 20), between 18–45 years of age were recruited from the Rutgers University community through an email distribution list. Subjects were screened for PROP taste responsiveness; only PROP non-tasters and super-tasters were admitted into the study. Subjects were also screened for general suitability (e.g., demographics, health information) and good oral health status, such as dental cleaning within the last six months, no ongoing oral or dental concerns, good oral hygiene (brushing teeth at least twice a day). Exclusion criteria included major metabolic diseases (diabetes, kidney disease, etc.), pregnancy, lactation, food allergies, use of medications that interfere with taste or smell functions (e.g., steroids, antihistamines, or anti-depressants), lack of routine oral health exam within last 6 months, incidence of dental/oral disease (such as periodontitis, infections, recent root canals etc.) or presence of oral piercings. Participants who were determined to be medium tasters for the PROP phenotype were also excluded.

The study was approved by the Rutgers University Arts and Sciences Institutional Review Board (Approval#13-309M) and trial was registered in the Clinical Trials database (ClinicalTrials.gov identifier: NCT04107688). All subjects provided written informed consent and were compensated monetarily for their participation.

4.3.2. PROP Taster Status

As part of the screening criteria, participants were classified according to PROP taster status using the paper disk method, which has been previously tested for validity and reliability (Zhao 2003). In brief, in this method, subjects place a filter paper disk impregnated with 1.0 mol/L NaCl (Sodium Chloride, S671-500, Fisher Scientific) on the tip of the tongue for 30 s. They rate the taste intensity of the disk using the labeled magnitude scale (LMS), a 100-mm scale anchored with the phrases "barely detectable" to "strongest imaginable." After a forced 5 min break, this procedure is then repeated with a second paper disk impregnated with 50 mmol/L PROP (6-n-propyl–2-thiouracil, P3755, Sigma-Aldrich). Subjects rinse with room-temperature spring water before and in between tasting each paper disk. Subjects are classified as non-tasters (NT) if they rate the PROP disk <15 mm on the LMS; they are categorized as super-tasters (ST) if they rate the PROP disk >67 on the LMS. All others are classified as medium tasters (MT). Since NaCl ratings do not vary with PROP taster status, these ratings are used as a reference standard to determine the taster status of subjects who give borderline ratings to PROP. This strategy is based on the rationale that NTs give higher ratings to NaCl than to PROP, MTs give equivalent ratings to both stimuli and STs give higher ratings to PROP than they do to NaCl.

4.3.3. Stimuli Preparation

Oral Rinse Samples:

An oral rinse solution was formulated using a carrier-free powdered cranberry polyphenol extract, CPE (Ocean Spray Inc. MA) added to spring water at a concentration of 0.75 w/v g/L. The control solution was spring water without any added CPE. These samples were dispensed in 20 mL aliquots in sealed amber glass vials. Samples were stored in an opaque aluminum lined bag and refrigerated until use. Each glass vial was brought to room temperature before use.

Taste Samples:

Briefly, cranberry juice (CJ) was made from fresh cranberries (donation, Ocean Spray Inc., Chatsworth, NJ) frozen at -20°C until use. CJ was made in small batches using a standard recipe where 300 g of berries were defrosted, washed, and cooked on a stovetop under medium heat for 10 min with 648 mL of spring water. The mixture was filtered through cheesecloth and cooled to room temperature and 8.75% w/v sugar was added to it to make cranberry juice cocktail (CJC).

CJC with various levels of astringency, as modulated by addition of cranberry polyphenol extract (CPE), was used as a taste stimulus. There were a total of 3 samples for sensory evaluation in this study: CJC (no added CPE), Low CPE (0.50 g/L CPE in CJC) and High CPE (0.80 g/L CPE in CJC). These concentrations were selected after extensive benchtop screening to select a range that demonstrates the lower and higher acceptable limits of broad consumer acceptance.

For the Low CPE and High CPE samples, CPE powder in the respective concentrations was mixed directly into freshly prepared CJC. These samples were

prepared as needed, the day before subject testing, and refrigerated at 4°C until 30 min before use. Samples were served at room temperature.

4.3.4. Experimental Procedures

Overall Study Design

Figure 4.1: Experimental Timeline

Subjects participated in a 2-week clinical trial (Figure 4.1), with a 3-day washout period followed by 11 days of intervention. From Day 1 to Day 3, subjects rinsed their mouths with the spring water control rinse twice a day (morning and evening after brushing their teeth). From Day 4 to 14, subjects used the CPE oral rinse, twice a day, following the same procedure as the control. On Days 3 and 14, subjects participated in a test session, in which they provided saliva for oral microbiome profiling as well as proteomic analysis immediately upon arrival to the test site. After saliva collection, subjects were given a short break (10 min) and then asked to evaluate cranberry beverage samples for key sensory attributes. Data resulting from Day 3 is considered the pre-intervention or baseline data, while Day 14 data is referred to as the 'post-intervention' data.

During this study, subjects were prohibited from using any other oral rinse products. However, they were not required to change their diet or routines in any

way except on saliva collection days. Subjects were instructed to not eat or drink anything except water, smoke, or chew gum in the morning of the test sessions. Test sessions were always conducted between 9:00 and 10:30 am for all subjects to minimize intra-day variation in saliva.

The testing sequence took two weeks to complete.

Saliva Collection and Treatment

At the beginning of the test session, subjects were given a cup to spit into directly. 1 ml of the saliva collected was immediately transferred to a 1.5 ml Eppendorf tube, which was labeled and immediately frozen at -80°C for oral microbiome profiling. The remaining saliva sample was transferred into two microcentrifuge tubes in an ice bath (0.5 ml per tube). One tube was treated with an acidic solution (0.2% Trifluoroacetic acid, TFA) in a 1:1 v/v ratio. These samples were centrifuged at 8000 g at 4°C for 15 min. The supernatant was separated from the pellet and stored at -80°C until analysis by HPLC-ESI-low resolution-IT-MS. A protease inhibitor cocktail solution (mix of 1 tablet/1.4 mL of cOmplete Protease Inhibitor Cocktail and ammonium bicarbonate 175 mM) (cOmplete®, Roche) was added to the second tube in a 1:2 v/v ratio. These samples were stored at -80°C until analysis by Dot-blot immunoblot procedure for alpha amylase and mucin quantification.

4.3.5. Oral Microbiome Analysis

A total of 40 saliva samples were collected from 20 subjects (2 time points each i.e. pre- and post-intervention). Insufficient genomic DNA was extracted from

one saliva sample which was then excluded from subsequent processing and analysis. Approximately 6.55 million usable 16S rRNA V4 amplicon reads were generated (average reads/sample = 167,958 ± 18,572). After denoising and abundance-based filtering, 1,031 reliable amplicon sequence variants (ASVs) were retained for further analysis.

Specifically, genomic DNA was extracted from 1 ml aliquots of saliva using protocol Q (Costea, Zeller et al. 2017) with minor modifications. Hypervariable region V4 of the 16S rRNA gene was amplified using the 515F and 806R primers modified by Parada et al (Parada 2016) and Apprill et al (Apprill 2015) respectively and sequenced using the Ion GeneStudio S5 (ThermoFisher Scientific, Waltham, MA). Primers were trimmed from the raw reads using cutadapt (Martin 2011) via QIIME 2 (Bolyen, Rideout et al. 2019). ASVs (Callahan, McMurdie et al. 2016) were obtained by denoising using the dada2 denoise-single command in QIIME 2 with parameters --p-trim-left 0 –p-trunc-len 215. Spurious ASVs were further removed by abundance filtering (Wang 2018). A phylogenetic tree of ASVs was built using the QIIME 2 commands alignment mafft, alignment mask, phylogeny fastree and phylogeny midpoint-root. Taxonomy assignment was performed by the q2-feature-classifier plugin (Bokulich, Kaehler et al. 2018) in QIIME 2 based on the silva database (release 132) (Quast, Pruesse et al. 2013). The data were then rarified to 76,000 reads/sample for subsequent analyses.

4.3.6. Salivary Protein Analyses

Total Protein Content Quantification

Bicinchoninic Acid (BCA) Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) was used to quantify total protein content of the saliva samples according to manufacturer's instructions. The total concentration was used to normalize protein levels for dot blot immunoblot analysis.

Immunoblot Analysis: Alpha Amylase

To purify amylase protein for use as a standard, a volume of 6 mL of whole saliva was collected and treated with 0.2% trifluoroacetic acid (TFA), as previously described. The sample was injected in an Ultimate 3000 Micro HPLC apparatus (Dionex, Sunnyvale, CA, USA) equipped with a photodiode detector (UV-VIS) and the chromatographic column was a Vydac-C8 with 5 μm particle diameter (column dimensions 250×10 mm) (Hesperia, CA, USA).

The following solutions were utilized for purification: 0.056% (v/v) aqueous TFA (eluent A) and 0.05% (v/v) TFA in acetonitrile-water 80/20 (eluent B) with flow rate at 2.8 mL/min. Salivary proteins were eluted using a linear gradient from 0 to 60% of B in 40 min, and from 60 to 100% of B in 5 min. Protein detection was carried out at a wavelength of 214 nm. The total injected saliva volume was 800 μL. Collected salivary fractions were analyzed in HPLC-ESI-IT-MS and the fraction containing amylase was lyophilized and then dissolved in 0.1% v/v TFA for a total volume of 500 μL. BCA assay was then performed, as previously described, to determine the concentration of amylase in the resulting solution.

The concentration of alpha amylase in the salivary samples was estimated semi-quantitatively by using dot-blot technique, where the protein samples were spotted directly onto a PVDF membrane (0.2 μm pore size; Immun-Blot® PVDF Membrane, Bio-Rad Laboratories, Inc., Italy). To set up a dot blot assay, the saliva samples treated with cOmplete protease inhibitor cocktail were first diluted with Tris Buffered Saline (TBS: 20mM Tris-HCl pH 7.6, 150mM NaCl) so that each diluted sample would have the same amount of total protein content (adjusted to 0.38 ug/ul). Before transferring the samples on to the PVDF membrane, it was pre-wetted with methanol for 1 min, then transferred to TBS for 2 min.

The amylase fraction, purified from whole saliva was used as a standard in 4 concentrations (0.01, 0.02, 0.05 and 0.1 μg/μL). All samples and standards were spotted onto the wet PVDF membrane in triplicate. Specifically, each test sample was spotted in a volume of 1μL (0.38 μg/μL of total protein content). The membrane was blocked with blocking agent 5% of BSA (Bovine serum albumin, Sigma Aldrich) in TBS-T buffer (20mM Tris-HCl pH7.6, 150mM NaCl, 0.05% Tween 20) for 1 hour at room temperature. Subsequently, the membrane was incubated with primary antibody (dilution 1:1000; Amylase G-10: sc-46657- Santa Cruz Biotechnology, Inc.) in 5% of BSA in TBS-T buffer, for 1 hour. Three washes for 5 min with TBS-T buffer were performed and the membrane was incubated for 1 hour with secondary antibody (dilution 1:5000; Rabbit anti-Mouse IgG, Secondary Antibody, HRP ThermoFisher Scientific). After three further washes with TBS-T, the membrane was incubated for 5 min with ECL substrate (Clarity Western ECL Substrate, Bio-Rad, Laboratories, Inc., Italy) for fluorescence signal development and captured on the

Chemidoc MP Imaging System (Bio-Rad, Hercules, CA, USA). Analysis of images obtained were performed using Image Lab 6.0.1 software (Bio-Rad Laboratories Inc.). Signals of samples were determined and shown as intensity values which were transformed by the software with volume tools in value of concentration (μg/μL) for each sample by using the standards as references. Each sample was analyzed in triplicate with acceptable coefficient of variation (CV%) set as below 15%.

Immunoblot Analysis: Mucins

All samples were spotted onto the wet PVDF membrane in triplicate for MUC5B and MUC7 quantification. Specifically, each test sample was spotted in a volume of 2μL (0.38 μg/μL of total protein content). The membrane was blocked with blocking agent 5% of BSA (Bovine serum albumin, Sigma Aldrich) in TBS-T buffer (20mM Tris-HCl pH7.6, 150mM NaCl, 0.05% Tween 20) for 1 hour at room temperature. Subsequently, the membrane was incubated with primary antibody (dilution 1:100; Mucin 7 (1C10): sc-517138 and Mucin 5B (5B#19-2E): sc-21768 - Santa Cruz Biotechnology, Inc.) in 0.5% of BSA in TBS-T buffer, overnight at 4°C. Three washes for 5 min with TBS-T buffer were performed and the membrane was incubated for 1 hour with secondary antibody (dilution 1:1000; Rabbit anti-Mouse IgG, Secondary Antibody, HRP ThermoFisher Scientific). After three further washes with TBS-T, one wash with TBS (5 min) the membrane was incubated for 5 min with ECL substrate (Clarity Western ECL Substrate, Bio-Rad, Laboratories, Inc, Italy) for fluorescence signal development and captured on the Chemidoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

HPLC-ESI-IT-MS Analysis

Table 3.1 shows the salivary proteins and peptides analyzed in each of the salivary samples collected using the HPLC-low resolution-ESI-IT-MS technique according to (Cabras 2012). 30 µL of the acidic soluble fraction corresponding to 15 µl of whole saliva was used (1:1 v/v dilution). Only proteins/peptides characterized in human saliva by applying the same analytical conditions in previous studies (Messana 2008, Castagnola 2012) were analyzed in the present investigation. Average mass values (Mav), obtained by deconvolution of averaged ESI-MS spectra automatically performed by using MagTran 1.0 software (Zhang 1998), and elution times of proteins/peptides were compared with those determined under the same experimental conditions in our previous studies (Messana 2008, Castagnola 2012).

Experimental Mav were also compared with the theoretical ones available at the UniProt-KB human data-bank (http://us.expasy.org/tools). The quantification of each protein and peptide was based on the area of the HPLC-ESI-IT-MS extracted ion current (XIC) peaks. The XIC analysis reveals the peak associated with the peptide of interest by searching, along the total ion current chromatographic profile, the specific multi-charged ions generated by the protein. The area of the ion current peak is proportional to concentration, and under constant conditions it may be used to perform relative quantification of the same analyte in different samples (Ong 2005).

4.3.7. Sensory Sample Evaluation

Intensity ratings for key sensory attributes of the cranberry juice cocktail were collected for three samples: cranberry juice cocktail with 0 (CJC), 0.5g/L CPE (Low) and 0.8 g/L CPE (High) of added CPE. A standard, 15-cm line scale end-anchored with the phrases "very weak" to "very strong" was used. Attributes of interest included sweetness, bitterness, sourness, astringency, thickness, cranberry flavor, and overall flavor. Sensory ratings were collected electronically using RedJade data collection software (Curion Insights, Redwood, CA), where the ballot is presented on a computer screen and subjects make their assessments electronically. All samples were served at room temperature. Order of presentation was randomized and there was a forced 5-min break in between sample evaluation.

4.3.8. Data Analysis

XLSTAT (Addinsoft, N.Y., USA) and R were used to perform all statistical analyses. Normality testing was conducted using Shapiro Wilk's test and homogeneity of variances was tested using Levene's test. Where the data was found to be non-normal, a non-parametric test was used.

Oral microbiome data analysis:

Alpha diversity indices (Shannon Index, observed ASVs, Faith's phylogenetic diversity and evenness) and beta diversity distance metrics (weighted and unweighted UniFrac distance (Lozupone, Lladser et al. 2011)) were used to evaluate the overall oral microbiota structure. Principal coordinates analysis (PCoA) and adjusted principal coordinates analysis (aPCoA) were performed by the R packages

"ape" (Paradis, Claude et al. 2004) and "aPCoA" (Shi, Zhang et al. 2020) respectively. ASVs shared by > 50% of the samples were considered prevalent and selected for guild-based analysis (Wu, Zhao et al. 2021). Specifically, pairwise correlations among the ASVs were calculated using the method described by Bland and Altman (Bland and Altman 1995). The correlation values were converted to a correlation distance (1 – correlation value) and the ASVs were clustered using the Ward clustering algorithm. From the top of the clustering tree, permutational multivariate analysis of variance (PERMANOVA; 9999 permutations; $p<0.001$) was used to sequentially determine whether the two clades were significantly different and the prevalent ASVs were clustered into guilds accordingly (Wu, Zhao et al. 2021).

Relationships of guilds in the oral microbiome with sensory ratings (using CPE High sample only) and salivary proteins were examined using the linear mixed effect model in the MaAsLin2 package (https://huttenhower.sph.harvard.edu/maaslin/), with subject as a random effect and adjusted for age, gender, and BMI. The correlations between sensory ratings and salivary proteins, and those between different guilds in the oral microbiome were determined as described by Bland and Altman (Bland and Altman 1995). An adjusted p-value (<0.25) was considered as significant for all correlation analysis between guilds, sensory ratings, and salivary proteins.

Immunoblot data and salivary proteins:

Samples from 4 subjects were determined to have unmeasurable levels of proteins and were therefore eliminated from the analysis. Friedman's ANOVAs for

each protein, comparing pre- and post-intervention data, were conducted to determine effect of intervention on salivary proteins (n=16).

Sensory ratings:

A repeated measures ANOVA was used (treatment and CPE concentration as within-subjects factors; PROP taster status as between-subjects factor) to determine effects of the intervention on intensity perception of flavor attributes (n=20).

4.4. Results

4.4.1. Oral Microbiome

Comparison of Oral Microbiome between PROP Taster groups

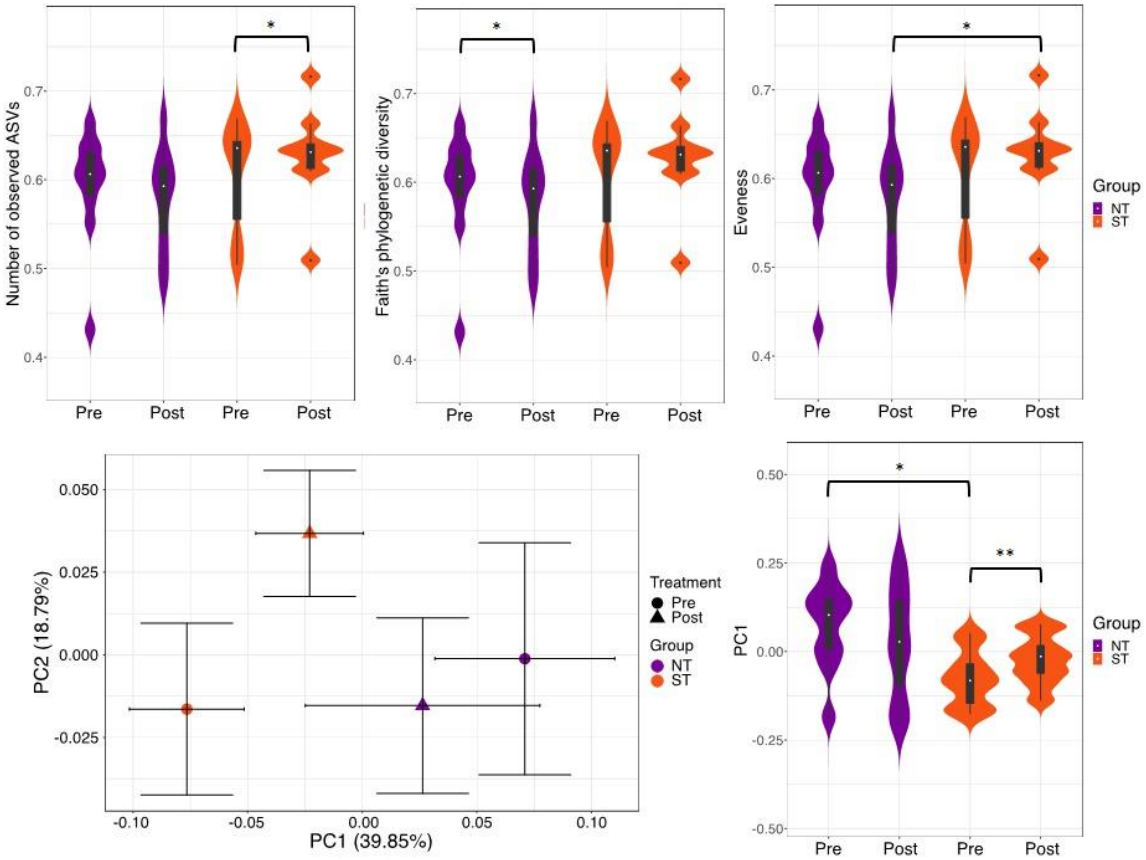

Figure 4.2: Oral microbiota was modulated by CPE oral rinse and showed differences between PROP NT and ST subjects in both alpha diversity (A-C; top 3 panels, sequentially) and beta diversity (D, E, bottom 2 panels, sequentially). CPE oral rinse altered the of oral microbiota as measured by Number of observed ASVs (A) Faith's phylogenetic diversity (B) and Evenness (C). NTs and STs differed in beta diversity based on Weighted UniFrac distance matrices shown in (D) as a 2D Principal Coordinate analysis (PCoA) plot and in (E) as violin plots. Each point in the PCoA plot represents the mean (± SEM) principal coordinate (PC) score of all individual subjects pre- and post-intervention. In the violin plots, rectangles show the medians and the interquartile ranges (IQRs), the whiskers denote the lowest and highest values that were within 1.5 times the IQR from the first and third quartiles. In NT/ST subjects, Wilcoxon matched-pairs signed-ranks test (two-tailed) was performed to compare pre- and post- intervention samples. Pre-/ Post-CPE oral rinse, Mann-Whitney test (two-tailed) was performed to compare NT and ST subjects. * $p < 0.05$ and ** $p< 0.01$. Pre-intervention: NT n=10, ST n=10; Post-intervention: NT n=10, ST n=9.

We first explored alpha diversity metrics to examine oral microbiome differences within NTs and STs. The two PROP taster groups did not differ in alpha diversity at baseline. However, we observed differences in alpha diversity as an effect of the intervention. Specifically, we observed that the CPE oral rinse altered the richness of the microbiome in PROP taster groups as measured by observed ASVs, Faith's phylogenetic diversity and evenness. Number of observed ASVs reflects the unique type of ASVs present in a community. Faith's phylogenetic

diversity (FPD) is a qualitative measure of community richness that considers the phylogenetic distances between ASVs, while evenness reflects the abundance distribution of distinct ASVs.

We observed a significantly higher number of ASVs (Fig. 4.2A) in STs (p= 0.027) and a similar, but non-significant trend in NTs (p=0.08, n.s.) at the end of the intervention. In contrast, there was a significant increase in FPD (Fig. 4.2B) in NTs (p= 0.049) but a non-significant trend in STs (p= 0.164, n.s.) after the intervention. We also observed that the intervention altered the evenness differences (Fig. 4.2C). At baseline there was no difference between the evenness of the oral microbiome in NTs and STs (p= 0.436, n.s.). However, STs had higher evenness in comparison to NTs at the end of the intervention (p= 0.035).

Next, we assessed beta diversity of NTs vs. STs using UniFrac distance. This index examines the oral microbiota structure by considering differences in bacterial classification and phylogenetic distances. Unweighted UniFrac distance is a qualitative measure which considers the phylogenetic distances between ASVs but not their abundances. On the other hand, weighted UniFrac distance incorporates relative abundance of ASVs data and the phylogenetic distances between them and is, therefore, a quantitative metric. We did not find a difference in unweighted UniFrac distance between NTs and STs at baseline or post-intervention in either subject group.

Community dissimilarities based on weighted UniFrac distance were then visualized using a PCoA plot (Fig. 4.2D) and violin plots (Fig. 4.2E). The PCoA model

captured 58.64% of the variation in the oral microbiome. PC1, alone, accounted for 39.85% of the variations of the oral microbial community, along which two significant separations between NTs and STs were observed. First, weighted UniFrac distance, confirmed that NTs and STs had distinct oral microbiota at baseline (p=0.0117) but that the groups did not differ at the end of the intervention (p=0.525, n.s.). Oral community composition among STs at baseline compared to STs post-intervention was significantly different (p=0.004, Fig. 4.2E); however, PERMANOVA analysis of the community composition was not (p=0.373). Similarly, PERMANOVA test on weighted UniFrac distance in NTs was also non-significant (p= 0.678).

Effect of the CPE Oral Rinse Intervention within PROP Taster Groups:

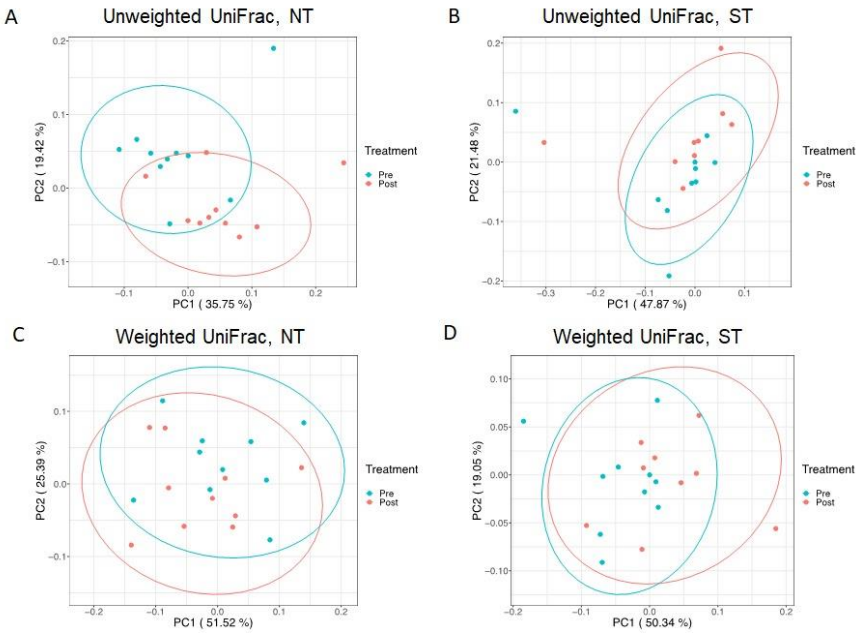

Figure 4.3: The effect of CPE oral rinse on the oral microbiota with inter-individual variation adjustment. Adjusted principal coordinates analysis (aPCoA) plots on (A)

unweighted UniFrac distance in NT subjects, (B) unweighted UniFrac distance in ST subjects (C) weighted UniFrac distance in NT subjects and (D) weighted UniFrac distance in ST subjects. Ellipses represent 95% confidence intervals.

Next, we explored the effect of the intervention on the oral microbiome within each PROP taster group while accounting for individual factors of variation. After adjusting for subject differences using an adjusted Principal Coordinates Analysis (aPCoA), we observed that the intervention significantly modified the oral microbiota composition in NTs (individual stratified PERMANOVA, p=0.023, Fig. 4.3A) but not in STs (individual stratified PERMANOVA, p=0.096, n.s., Fig. 4.3B) based on unweighted UniFrac distance. However, a similar effect was not observed when considering weighted UniFrac distance (individual stratified PERMANOVA test, p=0.416 for NTs and p=0.168 for STs; Figures 4.3C and 4.3D respectively).

These data suggest that PROP taster status is associated with distinct oral microbiota structures and the CPE oral rinse appears to have a greater impact on the oral microbiome of NTs than STs primarily by modifying bacterial membership.

Guild-based Analysis of the Oral Microbiome:

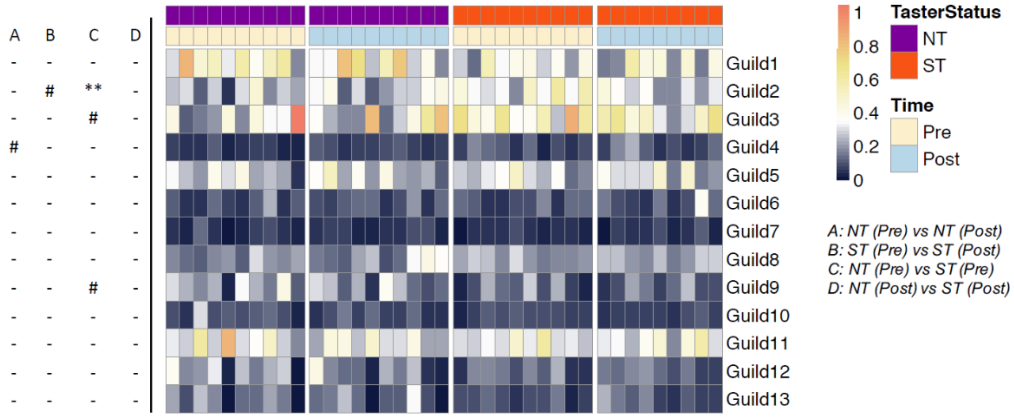

Figure 4.4: Heatmap showing guild-level differences in the oral microbiome pre- and post- intervention in PROP taster groups. The color of the heatmap represents the abundance of each guild in each sample (arcsine square root transformed). The left panel shows comparisons between pre- and post-treatment samples in PROP taster groups (A and B) or between NT and ST subjects at same timepoints (C and D). In NT/ST subjects, Wilcoxon matched-pairs signed-ranks test (two-tailed) was performed to compare pre- and post- treatment samples. Pre-/ Post- intervention, Mann-Whitney test (two-tailed) was performed to compare NT and ST subjects. # $p < 0.1$ and ** $p < 0.01$. Pre-intervention, NT n=10, ST n=10; Post-intervention, NT n=10, ST n=9.

We further characterized the differences in the oral microbiota associated with PROP taster status and CPE oral rinse by examining ecological interactions among the bacteria. Members of an ecosystem seldom exist in isolation; instead they develop local interactions and form inter-member organizations to influence the ecosystem's higher-level patterns and functions (Levin 1998). Such inter-member organization can be considered as guilds in which members exhibit co-abundant behavior (Wu, Zhao et al. 2021). We identified the guild structure in 108 prevalent

ASVs which were present in more than half of all saliva samples and accounted for ~90% of total abundance of the oral microbial community. Based on their co-abundance patterns, these 108 ASVs were clustered into 13 guilds (Figure 4.4).

Oral microbiota structure at the guild level further revealed differences between the PROP taster groups. When compared at baseline, NTs and STs differed in Guild 2 (7 ASVs from *Prevotella*, 2 from *Veillonella*, 1 from *Alloprevotella* and 1 from *Actinomyces*), whereby STs had higher abundance of this guild (p= 0.005). At the same time point, Guilds 3 and 9 were also found to have a non-significant trend. Guild 3 (3 ASVs from *Prevotella*, 2 from *Veillonella*, 1 from *Atopobium* and 1 from *Streptococcus*) trended higher in abundance in STs (p= 0.063, n.s.), while Guild 9 (2 ASVs from *Porphyromonas*, 1 from *Alloprevotella*, 1 from *Actinomyces graevenitzii*, 1 from *Mannheimia*, 1 from *Capnocytophaga* and 1 from *Actinomycetaceae*) trended lower in abundance in STs in comparison to NTs (p= 0.063, n.s.). Neither of these three guilds were found to be different between NTs and STs post-intervention. When comparing guilds post-intervention, the oral microbiome did not differ between taster groups.

When considering the effect of intervention, we observed two trends within PROP taster groups. Guild 2 decreased in abundance in STs (p= 0.098, n.s.), whereas Guild 4 (1 ASV from *Eubacterium infirmum*, 1 from *Ruminococcaceae*, 1 from *Leptotrichia* and 1 from *Lachnoanaerobaculum*) was increased in NTs (p= 0.084, n.s.), after the intervention. Consistent with the beta diversity analysis, NTs and STs harbored different oral bacterial guilds at baseline. While the intervention affected

some guilds, such effects were specific to PROP taster status. Generally, the intervention reduced overall dissimilarities in the oral microbial community between PROP taster groups.

4.4.2. Salivary Proteins

Using Friedman's ANOVAs, we evaluated the effect of treatment on salivary proteins. Only four of the nineteen proteins tested showed a significant difference at the end of the intervention: α-amylase and MUC-5B levels fell after the intervention (Figure 4.5) while, Cyst SN and Cyst SA levels rose at the end of the intervention (Figure 4.6).

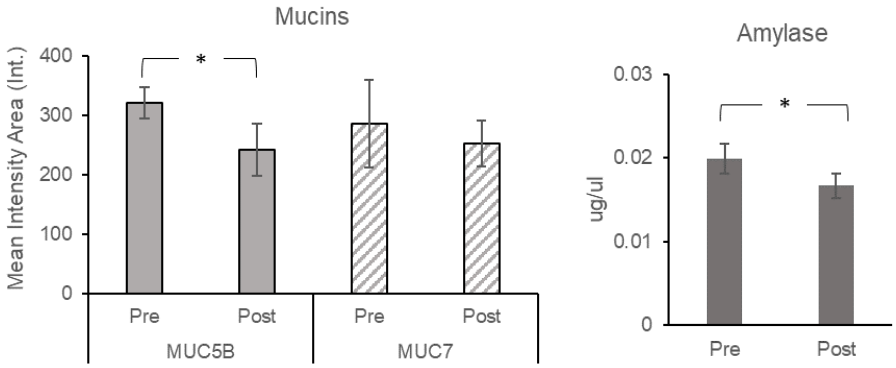

Figure 4.5: Immunoblot analysis of (a) MUC-5B and MUC-7 (Mean Intensity of chemiluminescent signal, Int. ± SEM) and (b) alpha amylase (μg/μL ± SEM) before and after the intervention (n=20). * shows significant effect of treatment ($p<0.05$).

For α-amylase mean levels were 0.02 μg/μL at baseline vs. 0.01 ug/ul after the intervention ($Q=3.841$, $p=0.025$) (Figure 4.5, right). Among mucins, MUC5B ($Q=3.841$, $p=0.025$) was higher at baseline than after the intervention (Figure 4.5, left). There was no effect of treatment on MUC7 levels.

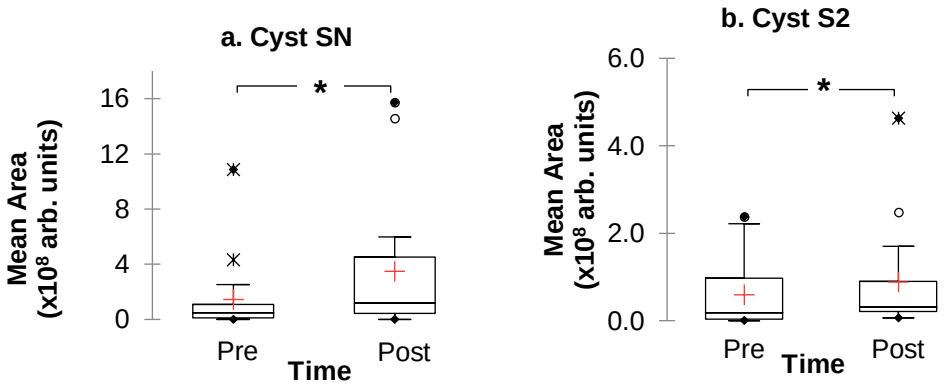

Figure 4.6: Levels of S-type Cystatins, (a) Cyst SN and (b) Cyst S2 before and after the intervention (n=16). The box-and-whisker plots show the minimum, first quartile, median, third quartile, and maximum of each set of data. * shows significant effect of treatment ($p<0.05$).

Among proteins measured via chromatographic analysis, only two selected S-type Cystatins had a significant effect of treatment (Figure 46). Specifically, Cyst SN ($p=0.02$) and Cyst S2 ($p=0.046$) had higher mean levels after the intervention.

There was no significant effect of taster status on differences in salivary proteins.

4.4.3. Sensory Ratings

Sensory ratings of CJC and CJC supplemented with CPE were analyzed at baseline and at the end of the study to examine if the oral rinse intervention altered sensory perceptions of the subjects. There were no significant differences between taste intensity ratings at the beginning or end of the intervention. There were also no differences observed with respect to PROP taster status.

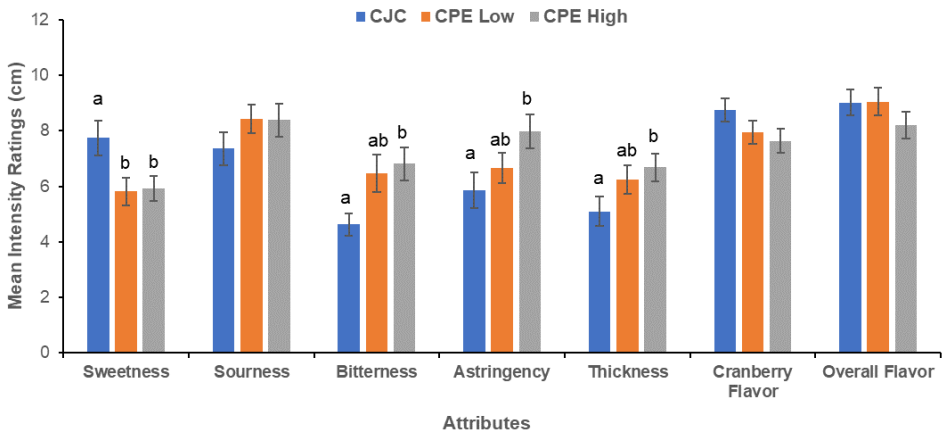

Figure 4.7: Evaluation of Taste Samples. Mean Intensity Ratings ± SEM (cm) for key flavor attributes present in cranberry juice cocktail with 0 (CJC), 0.5 g/L (CPE Low) and 0.8 g/L (CPE High) of added cranberry polyphenol extract (n=20). Different letters (a,b) indicate significant as a function of concentration at $p<0.05$, with post-hoc tests.

However, there was an overall significant effect of CPE concentration for four attributes showing that the subjects were able to distinguish the samples with added CPE. Specifically, there was a significant difference in the following attributes: sweetness, bitterness, astringency, and thickness (Figure 4.7). CJC was given a higher sweetness rating in comparison to the samples supplemented with CPE ($F_{[2,36]} = 3.97$, $p=0.028$). In contrast, CPE-supplemented samples were perceived as more bitter ($F_{[1.43,36]} = 5.18$, $p=0.021$), astringent ($F_{[18,36]} = 4.40$, $p=0.019$) and thicker in comparison to CJC ($F_{[2, 36]} = 3.67$, $p=0.035$).

4.4.4. Relationship between Oral Microbiome, Salivary Proteins and Sensory Ratings

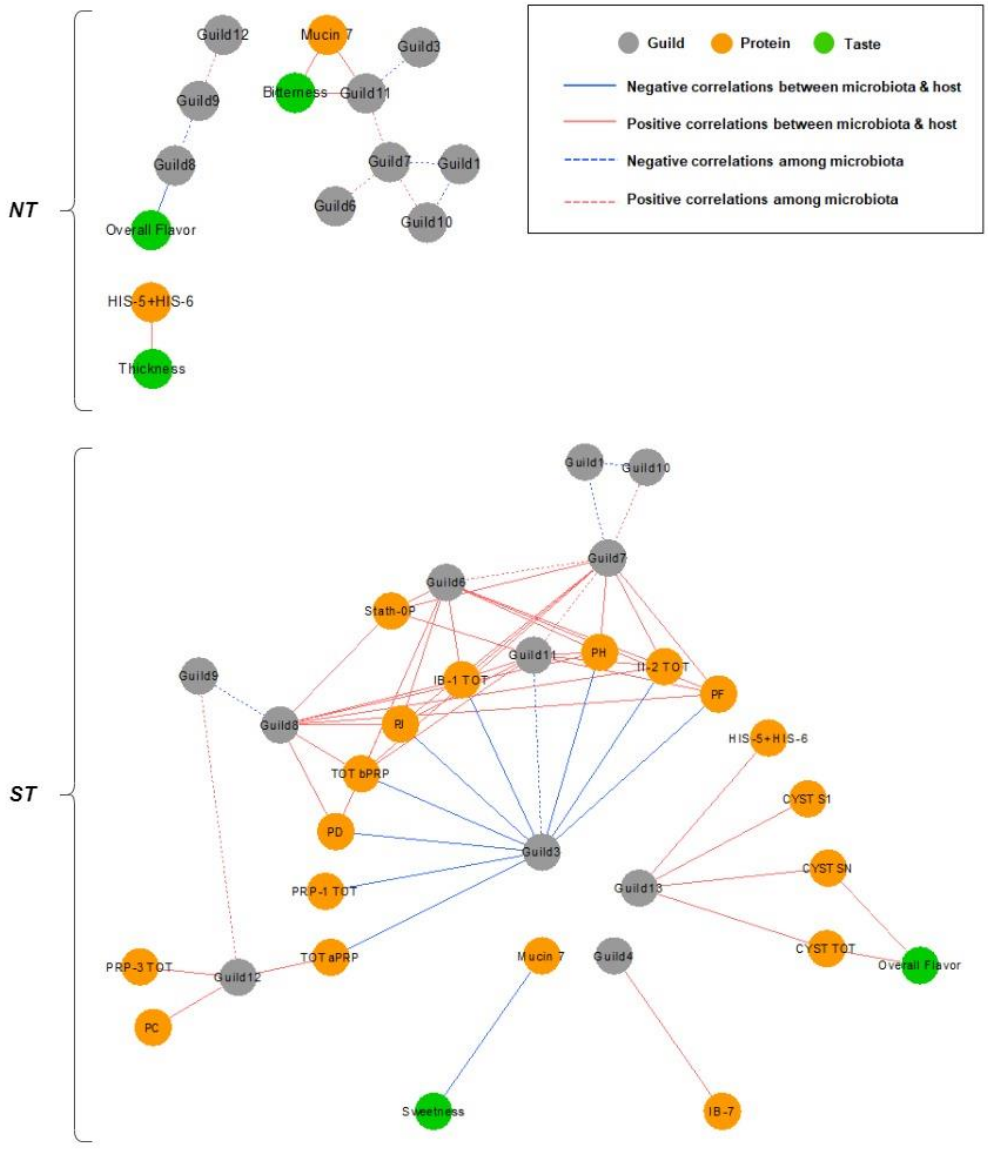

Figure 4.8: Microbial guilds, salivary proteins and taste measures correlation networks. The associations between taste, salivary proteins and microbial guilds were more complex in PROP STs than those in NTs. In the correlation networks, solid edges represent the correlations between taste and guilds, between salivary proteins and guilds, and between taste and salivary proteins. Dashed edges represent the correlations between guilds. The color of the edges represents the

positive (red) and negative (blue) correlations. All correlations with adjusted $p < 0.25$ were shown in the networks.

We constructed correlation networks, one for each PROP taster group, to examine relationships between oral microbiome guilds, salivary proteins, and sensory ratings (Figure 4.8). Overall, the network for STs was more complex than that for NTs, with more nodes (21 vs. 14 in NTs) and edges (58 vs. 13 in STs).

Microbial Guild- Salivary Protein Associations:

The ST network showed extensive interactions between oral bacterial guilds and salivary proteins. In total, 38 positive and 9 negative correlations among 11 guilds and 17 salivary proteins were observed. All 9 negative correlations were found between Guild 3 and specific proteins from the aPRP and bPRP families. In contrast, Guilds 4, 6, 7 and 8 had several positive correlations with the bPRPs while Guild 12 was positively correlated with aPRPs. In addition, several positive correlations between Guild 13 and selected S-type Cystatins and Histatins were found.

In contrast, there was only one such association in the NT network; Guild 11 was positively correlated with MUC-7. This specific association was not observed in the ST network despite both nodes being present in the network.

Microbial Guild- Sensory Attribute Associations:

The oral microbiome also showed interactions with sensory ratings for CJC with the highest CPE concentration, which were only observed in the NT network.

Specifically, Guild 11 abundance was positively correlated to bitterness ratings, while Guild 8 was negatively associated with Overall Flavor.

In the ST network, none of the sensory attributes were correlated with oral bacterial guild abundance.

Salivary Protein- Sensory Attribute Associations:

In the NT network, two attributes were observed to be correlated with salivary proteins. Bitterness was positively associated with MUC-7 while, Thickness was negatively associated with Histatin 5-6.

In the ST network, Sweetness was negatively correlated with MUC-7, while Overall Flavor was positively associated with Cyst SN and total S-type Cystatins.

These data show that PROP taster status was associated with distinct interactions between the oral microbiome and salivary proteins, with more complex bacterial guild to salivary protein interactions observed in the STs.

4.5. Discussion

The role of genetics in taste perception, salivary proteins and the oral microbiome is well known in separate areas of research, but in the present study, we studied them together for the first time. The first objective of this study was to determine if there is variation in the oral microbiome with respect to the PROP taster phenotype. We observed differences in alpha and beta diversity. When evaluating alpha diversity, NTs and STs did not differ in evenness at baseline but STs had a higher evenness at post-intervention than NT subjects. Beta diversity metrics

based on weighted UniFrac distance showed that PROP NTs and STs significantly differed in the oral microbiome composition at baseline. We then clustered co-abundant ASVs into guilds based on the principle that a group of ASVs that show consistent co-abundant behavior are likely to contribute to the same ecological function and thus are clustered into one bacterial guild (Wu, Zhao et al. 2021). Our guild-level analysis supported our observation that NTs and STs have different oral microbiome at baseline. Specifically, relative abundance of Guild 2, with membership from *Prevotella, Veillonella, Allprevotella* and *Actinomyces* genera, was significantly higher in STs than in NTs. Over the last decade, numerous studies have shown that NTs (both adults and children) experience more dental caries and gingival disease than STs (Wendell 2010, Pidamale 2012, Pidamale 2012). Subsequent in vitro work suggested that in the presence of oral pathogens, primary gingival epithelial cells derived from the PAV/PAV genotype could mount a stronger immune response, including induction of antimicrobial peptides, than cells from other TAS2R38 genotypes (Gil 2015). This suggests that the oral environment of STs may be better at resisting dysbiosis via regulation of innate oral immunity, while that of NTs may be more susceptible to oral disease. In the present study, we confirmed that the oral microbiomes of NTs and STs were different at baseline. Further metagenomic analysis is needed to determine the functional significance of the differences observed. Nevertheless, clustering bacteria in guilds based on exact ASVs as we did here could provide an early insight into ASVs most likely to be of functional significance to oral health. This awaits further investigation.

The second objective of this study was to determine if exposure to CPE oral rinse would alter the oral microbiome in NTs and STs. Using alpha diversity metrics, we observed some evidence that the CPE oral rinse increased the richness of the oral microbial community in NTs. Our adjusted PCoA model, based on unweighted UniFrac distance, confirmed that changes in microbiome composition were only observed in NTs. In effect, the oral microbiota of PROP NTs became more similar to that of STs by the end of the trial.

Studies from the oral health literature show that cranberry constituents effectively inhibit bacterial adhesion and co-aggregation in dental plaque and can also reduce abundance of oral pathogens (Weiss 1998, Khairnar MR and 6:35-9 2015). Specifically, cranberry constituents have been shown to lower counts of the cariogenic pathogen, *Streptococcus mutans*, in a human intervention (Weiss 2004). Thus, our observations showing that CPE shifted the composition of the oral microbiome are in line with expectations that cranberry polyphenols would affect the microbiome in some way. Interestingly, we found that the intervention altered the microbial membership in NTs but not in STs. The reasons for these differences are not fully understood. Nevertheless, we speculate that the NT microbiome can be characterized as having greater abundance of bacteria particularly responsive to the effect of cranberry polyphenols (i.e., less stable to environmental change), while the ST microbiome may be more stable to this environmental change, conferring beneficial oral health status to STs. The extent to which the stability or resilience of the oral microbiome equates with good oral health status is currently unknown (Rosier 2018, Wade 2021). Thus, it is essential to measure markers of host

immunity in the future to relate differences in oral microbiota features to oral health outcomes.

To our knowledge, only two previous studies have investigated the oral microbiome with respect to TAS2R38. Sandell and Collado (Sandell 2018) examined the oral microbiome of subjects classified by TAS2R38 genotypes who resided in Finland or Spain. The purpose of this analysis was to compare the oral microbiome of genotypic groups across the two geographic locations as a proxy for differences in diet and lifestyle. They observed that AVI/AVI and PAV/PAV genotypes (phenotypic PROP NTs and STs, respectively) differed in the oral microbiome at various taxonomic levels across these two geographic locations. In the second study, Cattaneo and colleagues (Cattaneo 2019) explored variation in the oral microbiome in subjects classified by PROP phenotype (in a single geographic location) and related it to taste sensitivity. Contrary to findings of the present study, Cattaneo et al. (2019) did not observe differences in alpha or beta diversity of the oral microbiome with respect to PROP taster status but found various ASV-based genus-level differences.

While both studies (Sandell 2018, Cattaneo 2019) found differences in the oral microbiome with respect to TAS2R38 genotype and phenotype, our work differs from these prior works in key aspects. First, there are fundamental differences in how microbiome samples were collected and analyzed in these three oral microbiome studies. The first difference is in the collection sites of the microbiome samples. Sandell and colleagues (Sandell 2018) used buccal swabs

while Cattaneo and colleagues (Cattaneo 2019) used swabs from the tongue dorsum. In contrast, we collected whole-mouth saliva. It is well-known that the microbial composition can vary drastically at different sites in the oral cavity e.g., microbial composition on the tongue surface can be different than that in saliva (Feng 2018).

A second major difference lies in the depth of the analysis. Here, we adopted a reference-free analysis of the oral microbiome by using ASVs as a functional unit of the microbiome instead of operational taxonomic units or reporting genus-level differences. By navigating away from a taxa-based approach, we examined microbiome dissimilarities at a finer resolution by leveraging the dada2 algorithm to differentiate bacterial genomes. Additionally, we showcase a guild-based analysis, which is based on the ecological framework that bacteria in the oral cavity do not exist in isolation from one another; instead, they may exist in functional communities, which utilize resources in a similar manner and thus proliferate together. Any change in the environment would then affect the entire guild in a similar fashion. Previous work reporting genus-level differences often lumped bacteria together based on taxonomic, but not functional, similarity. As a result, important differences may have been missed. Indeed, our guild-based approach demonstrates that even members belonging to the same genus may form co-abundant guilds with other bacteria and exhibit different distributions in NTs and STs. For instance, we observed that ASVs belonging to the same genus (e.g., *Alloprevotella)* were found in both Guilds 2 and 9. However, specific *Alloprevotella* ASVs were more abundant in STs in Guild 2, while other ASVs from the same genus

were less abundant in STs in Guild 9. Our study adopted an approach that overcomes various pitfalls of conventional microbiome analysis. We effectively reduced the dimensionality of a large microbiome dataset and also identified the exact ASVs in each guild. Going forward, this strategy will allow for direct comparison between these findings and future work emphasizing the functional ecology of oral microbial communities.

A final objective of the study was to examine potential changes in salivary proteins and taste perception after the intervention. We observed that amylase, MUC-5B and two S-type Cystatins (Cyst SN and Cyst S2) were altered after CPE oral rinse treatment. Amylase and MUC-5B were lower at the end of the intervention, while the Cystatins increased. Polyphenol ingestion has generally been linked with up-regulation of several salivary proteins (Torregrossa 2014, Lamy 2020) in animal models and most recently this has also been demonstrated in humans (Crawford 2020). Thus, the rise in the two Cystatin levels in our study seems to be consistent with previous findings in the literature. The decreases we observed in levels of alpha-amylase and MUC-5B were unexpected; the reasons for these outcomes are presently unknown. It is important to note that all previous studies examined daily polyphenol consumption whereas, our work tested daily oral exposure to polyphenols, not ingestion. Repeated ingestion of polyphenols may have different physiological effects on some salivary protein than oral exposure without swallowing. These differences should be examined in future studies.

In terms of taste perception, we found that CJC samples supplemented with CPE were different in sweetness, bitterness, thickness, and astringency compared to un-supplemented CJC. However, we observed no differences between PROP taster groups and no changes in the sensory ratings for any of the CJC samples after the intervention. The latter finding agrees with that of Crawford et al (2020) who reported only minimal changes in sensory perception after a polyphenol consumption intervention, despite seeing alteration in salivary proteins. It is possible that short-term, oral exposure to polyphenols as used in the present study may not be sufficient to alter taste perception. Based on current evidence, it is uncertain whether polyphenols alter taste perception in humans. This question warrants further attention.

Mechanisms regulating oral health are complex and interlocking. One line of evidence underscores the critical role that taste receptors play in oral health. For example, the TAS2R38 bitter taste receptor serves a protective function by detecting quorum-sensing molecules produced by bacteria and can also regulate oral immunity (Lee 2012, Gil 2015). Having a more functional bitter taste receptor (such as that expressed in PAV/PAV individuals and thus PROP ST individuals), may provide an advantage for oral health, as has been recently shown in animal models with other TAS2R receptors (Zheng 2019). Another line of evidence reveals that PRPs and Cystatins are involved in oral health and are reported to modulate bacterial attachment to oral mucosa and affect bacterial binding and aggregation (Marsh and Zaura 2017) (Levine 2011). We have previously shown that PROP STs have higher salivary levels of specific PRPs after stimulation with astringent stimuli

including cranberry-derived polyphenols (Melis 2017, Yousaf 2020). The exploratory association networks allowed us to combine this disparate information and visualize the interrelationships between the microbial guilds and the salivary proteins in NTs and STs. The networks reveal that interactions between oral microbial guilds and salivary proteins, especially for proline-rich proteins, appear far more complex in the ST oral environment than that in the NT oral environment. These provocative findings are preliminary and need to be confirmed in a larger more comprehensive study.

The present study has several limitations. First, this was a short intervention with a small sample size; a longer intervention with a larger sample size may have the potential to capture more profound changes. Secondly, we relied on self-reported information to screen subjects for good oral health. Future work should incorporate the DMFT (decayed, missing, filled teeth) index or similar metrics for an objective quantification of the oral health status. Third, subjects were instructed to maintain their habitual diets. We did not attempt to control the subjects' individual diets during the study or assess their dietary behaviors. Fourth, numerous other variables influence taste perception and the oral microbiome, such as salivary flow, pH and other biomarkers that were not measured in this pilot study. Accounting for these variables in future work, paired with the use of next generation sequencing would be essential for advancing our understanding of the functional significance of oral microbial communities and its relationship to oral health.

4.6. Conclusion

The present study, to our knowledge, is the first investigation into the interconnectedness of taste, salivary proteins, and the oral microbiome in PROP classified individuals. By adopting the latest tools in bioinformatics, we have demonstrated a way to study these relationships in concert. This presents a path forward for taste researchers interested in studying the highly intertwined nature of taste genetics, dietary behavior, and oral health. in addition to providing important insights for developing improved oral health interventions.

4.7. Acknowledgments

Conceptualization, N.Y.Y., B.J.T.; methodology, formal analysis, investigation, N.Y.Y., Me.M., Ma.M, C.C.; data curation, N.Y.Y., G.W., Me.M. and Ma.M.; writing—original draft preparation, N.Y.Y, G. W., Me.M., C.C., Y.Y.L., B.J.T.; writing—review and editing, N.Y.Y, G.W., Y.Y.L., Me.M., T.C., I.T.B., L.Z., B.J.T.; visualization, N.Y.Y, G.W.; supervision, project administration, Y.Y.L, T.C., I.T.B., L.Z., B.J.T.; funding acquisition, N.Y.Y., I.T.B., B.J.T.

5. CONCLUSION

The objectives of this research were to determine the role of PROP taster status as a marker for astringency perception and the salivary protein response that may be contributing to development of astringency.

In the first study, the focus was to evaluate if PROP taster status as well as other individual factors of variation play a role in development of astringency from cranberry-derived beverages. We chose cranberry polyphenols as a model stimulus instead of the traditional prototypical astringents such as tannic acid or alum to present a more ecologically relevant food model to consumers. Using traditional hypothesis testing and machine learning approaches, we determined that PROP classified groups did not perceive astringency differently from these samples. The findings are important for three major reasons. First, this highlights the need for use of real-world foods when studying sensory perception so that findings inform real-world behavior. As our data show, results may differ significantly when we move away from lab stimuli (such as tannic acid, alum, or grape seed tannin) to complex foods (such as fruit juices). Secondly, the three data analysis approaches (ANCOVA, regression trees and random forest modeling) together suggest a greater role for ethnicity in astringency development than previously considered. Whether ethnicity is influencing astringency due to specific physiological differences was beyond the scope of the current investigation. However, it is likely that ethnicity is acting as a proxy for differences in exposure, familiarity, and selection of diet. This shows that other individual factors, such as ethnicity or gender, may have a larger influence than PROP taster status in the perception of astringency from cranberry juice. Finally, the use of machine learning tools unmasked latent effects in an easy to

visualize framework, which demonstrated that each sensory attribute is influenced by different combination of factors. Thus, use of machine learning tools has the potential to advance the field of sensory science by making such analysis easier when many individual factors may be involved.

In the second study, we studied the time course of salivary proteins released after stimulation with cranberry-derived stimuli to understand if there would be any variation in salivary protein response with respect to PROP taster status. We observed robust effects of PROP taster status on how long the effects of polyphenol exposure linger, with STs having higher levels of key salivary proteins after exposure to cranberry-derived stimuli, when compared to NTs. We also observed a combined effect of PROP taster status and gender, whereby male STs had higher levels of key salivary proteins in comparison to other subgroups. This pattern was not observed among the female subjects. These findings support our previous observations (Melis 2017) that the oral environment in STs responds differently to astringent stimuli than it does in NTs and that this response is stimulus-dependent. In addition, this study demonstrated that gender is also an important factor to consider when studying astringency and the associated salivary protein response.

The final study explored the interaction between taste perception, salivary proteins, and the oral microbiome. We found that PROP NTs and STs had different oral microbiome at baseline but that exposure to cranberry polyphenols altered the microbiome in NTs. These novel findings are significant for several reasons. First, the study demonstrated that the oral microbiome is different in the two PROP

phenotypes. We know from the time-course experiment that the salivary protein response is different in STs than it is in NTs in response to environmental stimuli. Since, salivary proteins play a key role in maintaining a healthy oral environment, variation in their levels could be associated with variation in the oral microbiome. By using correlation networks, we demonstrated that the oral environment of STs may have more complex relationships between salivary proteins and the oral microbiome than in NTs. This may be the basis of why STs may be better protected against oral disease, as other studies report (Pidamale 2012, Gil 2015). This study suggests that PROP taster status has role to play in the variation in the oral microbiome.

Overall, this work proposes that individual factors of variation such as PROP taster status, gender and ethnicity may have a more significant impact on taste perception, salivary proteins and the oral health than previously considered. Observations from this dissertation suggest that characterizing the study population and accounting for individual factors of variation is essential to understanding astringency perception and relating the findings to dietary behavior and oral health outcomes.

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