A comparison of capsaicin and menthol as trigeminal modulators of salivary composition for use in oral care applications

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Contents

Abstract

The human oral cavity contains 700 types of bacteria. Gram-negative anaerobe bacteria are associated biofilm formation and oral malodour due to their abundance and metabolism producing volatile sulfur compounds. Human saliva contains Mucin, Proline-Rich Proteins (PRPs), Alpha-Amylase (α-Amylase), Cystatins, Histatins, and Statherin, which contribute to lubricate mouth, protect mucosal integrity and against microorganisms, and digest food. Due to their ability to stimulate the saliva and also have antibacterial activities. In this review, we investigate how menthol and capsaicin affect the salivary flow, oral protein composition, and also their effect on these oral bacteria. Following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, we conducted a literature search using Google Scholar from 1980 to 2023 in English form. The search query was based on the question "How do trigeminal modulators including capsaicin and menthol affect salivary flow, salivary protein composition, and oral microbiomes?" A total of twenty-seven articles were selected for analysis. The results showed that capsaicin still increased the saliva flow at 1 ppm, 5 ppm, 0.005M, $3x10^{-5}$M, and 0.3nM concentrations after the first minute of stimulation and then decreased after two minutes. In contrast, menthol did not change the saliva flow at 0.05M and 250 ppm doses and slightly changed the flow at 500 ppm only during the first minute. Regarding protein composition and its changes, MUC5B (above 188kDa), MUC7 (~150kDa), α-Amylase (50-60kDa), PRPs (40-50kDa), Cystatin (~14kDa), Statherin (~6kDa) were identified on 12% SDS-PAGE. Menthol did not increase protein content at 250 ppm and 0.05M whereas 500 ppm menthol increased slightly protein content and significantly increased cystatin S ($P < 0.05$). Capsaicin slightly increased protein content and significantly increased protein compositions, except MUC5B at 1 ppm and 0.05M. Regarding antibacterial activity measured by MBC and MIC methods, menthol decreased the growth of *Bacteroides, P. gingivalis,* and *F. nucleatum,* while capsaicin also decreased the abundance of *Bacteroides, P. gingivalis and* increased *Bacteroides*. In conclusion, based on their characteristics, menthol and capsaicin at some concentrations are potential ingredients for oral care applications.

1. Introduction

1.1. Human Saliva and Oral Health

Maintaining oral health is very important and necessary because we can prevent the occurrence of unpleasant breath, tooth decay, and gum diseases, which can contribute to maintaining healthy teeth as we get older. It has been scientifically proven that saliva, a naturally slimy fluid that is produced 90% from parotid, submandibular, and sublingual glands (Kaufman & Lamster 2002), plays an extremely crucial and indispensable role in keeping oral health. Menthol (extracted from peppermints) and capsaicin (extracted from chili peppers) are expected to give a positive effect on saliva flow and saliva composition besides their antibacterial properties that make them become potential ingredients for oral care products.

The biological functions of human saliva are diverse and essential for keeping oral health. These functions include lubricating, buffering, protecting against microorganisms, protection of mucosal integrity, and digestion of food (Huang, 2014). The sympathetic nervous system controls the production of saliva through the parasympathetic (or cholinergic) system and a and b fibres that connect receptor stimulation to ion transport and protein secretion mechanisms (Dodds et al., 2005; Arany et al., 2021). The number of different components present in saliva can differ from person to person depending on the individual's oral and overall health status (Saibaba et al., 2021). According to Mese H. and Matsuo (2007) study, a healthy person generally produces about 0.5 to 2 litres of saliva daily. The amount of saliva secreted tends to decrease during sleep but significantly increases when a person is talking or eating. The whole saliva is composed of water (99.5%), proteins (including enzymes and making up 0.3%), hormones, sugars, lipids, electrolytes (Na^+, Cl^-, HCO_3^-), and several other components (Liu & Duan, 2012). Thanks to recent advancements in proteomic technology, Si et al., (2015); Sun et al., (2016); and Wang et al., (2018) found that the salivary proteome contains a significant number of proteins, with up to 1166 proteins identified in total. Proteins in whole saliva are divided into a few families including Mucin, Proline-Rich Proteins (PRPs), Alpha-Amylase (α-Amylase), Cystatins, Histatins (small cationic histidine-rich peptides), and Statherin (Gonzalez-Begne et al., 2009; Amado, 2010; Zhang, 2013; Cabras et al., 2014), and the summary of their molecular weight, concentration, and functions in oral health are shown in Table 1.

Table 1: Characteristics of human salivary proteins

Human salivary proteins	Molecular weight (kDa)	Concentration (mg/mL)	Function(s) in oral care	References
Mucin	130 – 20000	0.88 (±0.11)	Form a protective layer over the oral cavity (lubrication) and gingiva to protect against invading pathogens and abrasions as well as help with speech, mastication, and the process of swallowing. Cause bacterial agglutination which facilitates their clearance from the oral cavity.	Tabak 1995; Aplin & Hey, 1995; Pedersen et al., 2002; Liu et al., 2002, Aas et al., 2005; Oppenheim et al., 2007
α-Amylase	62 (glycosylated form) 56 (non-glycosylated form)	1.91 (±0.05) (accounting for 40-50% of the salivary proteins)	Bind to bacteria, in plaque and prevent bacterial colonization. Cleave randomly at the alpha-1,4-glucosidic linkages of starch, glycogen, dextrin, and other complex sugars, which may provide glucose for plaque microorganisms used for metabolism.	Bank et al., 1991; Scannapieco et al., 1993; Ramasubbu et al., 1996; MacGregor et al., 2001; Mandel et al., 2010; Singh et al., 2015; Dinu et al., 2018.
PRPs	6 – 36	-	Bind to Ca^{2+} are involved in the acquired enamel pellicle formation. Function to ensure oral lubrication and bind to some types of oral microorganisms to regulate oral microbiomes.	Bennick 1982 & 1987; Hatton et al, 1985; Gillece-Castro et al., 1991; Amano et al., 1994 Schenkels et al., 1995; Ruhl 2004; Vitorino et al., 2007
Cystatins (family-2)	13 – 14	3.7×10^{-4} ($\pm 1.29 \times 10^{-4}$) for cystatin S	Possess potent antibacterial and antiviral activities. The phosphorylated forms of family-2 cystatins bind to hydroxyapatite (HA) and inhibit hydroxyapatite crystal growth which is important for enamel remineralisation; therefore, they may play a vital role in the formation of the acquired enamel pellicle.	Johnsson et al, 1991; Lamkin et al., 1991; Dickinson 2002; Koopaie et al., 2021
Statherin	5.380	9.6×10^{-4} (mean)	Along with PRPs, attach to calcium phosphate and calcium carbonate salts and prevent them from spontaneous precipitation, which promotes enamel remineralization. Act as a lubricant to create a barrier on the enamel surface.	Hay et al., 1986; Raj et al., 1992; Schwartz et al., 1992 Gururaja & Levine 1996; Humphrey & Williamson 2001; Pateel et al., 2017

Histatins	3 – 5	0.05 – 0.425	Possess antimicrobial and antifungal effects. Involve creating the acquired pellicle and contribute to the mineralisation process of oral fluids. Inhibit the release of histamine from mast cells, which manages oral inflammation.	Oppenheim et al., 1988; Oppenheim, 1989; Sabatini & Azen 1989; Troxler et al., 1990; Sugiyama et al., 1990; Van et al., 1997

More than 700 types of bacteria have been isolated from our oral cavity, which belongs to six phyla including *Firmicutes, Fusobacteria, Actinobacteria, Bacteroidetes, Proteobacteria,* and *TM7* (Aas et al., 2005; Jornet al., 2005; Cheng et al., 2009). These bacteria distribute in either specific or all oral sites, however, *Streptococcus mitis* is the most abundant species in all sites and subjects, and some species from *Granulicatella* (e.g. *Granulicatella adiacens*), *Streptococcus, Gemella*, and *Veillonella* are also commonly found in most sites (Aas et al., 2005). Although most bacteria in the oral cavity are harmless to the mucosal surfaces and teeth, normally 100 - 200 different bacteria in the healthy mouth of any person (Paster et al., 2006), a shift in the oral microbiome composition can result in oral malodour as well as in diseases specific to the oral cavity like gingivitis, dental caries, and oral thrush (Scannapieco, 1999; Gao et al., 2018). Most bacteria in oral flora can aggregate to form a slime layer (polysaccharide layer), which adheres and builds up to teeth and gum lines through receptors such as Statherin, bacterial cell fragments, sialylated Mucins, α-Amylase, PRPs, over time resulting in a thick layer called biofilm (also called bacterial plaque). The diagram of the representation of biofilm development and the order of coadhesion and coaggregation of bacteria on the tooth surface are shown in Figure 1. The acids generated by bacterial plaque can cause the enamel surface to demineralize and lose calcium normally at a pH level between 5 and 6 (Margolis et al., 1992), increasing the risk for cavities and enamel erosion and sensitive teeth. Oral malodour, also known as halitosis, is a condition characterized by unpleasant or foul-smelling breath. Oral malodour is mainly caused by volatile sulphur compounds (VSCs) such as hydrogen sulphide (H_2S) and methyl mercaptan (CH_3SH) which are the products from the metabolism of oral gram-negative anaerobes such as *Bacteroides spp.* (e.g. *B. gracilis, B. intermedius, B. loescheii, B. oralis*), *Prevotella intermedia, Porphyromonas gingivalis, Treponema*

denticola) Tannerella forsythia, Fusobacterium nucleatum, and *Porphyromonas endodontalis* (Persson et al., 1990; Rosenberg et al., 1991; Loesche & Kazor, 2000).

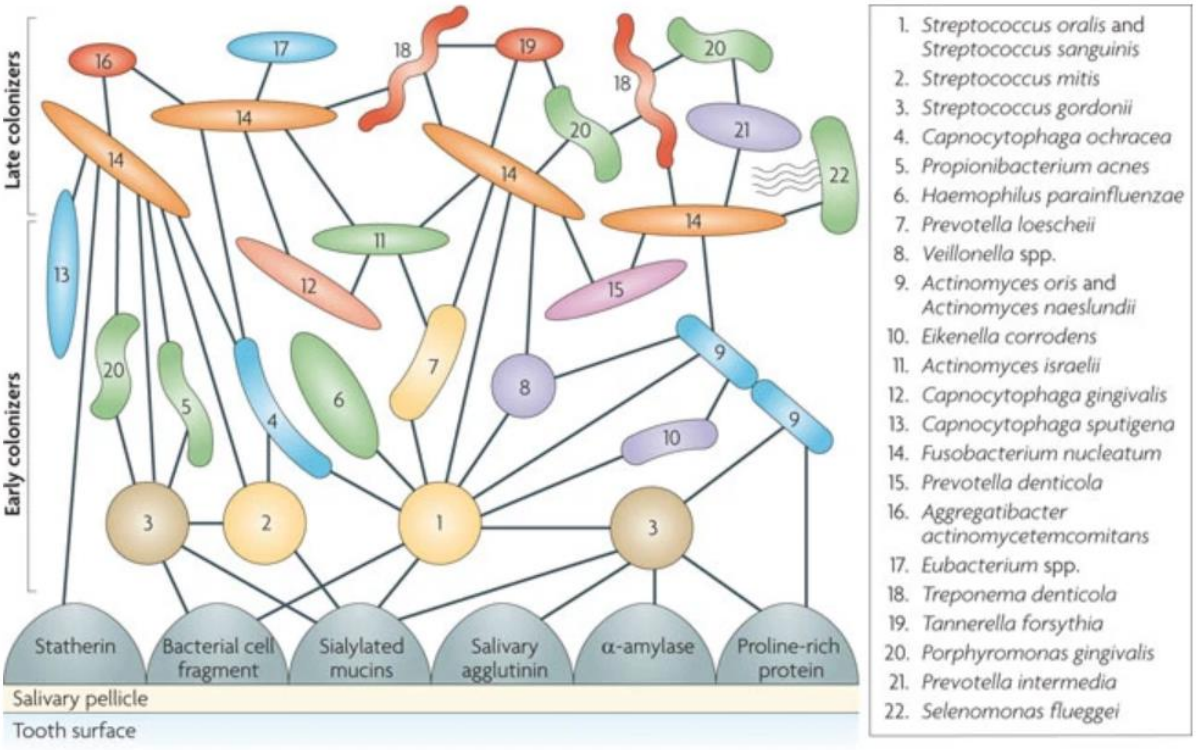


Figure 1: The diagram of the representation of biofilm development and the order of coadhesion and coaggregation of bacteria on the tooth surface (Kolenbrander et al., 2010). From bottom to top, acquired pellicles that cover the toothsurface will provide protects tooth enamel and also allows bacterial adhesion through complementary salivary receptors including Statherin, bacterial cell fragments, sialylated Mucins, α-Amylase, PRPs. Initial colonizers - commensal *Streptococcus* species (*Streptococcus gordonii, S. mitis, Streptococcus oralis, Streptococcus sanguinis*) and other early colonizers attach to the pellicle. The single sugars from the cleavage of alpha-Amylase provide food for *Streptococcus* to produce extracellular polymeric substances which allow the adhesion of late colonizers such as *F. nucleatum, P. gingivalis, P. intermedia, T. denticola, T. forsythia,* and *Aggregatibacter actinomycetemcomitans*. As a result of bacterial coaggregation, binding of bacteria in suspension (e.g. saliva), and coadhesion, adherence of microbial cells to immobilized bacteria, slime layers become biofilms presenting arround the teeth surface.

1.2. Trigeminal Modulators: Menthol and Capsaicin

The perception of hot and cold temperatures as well as pain are mediated by the transient receptor potential (TRP) family of nonselective cation channels (McKemy et al., 2007; Szallasi et al., 2007). One member of this family, the transient receptor potential vanilloid receptor 1 (TRPV1), can be activated by heat (at 43°C), low pH, and capsaicin (Caterina et al., 1997; Clapham 2003); whereas, the transient receptor potential melastatin 8 receptor (TRPM8) and the transient receptor potential subfamily A1 receptor (TRPA1), other members of TRP, are responsible for detecting cool temperatures (below 23°C) and can be activated by menthol (McKemy et al., 2002; Peier et al., 2002; Farco & Grundmann, 2013). TRPV1 and TRPM8/TRPA1 have been identified in the salivary gland and contribute to salivary flow (Liu et al., 2018).

1.2.1. Menthol

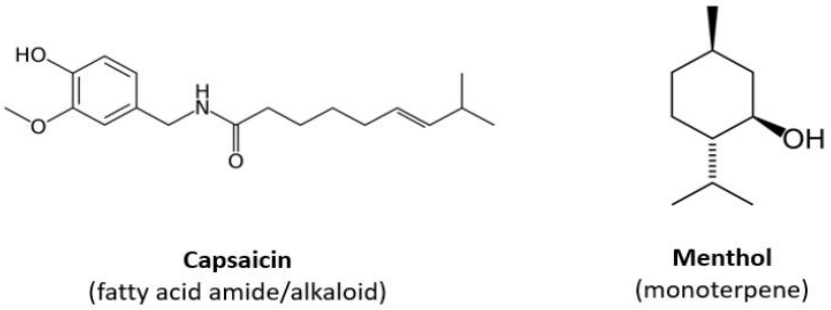
Menthol, also known as (1R,2S,5R)-2-isopropyl-5-methylcyclohexanol, is a cooling and soothing compound found in various plants such as peppermint and spearmint. Menthol in the form of crystal is poorly soluble in water (~0.46 mg/mL) but highly soluble in organic solvents such as 100% ethanol or methanol (~100 mg/mL). Due to its cooling properties, menthol is a widely used flavoring agent in foods and drinks, as well as a common ingredient in oral care products including toothpaste and mouthwash under essential oil form. It has a chemical backbone of monocyclic terpene (Figure 2) which can activate the peripheral cold receptors, which are present in the skin and mucous membranes, through its interaction with TRPM8 and TRPA1 (Schafer et al., 1986; McKemy et al., 2002; Peier et al. 2002). Menthol has been shown antibacterial properties when it can be active against a variety of microorganisms, including Gram-positive and Gram-negative bacteria (Schelz et al., 2006; Patel et al., 2007). A recent study has proven menthol inhibits the growth of *Streptococcus mutans, Streptococcus sobrinus, Streptococcus salivarius, Lactobacillus casei*, and several *Candida spp* (Mahzoon et al., 2022). In recent years, there has been growing interest in the potential role of menthol in promoting salivary flow (Pushpass et al., 2019; Gardner & Carpenter, 2019; Houghton et al., 2020). Studies have shown that menthol can increase the salivary flow rate and improve oral health by increasing the buffering capacity of saliva and reducing the growth of oral bacteria. The exact mechanisms underlying these effects are not yet

fully understood but are thought to involve the activation of TRPM8 and/or TRPA1 channels in the salivary glands. Besides its cooling effect, high concentrations of menthol can cause an irritating sensation. Normally, 0.01 - 0.1% of menthol is used for oral applications and the lethal dose (LD) ranges from 50–150 mg/kg according to Gosselin and his colleagues (1984). Therefore, it is important to understand the concentration and impact of menthol on the oral cavity.

1.2.2. Capsaicin

Capsaicin, also known as trans-8-methyl-N-vanillyl-6-nonenamide, is the primary pungent component of chili peppers. Capsaicin in the form of powder is poorly soluble in water (approximately 0.013 mg/mL) but highly soluble in organic solvents such as 100% ethanol or methanol (approximately 30 mg/mL). It is composed of vanillylamine and fatty acid (Figure 2), and its vanillyl residue can attach to the TRPV1. TRPV1 is present in various secretory epithelia, including salivary glands (Shin et al., 2016). Previous studies have shown that capsaicin has various effects on oral physiology, including changing saliva flow rate, and the concentration of salivary proteins through the TRPV1 pathway, particularly salivary secretory immunoglobulin A (SIgA), which plays a role in mucosal immunity (Gardner et al., 2020). In addition, capsaicin has been shown to have antibacterial activity. A recent study has proven that capsaicin inhibits the acid product from *Streptococcus sanguis, S. mutans, Actinomyces viscosus, and Lactobacillus spp.* (Gu et al., 2019). Capsaicin can also cause inflammation, and pain in the oral cavity, and impair sensory nerve endings have been observed with ingestion of capsaicin (Kono et al., 2018). Although capsaicin can trigger neurogenic inflammation in certain physiological circumstances, it also possesses analgesic and anti-inflammatory properties (Surh 2002). Normally, 0.02 - 0.025% of capsaicin is used for oral applications and the median LD is 47.2 mg/kg according to AAT Bioquest. Therefore, it is important to understand the concentration and impact of capsaicin on the oral cavity.

Figure 2: Chemical backbone of Capsaicin and Menthol



Capsaicin
(fatty acid amide/alkaloid)

Menthol
(monoterpene)

1.3. Ingredients of Oral Care Products and How They Work

Brushing and rinsing should be daily oral hygiene routine to maintain healthy teeth. Toothpaste that is present in a paste or a gel form is used in conjunction with a toothbrush, and mouthwash (also called mouth rinses) is another oral care product that has been particularly developed in aqueous solutions in the last few decades to maintain and improve oral health and appearance. The formulation of toothpaste and mouthwash is very intricate, with a wide range of active components such as fluorides, whitening agents, etc. that offer thorough mouth cleaning without harming the enamel or gum tissue. Flouride reduces the dissolution of calcium hydroxyapatite which is important for enamel remineralisation (Kanduti et al., 2016) and also functions as either an inhibitor of enzymes or creates metal-fluoride compounds that reduce the acid tolerance of bacteria (Hamilton & Bowden, 1996; Robert, 2011), resulting in reduction of tooth sensitivity, oral biofilm control, and tooth whitening. The whitening agent works either in mechanical, chemical, or optical ways to enhance the whiteness and aesthetics of teeth (Joiner et al., 2008; Joiner, 2010). Besides active ingredients that prevent tooth decay and gum diseases, inactive ingredients including sweeteners, flavours, surfactants, humectants, etc. also play an important role in the structural stability of toothpaste and mouthwash. Fluoride and abrasives normally give an unpleasant taste and therefore flavors (mostly menthol) and artificial sweeteners (mostly sodium saccharin) are employed. Surfactants (mostly sodium lauryl sulfate) help toothpaste to be nice and foamy during brushing, which allows other active ingredients to coat the teeth as long as possible (Lindenmuller & Lambrecht, 2011). Humectants (mostly a combination of glycerin and sorbitol, or propylene glycol) are used to keep toothpaste from drying out and becoming a homogenous delivery system. Sodium hydroxide is utilized to adjust the

pH, and ethanol acts as a solvent. Although mouth rinses possess antiseptic and cleaning properties, they are incapable of physically or mechanically eliminating plaque from the enamel and gingival surfaces as toothpaste, therefore, both should be used together to get the most efficient result.

1.4. Hypothesis

Based on the pungent and cooling properties of trigeminal modulators including capsaicin and menthol, their effect on oral stimulation, particularly in salivary flow rate and salivary protein changes, are still not well understood. Moreover, it remains unclear whether these trigeminal modulators significantly influence microbiomes that cause oral malodour. The aim of this review is to investigate how menthol and capsaicin, affect the salivary flow, oral protein composition, and also their effect target to bacteria *Bacteroides spp.* (e.g. *B. gracilis, B. intermedius, B. loescheii, B. oralis), P. intermedia, P. gingivalis, T. denticola, T. forsythia, F. nucleatum, P. endodontalis,* which contribute to both biofilm and oral malodour. We hypothesis that menthol and capsaicin would enhance salivary flow rate, change salivary protein concentrations, and also possess the antibacterial effect on targeted harmful oral bacteria because these influences of menthol and capsaicin make them potential ingredients for creating novel oral health products that could enhance saliva flow and provide protection against oral malodour.

2. Methods

To ensure transparency and comprehensiveness, this systematic review was conducted following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA). We conducted an electronic search on Google Scholar based on the specific question: “How do trigeminal modulators including capsaicin and menthol affect the salivary flow, salivary protein composition, and oral bacteria including *Bacteroides spp.* (e.g. *B. gracilis, B. intermedius, B. loescheii, B. oralis), P. intermedia, P. gingivalis, T. denticola, T. forsythia, F. nucleatum, P. endodontalis*?

We conducted a search for relevant articles published in English between 1980 and 2023 and also reviewed their reference lists for any additional relevant studies. A total of 11.787 articles were collected. After screening abstracts and titles, ninety-three were excluded because they were not for humans and/or mainly focused on blood flow, sensory perception, and oral bacteria causing dental diseases other than salivary flow, salivary protein, and targeted bacteria. Fourty-one articles were also excluded as it was difficult to access full-text, some even no longer exist. 171 articles were excluded as they were reviews, and sixteen articles were excluded as they were not in English. 4032 were excluded as they were not related to menthol, capsaicin, flow, salivary protein, and targeted oral bacteria, and some articles were duplicates. Twenty-seven full texts for the remaining articles were obtained. We examined the scientific names of oral microbiomes, concentrations of menthol, capsaicin, salivary protein, preparation types, and technology types as well as the study duration, evaluation indices, and subject characteristics, in the selected studies. Outcomes were compared between the effect on the salivary composition and targeted oral bacteria of menthol and capsaicin.

3. Results and Discussion

3.1. The Effect of Menthol and Capsaicin on Salivary Flow Rate

The volume of saliva can be collected in different ways, including holding cotton in the mouth with a set time and followed by cotton weighing (g/min) or simply splitting saliva in tubes with recorded time (mL/min), etc. The results of saliva flow rate may vary from individual to individual due to unclear physiological differences between individuals (Gardner & Carpenter, 2019). Motoi et al., (2019) have found that the resting saliva flow rate varied from 0.15 to 1.04 mL/min. These results are in agreement with other reported ranges which indicate that unstimulated and stimulated saliva typically falls within the range of 0.042 – 1.83 mL/min and 0.77 – 4.15 mL/min, respectively (Chen, 2009; Engelen et al., 2005). However, some studies have proven that menthol and capsaicin can stimulate additional salivary secretion, as shown in Table 2. Pushpass et al., 2019 have shown that when 31 participants rinsed their mouths with 1mL solution of 0,05M menthol, the salivary secretion increased but it was not significant compared to water rinse control (1.32 (± 0.22) g/min), while the flow rate rose to 2.37 (± 0.41) g/min when they use 1mL of 0.005M capsaicin ($p < 0.0001$). This trend is consistent with the finding of Gardner & Carpenter (2019) when only capsaicin significantly enhances the salivary flow rate. However, in 2020, Houghton et al. used a double concentration of menthol compared to the study of Gardner & Carpenter (2019), 500 ppm and 250 ppm respectively, the saliva was significantly secreted with 1.5 g/min ($p < 0.0001$). This finding suggests that increasing the menthol can increase the salivary flow rate. The finding of Dunér-Engström et al., (1986) is in agreement with the finding of Pushpass et al., (2019) that the saliva could be secreted around 2.5 g/mL although they used a lower concentration of capsaicin, 0.005M and 3×10^{-5} M respectively, whereas the finding of Yang et al., (2021) and Gardner & Carpenter (2019) have shown the same saliva flow rate when compared to the finding of Gardner & Carpenter (2019) although Yang et al., (2021) used a higher concentration of capsaicin, 5ppm compared to 1ppm. This may be because most participants from the study of Yang et al., (2021) belong to "low" flow individuals whose saliva will not be significantly stimulated by capsaicin (Gardner & Carpenter, 2019).

Table 2: Summary of the relation between menthol and capsaicin in salivary flow rate.

Trigeminal modulators	Doses and Methods	Flow Rate (g/min)	P-value	References
Menthol	Rinse 1 ml solution (0.05 M menthol dissolved in 1% propylene glycol and water) for 1 min, n = 31	No significant compared to water rinse control (1.32 ± 0.22)	-	Pushpass et al., 2019
	Hold 250 ppm menthol in mouth for 30s, n = 13	~ 1.15 (± 0.1)	P = 0.71	Gardner & Carpenter, 2019
	Rinse 10mL of 500 ppm menthol for 30s, n = 6	1.5	P < 0.0001	Houghton et al., 2020
Capsaicin	Rinse 1 ml solution (0.005 M capsaicin dissolved in 1% propylene glycol and water) for 1 min, n = 31	2.37 (± 0.41)	P < 0.0001	Pushpass et al., 2019
	Hold 1 ppm capsaicin in mouth for 30s, n = 13	~ 1.4 (± 0.2)	P = 0.046	Gardner & Carpenter, 2019
	Hold 10ml of 5ppm capsaicin in mouth for 10s and swallow after 60s, n = 15	1 (± 0.55)	P < 0.001	Yang et al., 2021
	Hold cotton swab containing 50µL solution ($3x10^{-5}$ M capsaicin) in mouth for 5 min, n = 27	2.67	P < 0.01	Dunér-Engström et al., 1986
	Hold 5 ml solution (0.3 mM capsaicin dissolved in 1ml of 99.5% ethanol and water) for 1 min, n = 18	2.6	P < 0.05	Kono et al., 2018

Menthol and capsaicin may increase salivary flow in the first few minutes and then the saliva secretion turns back to the initial flow (unstimulated). Houghton et al., (2020) have revealed that when six participants rinsed 10 mL of menthol (500ppm) for the 30s, their saliva was secreted 1.5 g/mL after the first minute and then the flow rate decreased to 0.75 g/min after two minutes and keep the same flow that similar to unstimulated saliva flow, for the rest of time experiment (5 min in total), as shown in Figure 3. However, capsaicin shows a longer time of stimulation in salivary flow. Kono et al., (2018) have indicated that by holding 5mL of capsaicin ($3x10^{-5}$ M) in the mouth, the mean saliva flow of 18 participants rose from 2.25 to 2.6 g/min during 1-

min stimulation and 1-min right after stimulation respectively, and these flow rates were significantly higher than the control ($P < 0.05$), as shown in Figure 4. This finding is in agreement with the finding of Hu et al., (2022) that after 1 min of holding 10 mL of capsaicin (5ppm), saliva secretion increased from 158% to 185%, and then decreased to 109% after the 80s of stimulation (Table 3). This trend is similar to oil capsaicin stimulation at the same concentration, however, it is two-fold lower in salivary flow rate than aqueous capsaicin stimulation, as shown in Table 3.

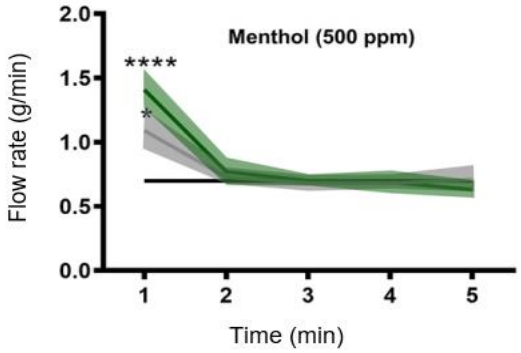


Figure 3: Influence of menthol (500ppm) on the salivary flow rate (Houghton et al., 2020). Green and grey shaded areas indicate the standard error of menthol and control, respectively. The black line reveals the mean unstimulated salivary flow rate. **** = $P \leq 0.0001$; * = $P \leq 0.05$.

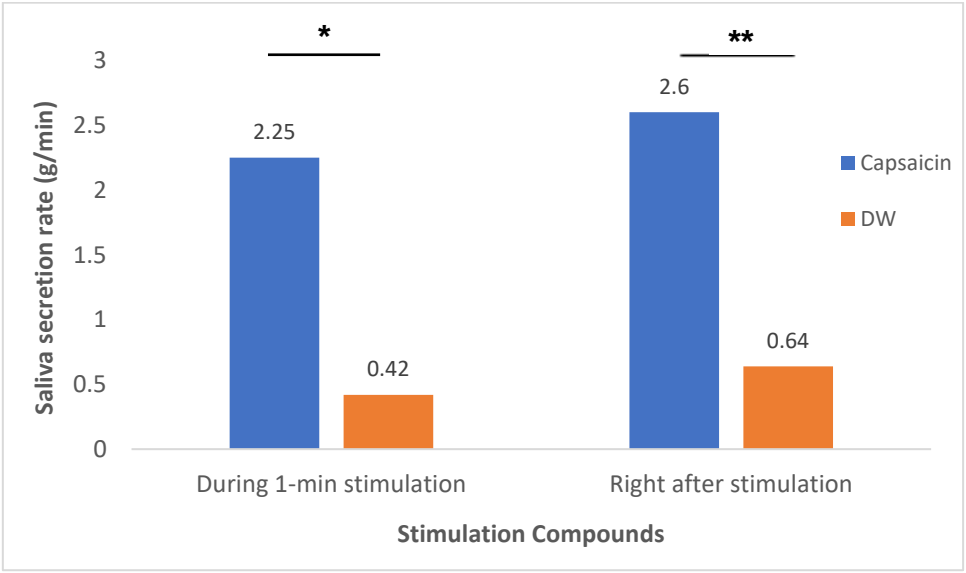


Figure 4: Influence of capsaicin ($3x10^{-5}$ M) on the salivary flow rate (Kono et al., 2018). DW: deionized water, * = $P < 0.05$, ** = $P < 0.001$.

Table 3: The percentage increase in saliva stimulated by aqueous and oil capsaicin compared to the control (water) (Hu et al., 2022). $P < 0.01$.

Capsaicin systems	During 1-min stimulation	Right after 40s stimulation	After 80s stimulation
Aqueous Capsaicin	158%	185%	109%
Oil Capsaicin	87%	82%	No change

3.2. The Effect of Menthol and Capsaicin on Salivary Protein Composition

The salivary protein composition was separated by 12% SDS-PAGE. In order to present each salivary protein type with its actual molecular mass and without any interactions between them, the whole saliva protein should be reduced by Dithiothreitol reagent before electrophoresis running. Figure 5A shows the main protein composition of saliva stimulated by capsaicin (1ppm), including MUC5B, MUC7, α-Amylase, Proline-rich proteins (PRPs), Cystatin, and Statherin. There are a similar number of bands with similar positions, shown in Figure 5B, when water-stimulated saliva was loaded, and a total of proteins in saliva was identified (Esser et al., 2008). Only in Figure 5C, gel with saliva of dental caries show fewer bands. According to Figures 5A and 5B, there was one band for Statherin (around 6 KDa), one clear band for Cystatin (around 14KDa), two clear bands and some faint bands for PRPs (ranging from 40 to 50 KDa), two clear bands for α-Amylase (ranging from 50 to 62 KDa), two clear bands for MUC7 (around 150 KDa), and band(s) for MUC5B at the well (above 188 KDa). Previous research has indicated that the apparent molecular weight, quantity, and intensity of the bands can differ among individuals due to genetic phenotypic polymorphism (Schwartz et al., 1995). Figure 5C showed highly intensive bands for α-Amylase (around 50 KDa), significantly fewer bands of PRPs, and no band of Cystatin. Besides showing the same pattern bands compared to the control, capsaicin also showed an increase in the intensity of proteins. Next step, a total protein composition should be measured to analyse how intensive these bands are; as a result, we understand how capsaicin affects protein composition.

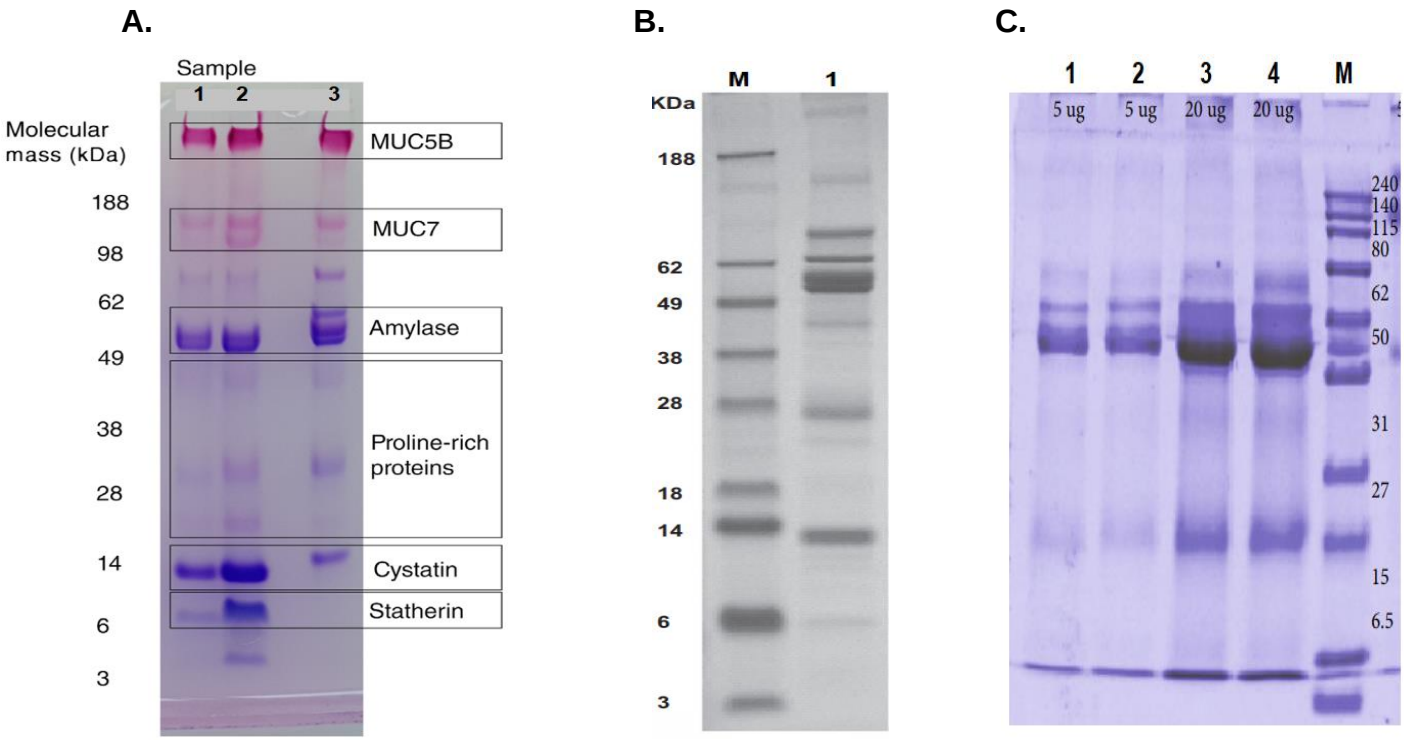


Figure 5. SDS-PAGE profile of saliva proteins. (A) lane 1: control stimulated saliva; lane 2: capsaicin stimulated saliva; lane 3: the standard reference saliva sample (Gardner et al., 2020). (B) lane 1: 10 µl of processed saliva (Esser et al., 2008). (C) lane 1, 2, ad 6: 5 *µ*g of saliva; lanes 3 and 4: 20 *µ*g of saliva (Khan et al., 2021). M: biomarker.

After detecting the protein composition of saliva, the subsequent objective was to estimate the total protein concentration and examine how the influence of menthol and capsaicin on specific salivary proteins based on Analytical Ultracentrifuge (AUC) techniques and analysis of band intensity from SDS-PAGE. AUC was used by Dinu et al., (2018) to understand differences in salivary compositions. The results of the experiment confirmed the existence of three main macromolecular components α-Amylase, Mucin, and secreted Immunoglobulin A (SIgA), and the α-Amylase mainly contributed to the changes in protein concentration (Dinu et al., 2018).

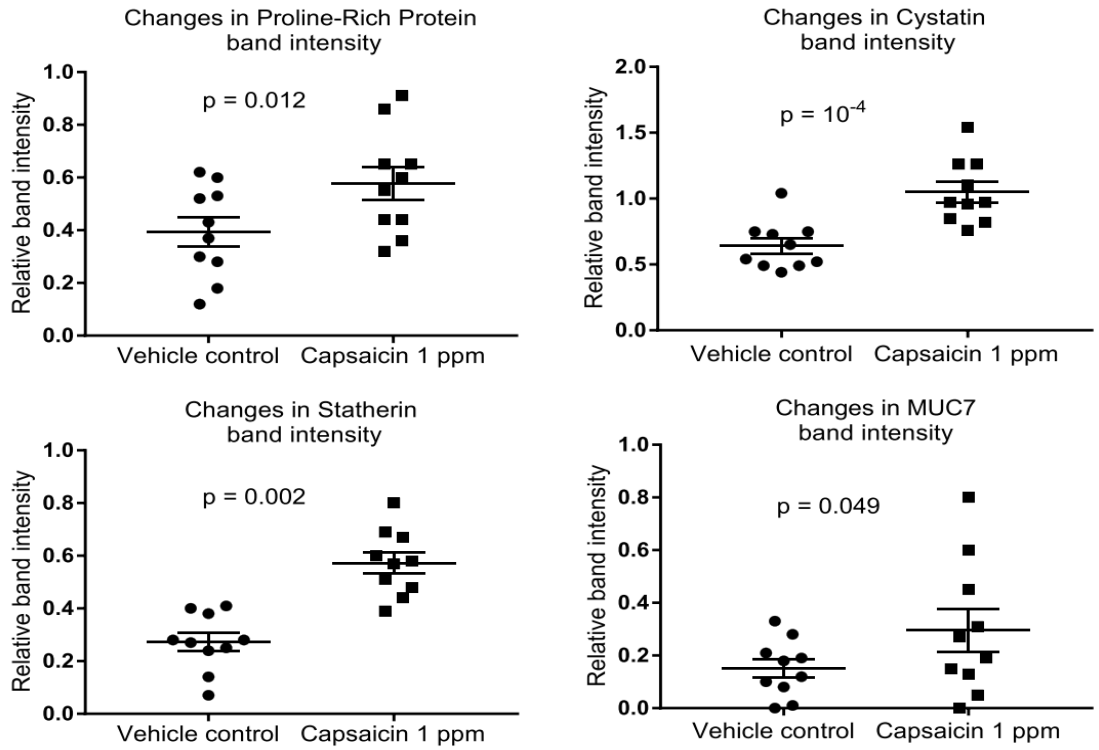
A study by Gardner and Carpenter (2019) has suggested that holding 10mL of menthol (250ppm) in the mouth for 30 seconds did not changed the total salivary protein concentration (1.04 mg/mL compared to 1.11 mg/mL), only stimulated with capsaicin (1ppm), there showed a little increase in the protein content (1.26 mg/mL), however, the rise was not significant. This finding is in agreement with the finding of

Pushpass et al., in the same year that the total protein has not changed dramatically, compared to unstimulated whole-mouth saliva (0.8 mg/mL) when participants rinsed 1 mL of 0.05M menthol and 0.005M capsaicin in 1 min. Houghton et al., (2020) also found the same result that there was no significant difference between the total salivary protein concentration in an unstimulated state (0.99 mg/min) and stimulated by 10mL of 500ppm menthol (1,17 mg/min). Although the salivary protein concentration slightly increases when an increase in menthol concentration, using 500 ppm menthol (Houghton et al., 2020) compared to 250 ppm menthol (Gardner and Carpenter, 2019), they are not significant. These recent findings are consistent with the discoveries a long time ago of the salivary concentration range in both unstimulated and stimulated (e.g., by water, parafilm) states, 0.72 to 2.45 mg/mL (Lin & Chang, 1989), and 0.5 – 2 mg/mL (Edgar, 1992), except the finding of Dinu et al., (2018) showing quite high protein concentration of both states in human saliva.

Some studies have shown that menthol does not significantly increase total protein concentration in human saliva, therefore there is little interest in digging into its influence on specific protein components. Only one article, in a time range of 1980 – 2023, has proved that the presence of menthol at a concentration of 500 ppm resulted in a stronger increase in the expression of salivary cystatins “S” family compared to propylene glycol control ($P < 0.05$) (Houghton et al., 2019). In contrast, capsaicin shows more increase in salivary protein concentration, therefore, Garner and Carpenter (2019) investigated further the changes in the concentration of each salivary protein composition based on the SDS-PAGE densitometry as shown in Figure 5A, and the results are shown in Figure 6A. After 30 seconds of holding 10mL of capsaicin (1 ppm) in the mouth, ten participants showed a significant increase in the intensity of PRPs, Cystatin, Statherin, and MUC7, especially Statherin and Cystatin with two-fold higher, compared to the intensity of the control. Only α-Amylase and MUC5B did not show any drastic changes, and this finding is in agreement with the study of Pushpass et al., (2019) that 1 min mouth rinse with 1 mL of 0.005M capsaicin only gives rise to MUC7 secretion, not MUC5B. However, one year later, Garner with other his colleagues conducted again the experiment with the same concentration of control and capsaicin (not to mention the same or different participants), the intensity of all salivary protein compositions including α-Amylase was statistically significant, only except for MUC5B, where the P-value approached

the threshold of significance (0.05). Furthermore, the MUC7 and PRPs bands exhibited lower intensity, three-fold and two-fold respectively, compared to Gardner's experiment in 2019.

A.



B.

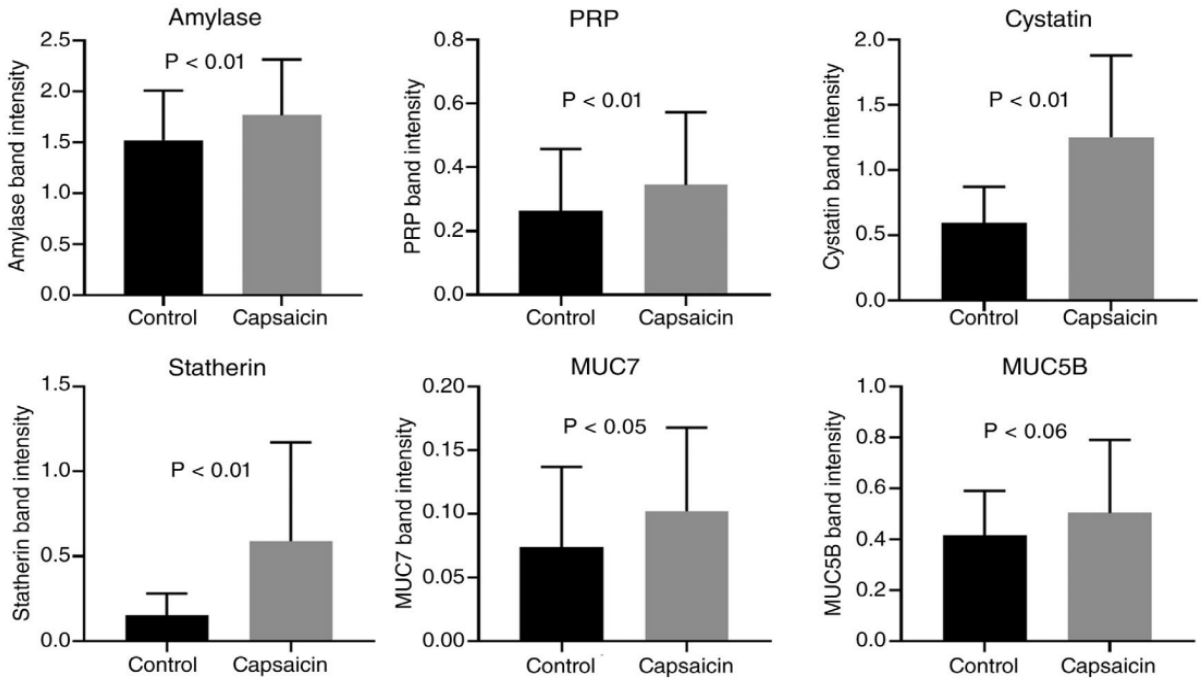


Figure 6: The changes in salivary protein band intensity stimulated by control (0.475% ethanol rinses) and capsaicin (1 ppm) based on SDS-PAGE densitometry. A. Conducted by Gardner and Carpenter 2019. B. Conducted by Gardner et al., 2020. These two experiments use the same concentration of control and capsaicin, except for participants. P-values obtained by paired t-tests, n = 10. Data are shown as means ± SD.

3.3. The Effect of Menthol and Capsaicin on Oral Microorganisms Causing both Biofilm and Oral Malodour.

Very few studies have shown the positive or negative effect of menthol and capsaicin on oral microorganisms related to both biofilm formation and oral malodour. Oral malodour is mainly caused by the release of volatile sulphur compounds (VSC), mainly H_2S and CH_3SH), from some abundant microbes in the mouth including *Bacteroides spp*, *P. intermedia, P. gingivalis, T. denticola, T. forsythia, F. nucleatum, and P. endodontalis* as shown in Table 4. Most studies have used two methods, Minimum Bactericidal Concentration (MBC) - the lowest concentration of an antibacterial agent needed to effectively eliminate a specific bacterium, and minimum inhibitory concentration (MIC) - the lowest concentration of antimicrobial substance agent that can prevent visible bacterial growth after being incubated overnight, to see whether menthol and capsaicin can lower or stop the oral bacterial growth at a specific level.

Table 4 reveals that only capsaicin can increase and decrease the *Bacteroides spp.* Hui et al., (2020) have shown that by using 1 μmol/L of capsaicin, the relative abundance of *Bacteroides* increased significantly ($P < 0.05$). This finding is in agreement with the finding of Shen et al., (2017) when using 0.01% capsaicin. However, the results of Song et al., (2017) show an opposite trend, although they used the same dose and method as the experiment of Shen et al., (2017). Furthermore, these three studies used mice models and targeted gut microbiomes, however, some *Bacteroides spp.* in the gut is also found in the mouth. Therefore, more experiments need to be conducted to verify the antibacterial activity of capsaicin against *Bacteroides spp*., especially the human and oral targets. *P. gingivalis* has also been shown to be negatively affected by capsaicin. Zhou et al., (2014) have indicated that 16 mg/mL and 64 mg/mL were the capsaicin MIC and

MBC of *P. gingivalis*, respectively, in planktonic culture. Besides, 64mg/L dose also showed a reduction in the viability of biofilm cells (31.2 ± 5.2%, $P < 0.05$) and the thickness of the biofilm uniform (30.1 ± 3.7 µm, $P < 0.05$). However, this experiment was conducted in vitro, the MIC and MBC may not be accurate if applied clinically.

Menthol has been proven to be antibacterial activity as shown in Table 4. Thapa et al., (2022) have indicated that using 450 mg of peppermint oil can lower the *Firmicutes/Bacteroides* ratio compared to using 180 mg dose ($P = 0.04$). Although their experiment was conducted in the human gut, their result may be consistent with *Firmicutes/Bacteroides* that are present in the mouth, however, we need more evidence/experiment to prove that. Kraivaphan et al., (2013) have shown that *Mentha cordifolia* (kitchen mint) shows higher MBCs in both *P. gingivalis planktonic* and *P. gingivalis* biofilm than *Mentha arvenis* (Japanese mint) (Table 5). In addition, the results have indicated that the biofilm *P. gingivalis* is less sensitive and requires a higher concentration of mint than planktonic *P. gingivalis* to kill the bacteria. Lagha et al., (2020) have proved that peppermint oil can be against *F. nucleatum* at 0.25% (v/v) for MIC and 1% (v/v) for MBC. They have also shown that peppermint at 1% (v/v) can significantly reduce the viability of biofilm by 69.1% ($P < 0.01$) and at 0.015% and 0.03125% can decrease drastically VSC by 12.2% and 43,9%, respectively ($P < 0.01$). Although menthol is the main component in most types of mint leaves (accounting for 30-50%), the essential oil extracted from these mint leaves may contain other antibacterial agents, which can lead to deviations in MIC and MBC values if these values are applied only to pure menthol. Therefore, there should be experiments using only purified menthol concentrations to obtain the most accurate MIC and MBC values.

Table 4: influence of menthol and capsaicin on common bacteria related to both biofilm and intra-oral halitosis (IOH) (Persson et al., 1990). "–": no found, "+": found.

Volatile sulphur compounds	Bacterial species	Effect by capsaicin		Effect by menthol		References
		Increase	Decrease	Increase	Decrease	
H_2S	*Bacteroides spp.* (e.g. *B. gracilis, B. intermedius, B. loescheii, B. oralis)*	+	+	-	+	Song et al., 2017; Shen et al., 2017; Hui et al., 2020; Thapa et al., 2022
	Prevotella intermedia	-	-	-	-	-
	Porphyromonas gingivalis	-	+	-	+	Zhou et al., 2014; Kraivaphan et al., 2013
	Treponema denticola	-	-	-	-	-
	Tannerella forsythia	-	-	-	-	-
CH_3SH	*Fusobacterium nucleatum*	-	-	-	+	Lagha et al., 2020
	Treponema denticola	-	-	-	-	-
	Porphyromonas endodontalis	-	-	-	-	-

Table 5: the MBC values of Kitchen mint and Japanese mint for *P. gingivalis* planktonic and biofilm (Lagha et al., 2020).

MBCs (mg/mL)	Kitchen mint	Japanese mint
Planktonic *P. gingivalis*	0.821	6.537
Biofilm *P.gingivalis*	6.568	26.150

4. Conclusion

In conclusion, the results from menthol and capsaicin supported the hypotheses we proposed in this study: menthol and capsaicin i) increased saliva flow rate, ii) also increased the salivary protein concentrations, and iii) possess the antibacterial effect on *Bacteroides spp., P. gingivalis,* and *F. nucleatum*. However, at a concentration of 500 ppm, the impact of menthol on saliva flow is low, and even at lower concentrations, there are no noticeable alterations in the flow or total protein content. In addition, two articles have shown the opposite effect of capsaicin on *Bacteroides spp.,* at the same dose and method. Furthermore, regarding the MBC and MIC results of menthol and capsaicin on *Bacteroides spp., P. gingivalis, F. nucleatum*, the scientists used different types of mint, and mice models and targeted these bacteria in the gut. Since menthol is the main component of mint and these bacteria are also present in the human mouth, these results constitute a valuable initial endeavor in investigating the biological roles of pure menthol on human saliva proteins and oral bacteria. Therefore, further exploration could be conducted to investigate the enduring effects of menthol (should be higher than 500 ppm) and capsaicin (no more than 5 ppm) on oral compositions, which will make them to be potential ingredients for oral care applications.

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A comparison of capsaicin and menthol as trigeminal modulators of salivary composition for use in oral care applications

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Contents

Abstract

Menthol and capsaicin, derived from peppermint and chili, exhibit bioactive attributes that could enhance oral antibacterial defenses and alleviate halitosis. This study examined the effect of menthol and capsaicin on the properties of the oral cavity such as their effect on bacterial growth (mainly *Bacteroides, Prevotella, Lactobacillus, Streptococcus spp)*, saliva flow rate, interactions with salivary proteins and oral malodour. Employing viable cell counts, SDS-PAGE, AUC, GC-MS, the effect of menthol and capsaicin on the saliva properties of a healthy woman was investigated. Generally, capsaicin exerted a stronger effect than menthol. Although not statistically significant within the sample population, both menthol and capsaicin reduced bacterial counts and odour compounds, with some exceptions in-vivo. However, capsaicin exhibited no significant odour compound reduction in the in-vitro experiment. Both menthol and capsaicin increased saliva flow rates and altered the salivary protein composition. Interestingly, menthol prompted a significant salivary secretion within the initial two-minute, whereas capsaicin significantly continued to build up the saliva flow during the 10-minute experimental time. SDS-PAGE revealed five main salivary proteins, corresponding to MUC(s) (above 188kDa), MUC7 (150kDa), α-Amylase (50-62kDa), PRPs (16-50kDa), and Cystatin (10-14kDa). SV-AUC highlighted two distinct populations at ~1.8S and ~4.2S, correlating with previous reports. Although the analysis indicated no significant differences in the total salivary concentration, some lower molecular weight compounds such as PRPs and Cystatins were significantly affected upon stimulation with the two trigeminal compounds ($P > 0.05$). A thorough clinical trial would therefore be recommended to confirm the effects observed in this proof of concept study

1. Introduction

Maintaining good oral health is crucial for preventing tooth decay, gum diseases, and unpleasant breath which contribute to overall oral and dental health. Saliva, an essential fluid produced mainly by salivary glands (Kaufman & Lamster, 2002), together with its proteins, plays a vital role in oral health.

Human saliva possesses diverse and essential biological functions in maintaining oral health, including lubrication, buffering, protection against microorganisms, preservation of mucosal integrity, and digestion of food (Huang, 2004). The sympathetic nervous system regulates saliva production through the parasympathetic (cholinergic) system and specific nerve fibers that connect receptor stimulation to ion transport and protein secretion mechanisms (Dodds et al., 2005; Arany et al., 2021). The flow and composition of saliva can vary from person to person depending on their oral and overall health status (Saibaba et al., 2021). On average, a healthy individual produces about 0.5 to 2 liters of saliva daily (Mese & Matsuo, 2007), with secretion decreasing during sleeping and increasing significantly during talking or eating. Whole saliva is primarily composed of water (99.5%), proteins and enzymes, hormones, sugars, lipids, electrolytes (such as sodium, chloride, and bicarbonate), and several other components (Liu & Duan, 2012). With recent advances in proteomic technology, Si et al., (2015); Sun et al., (2016); and Wang et al., (2018) have revealed that the salivary proteome contains a significant number of proteins, with up to 1166 proteins identified in total. Salivary proteins can be categorized into several families, including Mucin, Proline-Rich Proteins (PRPs), Alpha-Amylase (α-Amylase), Cystatins, Histatins, and Statherin (Gonzalez-Begne et al., 2009; Amado, 2010; Zhang, 2013; Cabras et al., 2014). These proteins have specific molecular functions that contribute to oral health as shown in Table 1.

Table 1: Characteristics of human salivary proteins

Human salivary proteins	Function(s) in oral care	References
Mucin	Form a protective layer over the oral cavity (lubrication) and gingiva to protect against invading pathogens and abrasions as well as help with speech, mastication, and the process of swallowing. Cause bacterial agglutination which facilitates their clearance from the oral cavity.	Aplin & Hey, 1995; Pedersen et al., 2002, Aas et al., 2005; Oppenheim et al., 2007
α-Amylase	Bind to bacteria, in plaque and prevent bacterial colonization. Cleave randomly at the alpha-1,4-glucosidic linkages of starch, glycogen, dextrin, and other complex sugars, which may provide glucose for plaque microorganisms used for metabolism.	Scannapieco et al., 1993; MacGregor et al., 2001; Mandel et al., 2010; Singh et al., 2015;
PRPs	Bind to Ca^{2+} are involved in the acquired enamel pellicle formation. Function to ensure oral lubrication and bind to some types of oral microorganisms to regulate oral microbiomes.	Gillece-Castro et al., 1991; Amano et al., 1994; Schenkels et al., 1995; Ruhl 2004; Vitorino et al., 2007
Cystatins (family-2)	Possess potent antibacterial and antiviral activities. The phosphorylated forms of family-2 cystatins bind to hydroxyapatite (HA) and inhibit hydroxyapatite crystal growth which is important for enamel remineralisation; therefore, they may play a vital role in the formation of the acquired enamel pellicle.	Johnsson et al, 1991; Lamkin et al., 1991; Dickinson 2002; Koopaie et al., 2021
Statherin	Along with PRPs, attach to calcium phosphate and calcium carbonate salts and prevent them from spontaneous precipitation, which promotes enamel remineralization. Act as a lubricant to create a barrier on the enamel surface.	Hay et al., 1986; Raj et al., 1992; Gururaja & Levine 1996; Humphrey & Williamson 2001; Pateel et al., 2017
Histatins	Possess antimicrobial and antifungal effects. Involve creating the acquired pellicle and contribute to the mineralisation process of oral fluids. Inhibit the release of histamine from mast cells, which manages oral inflammation.	Oppenheim, 1989; Sabatini & Azen 1989; Troxler et al., 1990; Sugiyama et al., 1990; Van et al., 1997

Over 700 different types of bacteria have been identified in the human oral cavity, belonging to six phyla: *Firmicutes, Fusobacteria, Actinobacteria, Bacteroidetes, Proteobacteria*, and *TM7* (Aas et al., 2005; Jornet al., 2005;

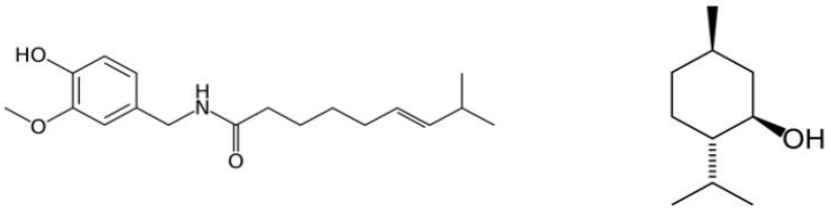
Cheng et al., 2009). Among them, *Streptococcus mitis* is the most abundant species across all oral sites and subjects, and some species from *Granulicatella* (such as *Granulicatella adiacens*), *Streptococcus, Gemella*, and *Veillonella* are commonly present in most oral sites (Aas et al., 2005). Although most oral bacteria are harmless to the mucosal surfaces and teeth, a shift in the composition of the oral microbiome can lead to oral malodor and specific oral diseases like gingivitis, dental caries (cavities), and oral thrush (Scannapieco, 1999; Gao et al., 2018). Most bacteria in the oral flora have the ability to aggregate, adhere, and builds upon teeth and gum lines through receptors such as Statherin, bacterial cell fragments, sialylated Mucins, α-Amylase, PRPs, over time resulting in a thick layer called biofilm (also known as bacterial plaque). The acids produced by the bacterial plaque can cause demineralization of the enamel surface, resulting in the loss of calcium. This demineralization occurs when the pH level in the mouth is between 5 and 6. The demineralization process increases the risk of cavities, enamel erosion, and tooth sensitivity. Oral malodor, also known as halitosis, is characterized by unpleasant or foul-smelling breath. It is primarily caused by the accumulation of food debris and bacterial plaque. These bacteria, particularly *Bacteroides spp., Prevotella intermedia, Porphyromonas gingivalis,* and *Fusobacterium nucleatum*, consume food debris and release odour compounds, particularly volatile sulfur compounds (VSCs) such as hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH) causing oral malodour (Persson et al., 1990; Rosenberg et al., 1991; Loesche & Kazor, 2000). In one study by Phillips and co-researchers (2005), there were 30 abundant volatile compounds detected in the oral cavity of halitosis patients, and these compounds belonged to alkanes or alkane derivatives, therein methyl benzene, tetramethyl butane, and ethanol. Another study has shown that VSCs and amines (such as cadaverine, putrescine, and trimethylamine) are the most abundant volatile organic compounds in halitosis patients (Dadamio et al., 2011). Bad breath in addition to causes results in shyness and social difficulties, these bacteria also cause periodontitis diseases (Hampelska et al., 2020).

Menthol and capsaicin act as trigeminal modulators by activating the somatosensory system associated with the trigeminal nerve, leading to sensations of cooling and pain. The perception of hot and cold temperatures, as well as pain, is mediated by transient receptor potential (TRP) channels, which are a family of nonselective cation channels (McKemy et al., 2007; Szallasi et al., 2007) identified in the salivary glands and contribute to salivary flow (Liu et al., 2018).

Menthol, also known as (1R,2S,5R)-2-isopropyl-5-methylcyclohexanol, is a compound with cooling and soothing properties found in plants such as peppermint and spearmint. Cooling properties make it a popular flavoring agent in food and beverages, and it is commonly used in oral care products such as toothpaste and mouthwash in the form of essential oil. It has a chemical structure consisting of a monocyclic terpene backbone, as depicted in Figure 2, which can activate peripheral cold receptors found on TRP channels (Schafer et al., 1986; McKemy et al., 2002; Peier et al., 2002). The activation of TRP channel may stimulate saliva flow and improve oral health (Pushpass et al., 2019; Gardner & Carpenter, 2019; Houghton et al., 2020). However, the exact mechanisms underlying these effects are not fully understood. In addition to its cooling effect, menthol has antibacterial properties and can inhibit the growth of various microorganisms (Schelz et al., 2006; Patel et al., 2007), including *Streptococcus mutans, Streptococcus sobrinus, Streptococcus salivarius, Lactobacillus casei*, and several *Candida spp.* (Mahzoon et al., 2022). The reduction in bacteria population may potentially mitigate halitosis. Therefore, it is important to use appropriate concentrations of menthol in oral applications to avoid any irritating sensations or adverse effects, 0.1 – 0.4% of menthol is normally used for oral applications (Fatmawati et al., 2022) and the lethal dose (LD) ranges from 50–150 mg/kg according to Gosselin and his colleagues (1984).

Capsaicin, also known as trans-8-methyl-N-vanillyl-6-nonenamide, is the main compound responsible for the pungent taste of chili peppers.

Capsaicin with its vanillylamine and fatty acid backbone (Figure 2) can activate peripheral cold receptors found on the TRP channel, which is present in salivary glands and other secretory epithelia (Shin et al., 2016). The activation of TRP channel may lead to the changes in saliva flow rate and the concentration of salivary proteins (Gardner et al., 2020). It also exhibits antibacterial activity against certain oral bacteria, incuding *Streptococcus sanguis, S. mutans, Actinomyces viscosus*, and *Lactobacillus spp.* (Gu et al., 2019). And based on its antibacterial properties, capsaicin contributes in reducing oral malodour. However, capsaicin can cause inflammation, pain, and impair sensory nerve endings in the oral cavity (Kono et al., 2018). Despite its potential to induce neurogenic inflammation, capsaicin also possesses analgesic and anti-inflammatory properties (Surh 2002). Therefore, appropriate concentrations of capsaicin should be used in oral applications to avoid any adverse effects, 0.02 - 0.025% of capsaicin is normally used for oral applications and the median LD is 47.2 mg/kg according to AAT Bioquest.



Capsaicin
(fatty acid amide/alkaloid)

Menthol
(monoterpene)

Figure 1: Chemical backbone of Capsaicin and Menthol

Maintaining healthy teeth requires a daily oral hygiene routine of brushing teeth with toothpaste and rinsing mouth with mouthwash (also called mouth rinse). Menthol is a flavoring agent found in most oral health applications, especially in toothpaste and mouthwash with a range of concentration from 0.1 - 0.4% depending on the brand (Fatmawati et al., 2022). Besides its main effect of fresh breath after rinsing the mouth and brushing teeth, menthol has also been shown to refresh the mind, ease mental fatigue, be pain-relieving, anti-inflammatory, and anti-bacterial

(Eccles, 1994; Cliff & Green, 1996; Sakai et al., 2011; Thosar et al., 2013). Due to its anti-inflammatory properties, capsaicin is one of the main ingredients in mouthwash for burning mouth syndrome, commonly used in concentrations of 0.02% - 0.025% (Menicagli et al., 2020; Jankovskis & Selga, 2021). In addition to its anti-inflammatory properties, capsaicin has also shown anti-microbial properties (Zhou et al., 2014) that make capsaicin a potential agent for reducing oral malodour. Although compared to menthol, capsaicin is still in the early stages of potential oral health research.

Menthol and capsaicin hold promise as potential active ingredients in oral health applications and should be further studied to understand how, besides their role as trigeminal stimulants, they might affect the functionality of saliva in developing future oral health applications. In this experiment, we investigated the influence of menthol and capsaicin (0.02%) on salivary flow, oral protein composition, oral malodour and their effects on some oral bacteria including *Bacteroides spp., Prevotella spp. Lactobacillus spp., Streptococcus spp*., and some other fastidious anaerobes at the genus level. Our hypothesis suggests that 0.02% menthol and capsaicin could potentially boost salivary flow rate, alter salivary protein concentrations, reduce oral bacteria, and 0.01% and 0.5% menthol and capsaicin also reduce oral malodour in-vivo and in-vitro.

2. Materials and Methods

2.1. Preparations of aqueous menthol and capsaicin solutions

For the preparation of 0.5% aqueous solutions, 90 mg each DL-menthol crystal and natural capsaicin powder (Sigma-Aldrich, UK) was first dissolved in 9 mL of ethanol (99.8%), which was then added up to 9 mL of pure water (Suez Purite Fusion 160/320) to make the final stock menthol and capsaicin solutions. For the control solution, 9 mL of ethanol (99.8%) were mixed with 9 mL of pure water (Suez Purite Fusion 160/320). Stock solutions were then diluted with pure water (Suez Purite Fusion 160/320) to prepare 0.01% and 0.02% aqueous solutions and relative control solutions for different experiments.

2.2. Collections of whole saliva

This proof-of-concept study of a healthy female participant (age range of 23 to 30) which has followed a rigorous and well standardized saliva collection protocol. Saliva was collected by chewing a squared parafilm (5 cm x 5 cm, Bemis) that wrapped 200 µL (0.02%) of control, menthol, and capsaicin solutions between 10.30 AM to 12.30 AM and 1.30 PM to 3.30 PM. The collection was conducted in a 15 mL centrifuge tube (Started AG & Co. KG) that was placed on the ice. The total time for collection was 10 min for each solution with recorded saliva flow rate (mg) every min, and 30 min break between each solution. The data (mg/min) was analysed by using ANOVA in OriginLab software. The percentage increase in total saliva stimulated by menthol and capsaicin solutions compared to the control solutions were calculated by:

$$Percentage\ increase = \frac{Volume\ of\ stimulated\ saliva\ from\ Trigeminal\ solution - Volume\ of\ stimulated\ saliva\ from\ Control}{Volume\ of\ stimulated\ saliva\ from\ Control} \times 100$$

The collected saliva samples were then centrifuged for 5 minutes at 1400 g to eliminate the precipitated mucins and render the sample acellular. Finally, the pellet (debris) was discarded, and the supernatant was stored at -20°C until the next analysis.

2.3. Analysis of rinsing (in-vivo) and adding (in-vitro) effect on oral malodour formation by Gas Chromatography–Mass Spectrometry (GC-MS)

For the in-vivo experiment, the participant rinsed her mouth with 5 mL of control, capsaicin, and menthol solutions at 0.01% concentration for 30 seconds and then was removed from the mouth. Saliva was then collected and divided evenly into GC tubes (2.5 mL/tube) for each solution. For in-vitro method, the participant collected morning saliva (no eating and brushing teeth) into 6 GC tubes (2.5 mL/tube). Control, menthol, and capsaicin solutions were in turn added into every 2 GC tubes with 50 µL at 0.5% concentration per tube. All prepared tubes were finally run by GC-MS machine to detect the quantity of odour compounds.

A pool data detected by the Tracefinder software was selected based on its relation to oral malodour and oral health. Twenty-three compounds were selected and statistically analysed (ANOVA, OriginLab).

2.4. Analysis of oral bacteria growth

Brain Heart Infusion (BHI), *De Man, Rogosa* and *Sharpe* (MRS), M17, and Nutrient Agar (NA) plates were provided by Dr. Jianhua Jia, University of Nottingham, UK. Specific types of agar plate used for isolating *Bacteroides spp., P. gingivalis, Lactobacillus spp., Streptococcus spp.*, and the suitable serial dilution of saliva samples (collected only after 2 min without centrifugation step) for each type of agar plate were shown in Table 2. 100 µL of appropriate dilution was spread into each plate with two replicates for each dilution and NA plates with an anaerobic sachet and BHI, MRS, M17 plates were incubated at 37°C in 24 hours.

The total number of colony-forming units (CFU) in 1ml of the saliva sample was calculated by: $CFU/mL = \frac{number\ of\ colonies \times dilution\ factor}{volume\ of\ culture\ plate\ (mL)}$

Then, log10 (CFU/ml) was applied to plot the graph and statistical analysis (ANOVA, OriginLab) was also applied to test the null hypothesis.

Table 2: Media, serial dilutions of uncentrifuged saliva samples used for culturing oral microbes.

Identified bacteria	Media	Dilution factors
Bacteroides spp. and *other anaerobes*	Anaerobic NA	10^3, 10^4
Bacteroides spp. and *Prevotella spp.*	BHI	10^3, 10^4
Lactobacillus spp.	MRS	10^0, 10^1
Streptococcus spp.	M17	10^1, 10^2

2.5. Analysis of salivary protein compositions by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For preparation of sample buffer, 2X-Laemmli Sample Buffer (Bio-Rad Laboratories, UK) was mixed with β-mercaptoethanol (Bio-Rad Laboratories, UK) at 19:1 ratio. The saliva samples was then mixed with sample buffer at 1:1 ratio. 30 µL of processed saliva samples, model Bovine Serum Albumin (BSA) solutions containing 2.5 µg, 5 µg, and 10µg (Sigma-Aldrich, UK), and 5 µL of Precision Plus Protein biomarker (Bio-Rad Laboratories, UK) were loaded into 12% SDS-PAGE gels (Bio-Rad Laboratories, UK). Electrophoresis was conducted at 80V for 5 min and then at 180V for 20 – 35 min. After electrophoresis completion, the gels were stained overnight in InstantBlue Coomassie Protein Stain (Abcam, UK). The gels was then washed 3 times with pure water (Suez Purite Fusion 160/320) and the protein bands were visualised using a ChemiDoc MP imaging system (Bio-Rad, UK). Band intensities of major salivary proteins were analysed by ImageJ software based on the band intensity of the BSA model and statistical analysis (ANOVA, OriginLab).

2.6. Analysis of major salivary proteins by Analytical Ultracentrifuge (AUC)

The sedimentation velocity experiment was performed in an Optima XL-I analytical ultracentrifuge (Beckman Coulter, USA) at 20°C. Reference

buffer solution (0.45 mL) and processed saliva solutions (0.44 mL) were loaded into double-sector cells with sapphire windows and mounted in an 8-hole rotor. Sample solutions were run at 35000 rpm and the scans were taken at 2 minutes intervals. The interference and absorbance system produced data seven different concentrations was centrifuged at 35000 rpm at 20.0°C. The data generated by the interference and absorbance systems was obtained by measuring alterations in concentration (in fringe units) versus radial displacement. The results were analysed using the diffusion corrected c(s) and lg*(s) models in SEDFIT algorithm, which generated sedimentation coefficient distributions (in Svedberg units, $S = 10^{-13}$ sec).

3. Results and Discussion

3.1. Demonstration that menthol and capsaicin reduced the number of oral bacteria.

The investigation aimed to demonstate the effect of dilutions of menthol and capsaicin on oral bacteria through the utilization of the viable cell count log10 (CFU/mL) method. Diverse media, including anaerobic NA, BHI, MRS, and M17), were employed to conduct the isolation of species encompassing *Bacteroides, Prevotella, Lactobacillus, Streptococcus*, and other anaerobes, and the outcomes are depicted in Figure 2. Upon analysis, it was observed that the CFU/mL obtained from chewing menthol-infused parafilm showed a lower count compared to the control (water-containing parafilm), and a higher count compared to capsaicin-containing parafilm. Nevertheless, these disparities were not found to be statistically significant. Notably, Figure 2 unveiled an interesting trend where the CFU/mL values for "*Bacteroides spp.* and other anaerobes" closely paralleled those of "*Bacteroides spp*. and *Prevotella spp.*" under all conditions - control, menthol, and capsaicin exposure. Furthermore, both "*Bacteroides spp*. and other anaerobes" and "*Bacteroides spp*. and *Prevotella spp*." demonstrated the highest CFU/mL values, significantly surpassing other groups in the experiment. Along with *Prevotella spp*., falls within the category of anaerobic gram-negative bacteria, these findings signify the substantial prevalence of *Bacteroides* and *Prevotella spp*. within saliva. Corroborating this, existing research studies (Aas et al., 2005; Preza et al., 2008; Keijser et al., 2008) have underscored the prominence of *Bacteroidetes* phyla, while the work by Xu et al. (2015) concurs with the high prevalence of *Prevotella* species in both saliva and dental plaque among individuals who are in good health.

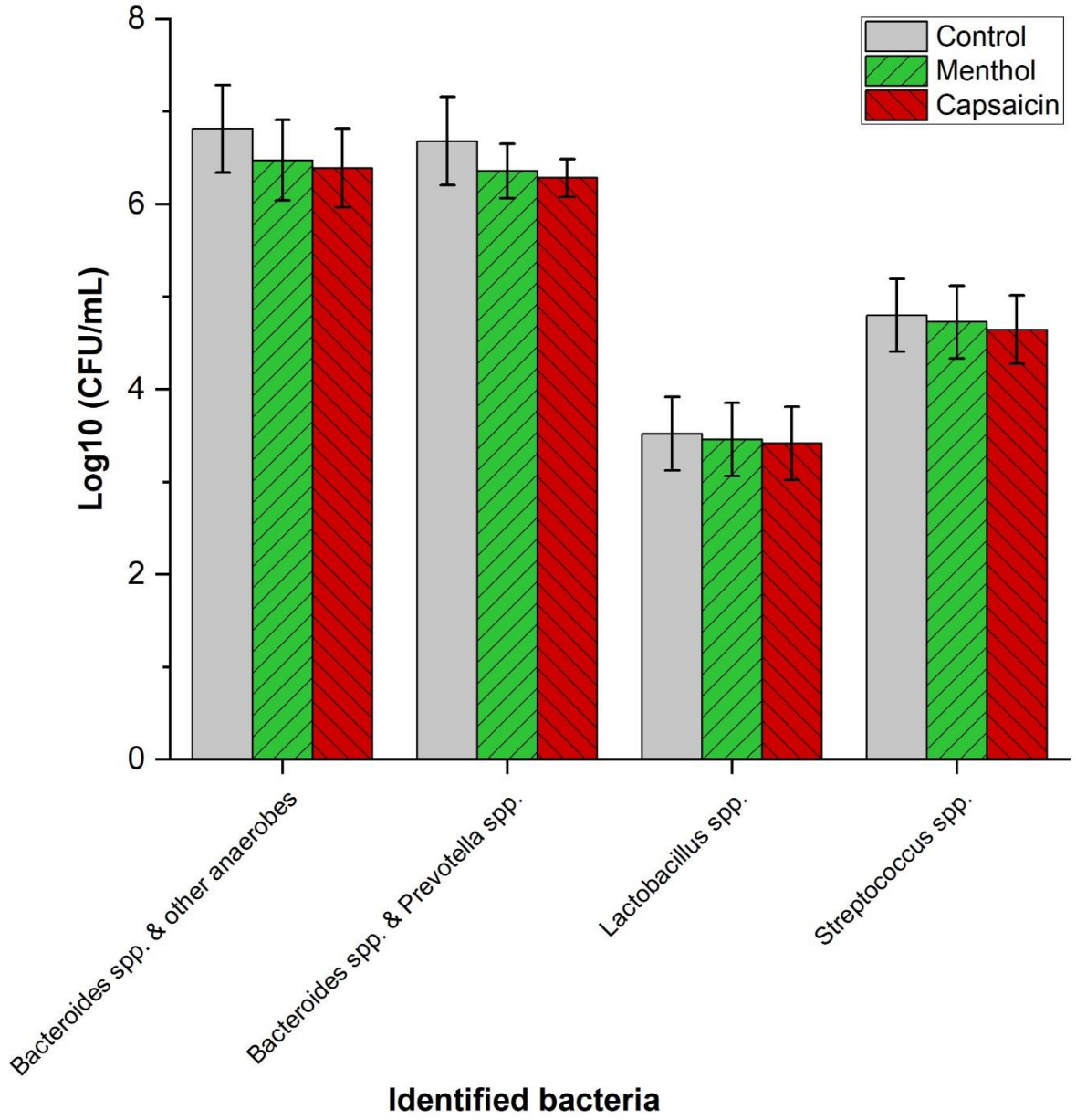


Figure 2: Difference in total viable cells count log10 (CFU/mL) of untreated saliva (control) and saliva treated with 200 µL of menthol and capsaicin (0.02%). P > 0.05 (means ± SD, n = 4).

The quantification of oral bacterial densities typically falls within the range of 10^5-10^8 CFU/ml, with variations observed across distinct oral niches; however, plaque tends to exhibit higher counts (Bloomquist et al., 1996). For each genus of oral bacteria, a specific range is indicative of the oral health status. For example, species like *Streptococcus spp.* (e.g., *S. mutans, S. mitis, S. oralis*) and *Lactobacillus spp.* (e.g., *L. rhamnosus, L.*

paracasei, L. fermentumcao) are pivotal pathogens implicated in the initiation and progression of dental caries (Badet 2008; Chokshi et al., 2016). Elevated levels of *Streptococcus spp.* and *Lactobacillus spp.* in saliva signify an escalated risk of dental caries development (Gábris et al., 1998; Messer, 2000; Badet 2008). In the context of our findings, the level of *Lactobacillus spp.* density was lower than that of *Streptococcus spp*. (~0.43 x 10^3 CFU/mL compared to ~8.38 x 10^4 CFU/mL) after treatment with both menthol and capsaicin solutions. This underscores the potential for biofilm formation during overnight periods and accentuates the importance of early-morning tooth brushing to deter biofilm accumulation.

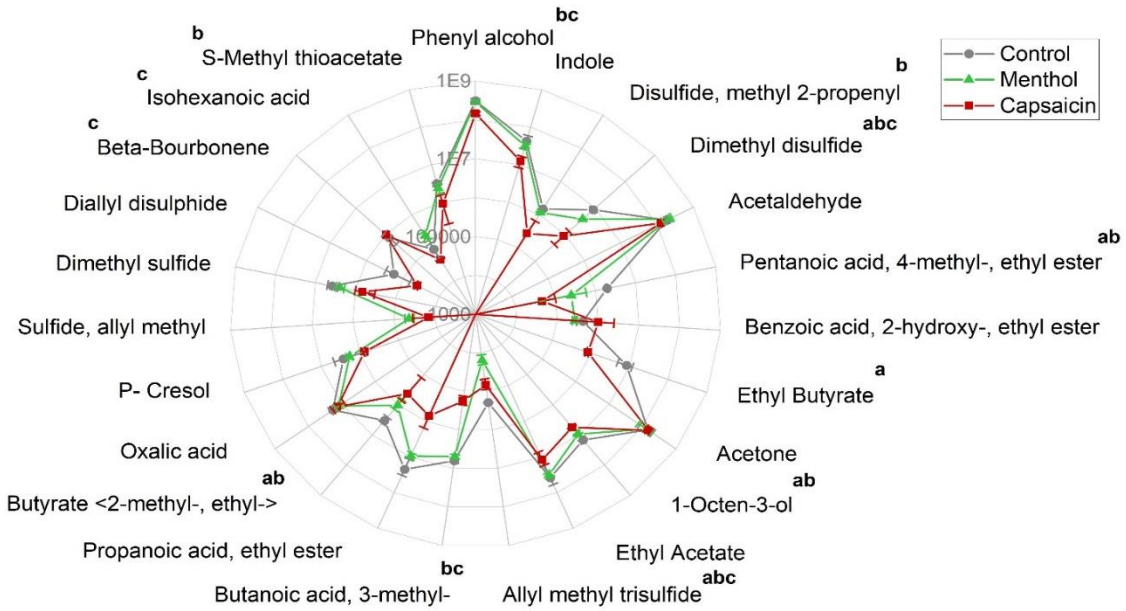
Concurrently, certain oral bacteria, notably *Prevotella spp.* (e.g., *P. intermedia*) and other anaerobes including *P. gingivalis, F. nucleatum*, and *T. denticola*, are implicated in halitosis formation (Persson et al., 1990; Rosenberg et al., 1991; Loesche & Kazor, 2000). Although not severe in terms of oral health, halitosis may cause social discomfort and interpersonal interactions. As a result, proactive measures are essential to mitigate these sources of halitosis. The ensuing section elaborates on the influence of menthol and capsaicin on the development of halitosis.

3.2. Demonstrating the effect of menthol and capsaicin on oral malodour formation (in-vivo and in-vitro)

The investigation centered on the influence of menthol and capsaicin on oral malodour, employing comprehensive GC-MS analysis encompassing both in-vivo and in-vitro methods. The results garnered from the in-vivo method, involving mouth rinsing, revealed distinctive trends in the modulation of malodorous compounds. Notably, both menthol and capsaicin demonstrated efficacy in reducing the levels of various compounds, except Acetaldehyde, Acetone, Oxalic acid, and Beta-Bourbonene, compared to the control (Figure 3A). Of particular interest, capsaicin consistently exhibited a more pronounced capacity to diminish compound levels in comparison to menthol, except in the case of Allyl

methyl trisulfide. Both menthol and capsaicin yielded significant reductions in Dimethyl sulfide, Pentanoic acid, 4-methyl-, ethyl ester, 1-Octen-3-ol, Allyl methyl trisulfide, and Butyrate <2-methyl-, ethyl-> compounds when compared to the control group. In parallel, Figure 3B showed the highest detected quantities of Phenyl alcohol, Acetaldehyde, and Acetone in saliva samples. Intriguingly, the heatmap underscored the superior efficacy of menthol and capsaicin in mitigating the levels of malodorous compounds through less dark colour, thereby suggesting its potential as a candidate in combating oral malodour.

A.



B.

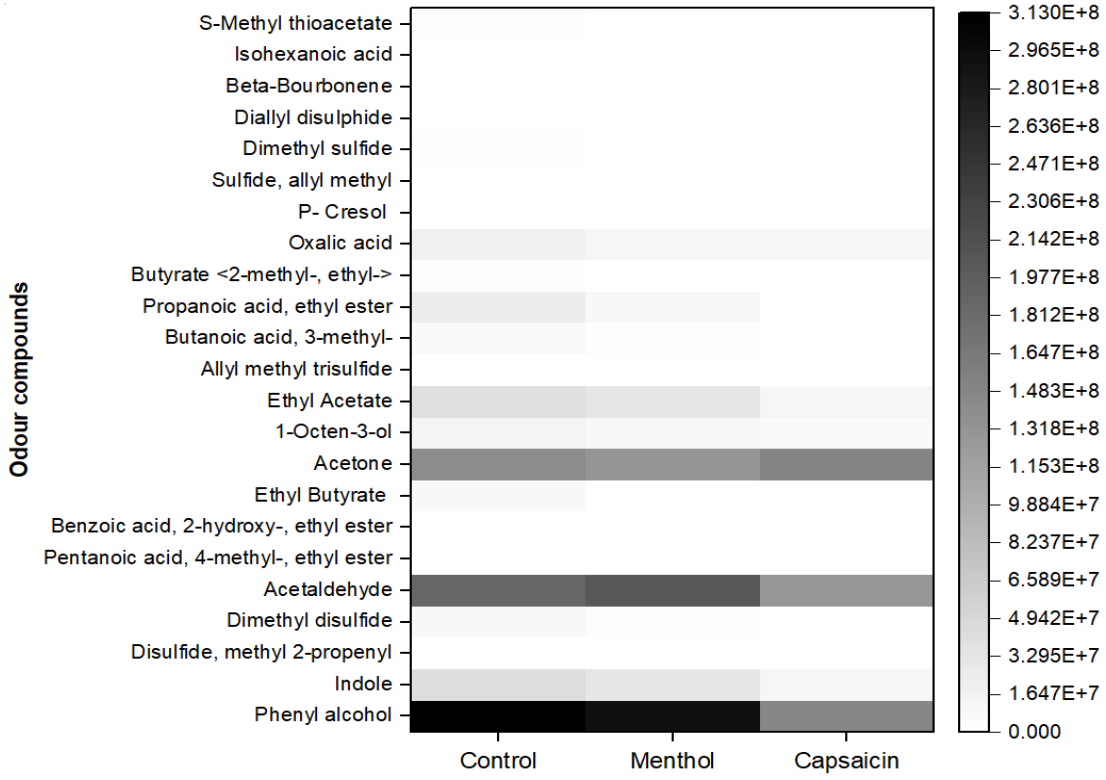
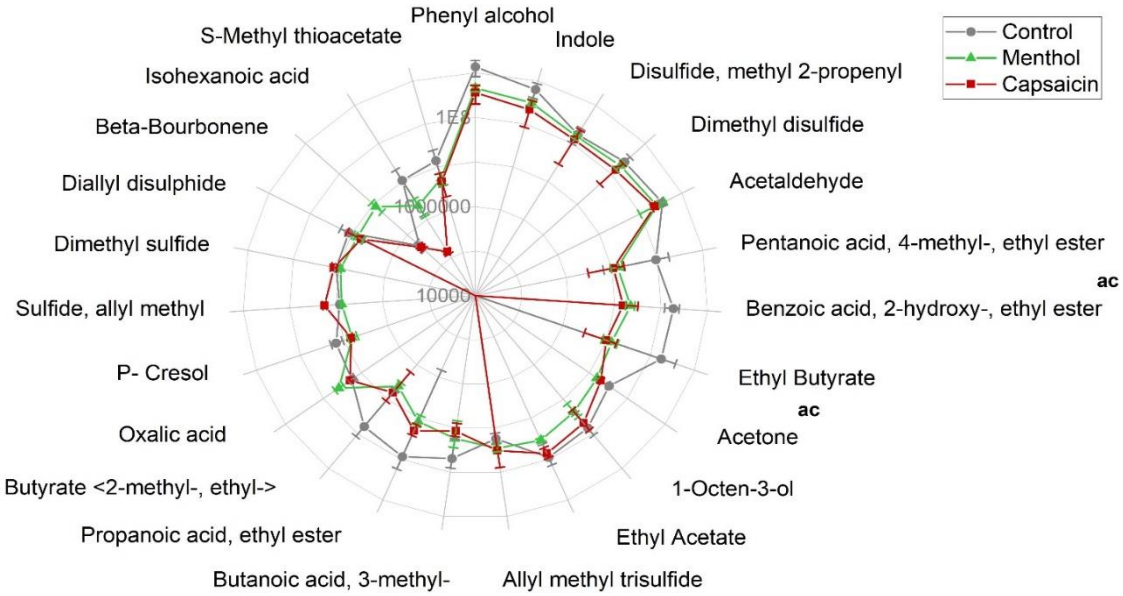


Figure 3: The rinsing effect of menthol and capsaicin (5 mL, 0.01%) on oral malodour formation (in-vivo). A: Presented by spider chart. B: Presented by heatmap. Letter "a", "b", and "c" indicate the significant difference among Menthol-Control, Capsaicin-Control, and Capsaicin-Menthol groups, respectively, $P < 0.05$ (means ± SD, n = 2).

The in-vitro experiment encompassed the introduction of menthol and capsaicin into collected saliva within GC vials. Intriguingly, although the concentrations of menthol and capsaicin were notably elevated at 0.5% as compared to 0.01% in the in-vivo method, the in-vitro outcomes revealed higher levels of most compounds. Diverging from the in-vivo trends, menthol and capsaicin showcased a propensity to primarily reduce the levels of several compounds, including Phenyl alcohol, Indole, Pentanoic acid, 4-methyl-, ethyl ester, Benzoic acid, 2-hydroxy-, ethyl ester, Ethyl Butyrate, Propanoic acid, ethyl ester, Butyrate <2-methyl-, ethyl->, Isohexanoic acid, and S-methyl thioacetate (in comparison to the control). However, the distinction between the effects of menthol and capsaicin on these compounds was not statistically significant, barring instances such as Benzoic acid, 2-hydroxy-, ethyl ester, and Acetone. The heatmap (Figure 3B), similar to Figure 4B, reaffirmed the prominence of Phenyl alcohol and Acetaldehyde, registering the highest detected levels in saliva samples. Besides, compounds like Indole, Disulfide, methyl 2-propenyl, and Dimethyl disulfide displayed high detection levels across control, menthol, and capsaicin solutions. Furthermore, the heatmap underscored a significant reduction in the levels of the compounds, positioned at the center of the heatmap, induced by the applications of menthol and capsaicin in comparison to the control.

A.



B.

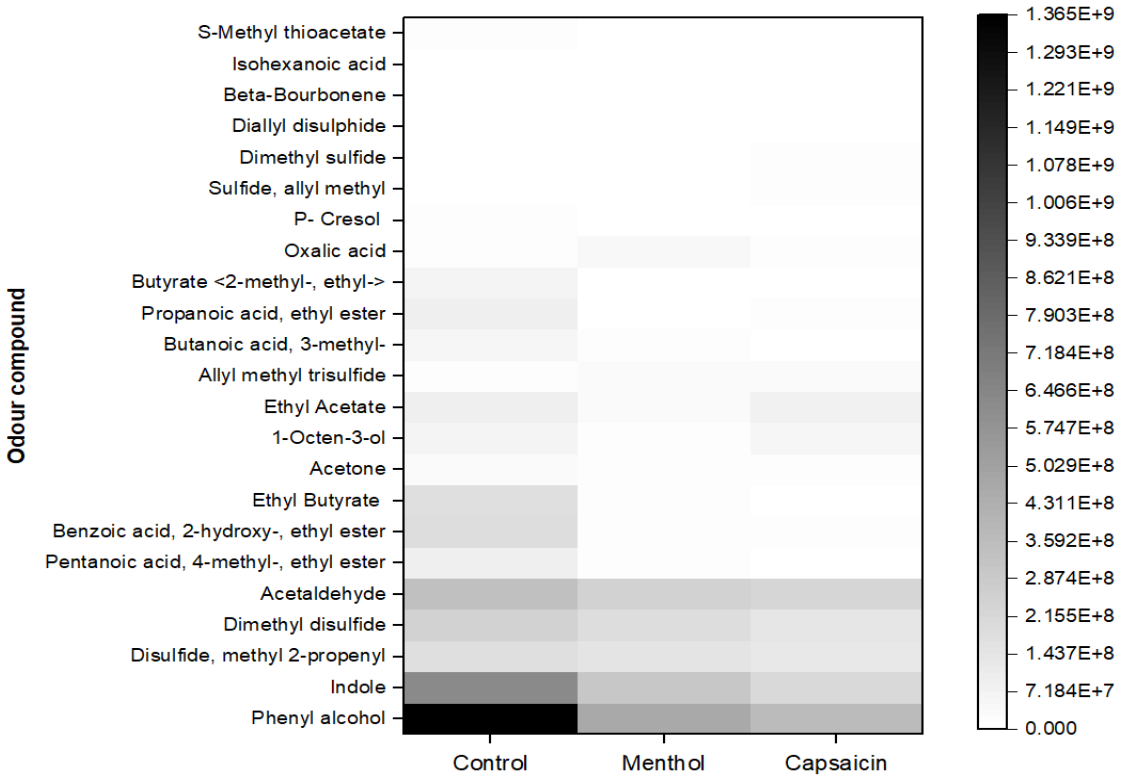


Figure 4: The adding effect of menthol and capsaicin (50μL, 0.5%) on oral malodour formation (in-vitro). A: Presented by spider chart. B: Presented by heatmap. Letter "a", "b", and "c" indicate the significant difference among Menthol-Control, Capsaicin-Control, and Capsaicin-Menthol groups, respectively, $P < 0.05$ (means ± SD, n = 2).

The detected compounds were classified into three groups based on their relevance to oral malodour as shown in Table 3. Like body odor, oral malodour predominantly arises from bacterial origins, accounting for approximately 85% of cases (Rosenberg, 1995; Rosenberg & Leib, 1995). Bad breath is primarily caused by the by-products, mainly VSCs, from the metabolism of oral anaerobic bacteria (Persson et al., 1990; Rosenberg et al., 1991; Loesche & Kazor, 2000). Based on the result from the GC-MS experiment, there were seven VSCs detected in both in-vivo and in-vitro methods (Table 3). Most of these VSCs were low compared to other compounds in the in-vivo method, and most of these VSCs were significantly reduced by 0.01% capsaicin ($P < 0.05$), except Diallyl disulfide, Dimethyl sulfide, Sulfide, allyl methyl. This suggests that the presence of menthol and capsaicin at 0.01% concentration in the oral cavity can effectively mitigate oral malodour. Interestingly, the in-vitro observations presented a contrasting scenario, with VSCs obtained at notably elevated levels as other compounds (Figure 4A). However, neither menthol nor capsaicin elicited significant reductions in VSC levels. This outcome potentially underscores the presence of a substantial quantity of oral bacteria, actively generating VSCs during the saliva collection process. Even when administered at elevated concentrations (0.5%), menthol and capsaicin could not to counteract the elevated VSC levels produced by these bacteria.

In addition, a cluster of volatile organic compounds (VOCs) also substantially contributes to halitosis, including Indole (foul odor of resembling feces), Butanoic acid, 3-methyl- (foul odor resembling rancid butter or sweaty socks), P- Cresol (foul odor of a fecal or urine-like smell) and 1-Octen-3-ol (distinct musty and mushroom-like smell), etc (Table 3). These VOCs that cause haitosis are also byproducts of metabolism of some oral bacteria and have high levels in saliva in both methods; however, only Acetone, 1-Octen-3-ol, and Butanoic acid, 3-methyl- exhibited significant reductions in the presence of either menthol or capsaicin (Figures 3A and

4A). For instance, *F. nucleatum*, a known periodontal bacterium, contributes to the production of Indole (Sasaki-Imamura et al., 2010). The presence of Indole and its derivatives in human saliva (Cooke et al., 2003), in conjunction with the activity of *S. mutants*, contribute to the biological biofilm formation (Hu et al., 2010). In addition, levels of Acetaldehyde were very high in both methods, possibly through the metabolism of *Streptococcus* species and oral microflora such as *Candida* species that converts ethanol in the menthol and capsaicin solutions to Acetaldehyde (Homann et al., 1997; Homann et al., 2000; Kurkivuori et al., 2007). Furthermore, Acetone, while not directly produced by oral bacteria, can emerge through fatty acid breakdown in the liver (Chakravartty et al., 2022). Consequently, the Acetone levels found within the in-vivo method were higher than those detected in the in-vitro approach. The heightened concentrations of Acetaldehyde and Acetone pose potential risks, including the development of oral cavity cancer (Homann et al., 2000) and throat and nasal irritation (Atlanta et al., 2022), respectively. Additionally,the level of Acetone in the oral cavity is also used as a biomarker for the detection of various metabolic conditions such as diabetes mellitus (Saasa et al., 2018), lung cancer (Ruzsányi et al., 2017), etc.

However, besides those compounds that cause unpleasant odors, there are some compounds that produce pleasant aromas (Phenyl alcohol, Benzoic acid, 2-hydroxy-, ethyl ester, Beta-Bourbonene), or fruity odor (Pentanoic acid, 4-methyl-, ethyl ester, Ethyl Butyrate, Ethyl Acetate, Butyrate <2-methyl-, ethyl->). Contrary to the detrimental effects of VOCs causing halitosis, these favourable-scented VOCs bear anti-bacterial and anti-inflammatory attributes, potentially contributing to oral health improvement. For example, thanks to the antibacterial properties of Ethyl acetate, the growth of *L. acidophilus*, *Candida albicans*, and *Aggregatibacter actinomycetemcomitans*, common oral pathogens related to dental caries and periodontitis, was significantly reduced ($P < 0.05$) (Owusu-Boadi et al., 2021). However, these non-malodorous VOCs are also

reduced by menthol and capsaicin. Furthermore, Oxalic acid, while neutral in terms of its impact on oral malodour, when consumed through oxalate-rich foods, can potentially lead to the formation of minute crystals or deposits within the oral cavity, instigating oral tissue irritation and associated dental ailments (Alan et al., 2013).

Table 3: Classification of detected compound based on relation to halitosis.

Volatile sulfur compounds (VSCs)	**Other compounds can relate to halitosis**	**Compounds may not relate to halitosis**
Disulfide, methyl 2-propenyl	Indole	Phenyl alcohol
Dimethyl disulfide	Acetaldehyde	Pentanoic acid, 4-methyl-, ethyl ester
Allyl methyl trisulfide	1-Octen-3-ol	Benzoic acid, 2-hydroxy-, ethyl ester
Sulfide, allyl methyl	Butanoic acid, 3-methyl-	Ethyl Butyrate
Dimethyl sulfide	P- Cresol	Ethyl Acetate
Diallyl disulfide	Isohexanoic acid	Beta-Bourbonene
S-Methyl thioacetate	Propanoic acid, ethyl ester	Butyrate <2-methyl-, ethyl->
	Acetone	

In the in-vitro experiment, no saliva stimulation took place which means no more salivary proteins were secreted. Therefore, the in-vitro experiment aimed to highlight the influence of menthol and capsaicin on metabolic activity within the aerobic timeframe of oral bacteria. Because in in-vitro experiments, the effects of menthol and capsaicin were not as significant as the results in in-vivo experiment, the subsequent sections explore the effects of menthol and capsaicin on oral cavity saliva flow rates as well as salivary proteins.

3.3. The effect of menthol and capsaicin on salivary flow rate

Investigating the effect of menthol and capsaicin at 0.02% on saliva flow rate was conducted, the control trend line in Figure 5 showed that chewing a parafilm infused with water, containing a trace of ethanol, yielded an approximate saliva flow rate of 1.25 g/mL. This outcome aligns with previously reported ranges, showing that stimulated saliva production typically ranges 0.77 – 4.15 mL/min (Engelen et al., 2005; Chen, 2009). Saliva flow rate may vary from individual to individual due to unclear physiological differences between individuals (Gardner & Carpenter, 2019). In general, the application of menthol and capsaicin increased high levels of saliva production as contrasted with the control over a 10-minute interval (Figure 5). Table 4 shows the percentage increase in total saliva stimulated by menthol and capsaicin compared to control. In the initial minute, menthol and capsaicin induced an average surge of approximately 60% and 100%, respectively; however, this enhancement was reduced by approximately one-third and one-half, respectively, by the conclusion of the 10-minute experimental time. Notably, the elevation in saliva flow rate achieved statistical significance ($P < 0.05$) solely within the first two minutes of menthol exposure. In contrast, capsaicin demonstrated a notably higher level of saliva secretion throughout the experimental duration, establishing statistical significance ($P < 0.05$). Moreover, a discernible disparity ($P < 0.05$) in saliva production between menthol and capsaicin persisted until the 7-minute mark. Furthermore, menthol increased saliva production in comparison to the control until the 5-minute juncture, where the stimulated saliva level consistently approximated the control, at around 1 – 1.25 g/mL. Likewise, capsaicin-triggered salivary secretion maintained a relatively stable pattern during the last 3 minutes, approximating 1.5 mg/mL.

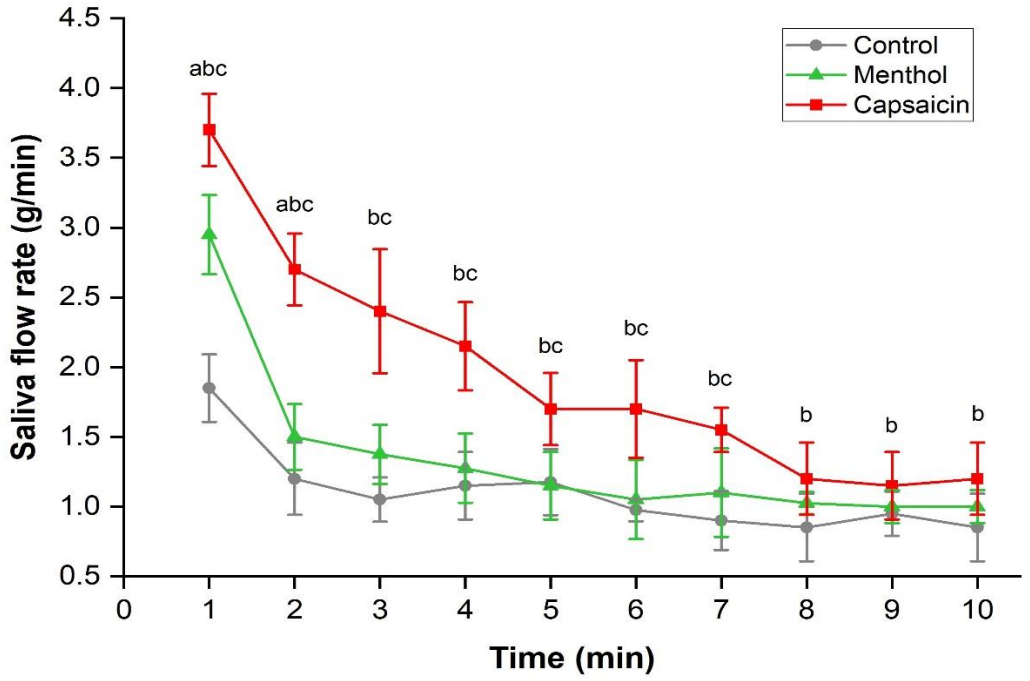


Figure 5: Influence of 200µL of menthol (0.02%) and capsaicin (0.02%) solutions on saliva flow rate. Salivary secretion after every 1 min by using 200µL of trigeminal modulators. Letter "a", "b", and "c" indicate the significant difference among Menthol-Control, Capsaicin-Control, and Capsaicin-Menthol groups, respectively, $P < 0.05$ (means ± SD, n = 10).

Table 4: The percentage increase in total saliva stimulated by menthol (0.02%) and capsaicin (0.02%) solutions compared to the control solutions.

Aqueous systems	After 1-min stimulation	After 10-min stimulation
Menthol (0.02%)	59.46%	17.65%
Capsaicin (0.02%)	100%	41.18%

In 2019, Gardner and Carpenter conducted a study to explore the impact of menthol on saliva flow rate at a closely related concentration of 250 ppm (equivalent to 0.025% in our context). Their findings revealed an average saliva production of approximately 1.15 ± 0.10 g/mL, with no statistically significant effect of menthol observed ($P > 0.1$). Intriguingly, the outcomes diverge from our present study, wherein menthol induced a significant increase in salivary volume during the initial two minutes, 2.95 ± 0.28 g/mL

at the first minute and 1.50 ± 0.24 g/mL at the second minute. However, by the fifth minute, the saliva flow rate regressed to 1.15 ± 0.24 g/mL (Figure 5). On the other hand, the investigations into the impact of capsaicin on saliva flow rate operated at lower concentrations of 1 ppm (0.0001% equivalent) (Gardner & Carpenter, 2019) and 5 ppm (0.0005% equivalent) (Yang et al., 2021). Interestingly, these experiments yielded comparable saliva flow rates of approximately 1.4 ± 0.2 g/mL (0.01%, $P < 0.05$) and ~1 ± 0.55 g/mL (0.05%, $P < 0.05$) respectively, and these results were lower than the results obtained in our study, 3.70 ± 0.26 g/mL (0.02%). This observed variance might potentially be attributed to several factors. The participation of individuals with "low" saliva flow rates in our study could be a contributing factor (Gardner & Carpenter, 2019). Discrepancies in physiological attributes and variances in individual "cold" and "hot" tolerance levels could also potentially account for these distinctions. Notably, the augmented saliva volume induced by both menthol and capsaicin holds a crucial benefit—enhanced bacterial clearance from the oral cavity. At the same time, increased saliva can also increase salivary proteins, which have properties that can reduce the number of bacteria in the oral cavity, making the oral cavity healthier. Therefore, the ensuing section delves into an evaluation of the effects of menthol and capsaicin on the composition and intensity of salivary proteins to understand their potential implications.

3.4. Differences in relative concentration of salivary protein composition.

The separation of salivary protein composition, induced by the stimulation of menthol and capsaicin at a concentration of 0.02%, was accomplished through 12% SDS-PAGE. Figure 6 shows insight into the prominent protein constituents within the saliva, showed by distinct bands including MUC(s), MUC7, α-Amylase, PRPs, and Cystatin. The profile revealed two distinct bands for Cystatin, ranging within 10 - 15 kDa, followed by five prominent

bands alongside several faint ones for PRPs (16 to 50 kDa), two dark bands for α-Amylase (50 to 62 kDa), a clear band for MUC7 (approximately 150 kDa), and other mucin family's diverse constituents appearing above 188 kDa. Notably, the bands attributed to α-Amylase showed the highest intensity, characterized by the size and darkness, followed by Cystatin with the second-most intense band. These findings, encompassing the main salivary protein composition, their respective molecular weight, and the big intensity of α-Amylase and Cystatin principal protein constituents, exhibit concordance with the results obtained by Gardner and colleagues in 2020. However, the number of bands of each protein differs markedly, particularly PRPs. This difference could potentially stem from disparities in gel composition and attributes or may be attributed to differences among individuals with different genetic phenotypic polymorphism (Schwartz et al., 1995).

In addition, only capsaicin increased the band intensities of Cystatin, compared to menthol and control. This phenomenon was similar for bands of PRPs featuring molecular weights approximating 37 and 18 kDa. In contrast, the intensity of PRP bands (approximately 41, 26, 29, and 31 kDa) experienced a reduction subsequent to both menthol- and capsaicin-induced salivary stimulation, clear observation at replication 2, when compared with relative intensity bands of control. These are consistent with the findings of Gardner and co-researchers (2020) wherein the relative intensities of Cystatin bands exhibited augmentation and the PRPs bands displayed alterations consequent to capsaicin (1 ppm) stimulation.

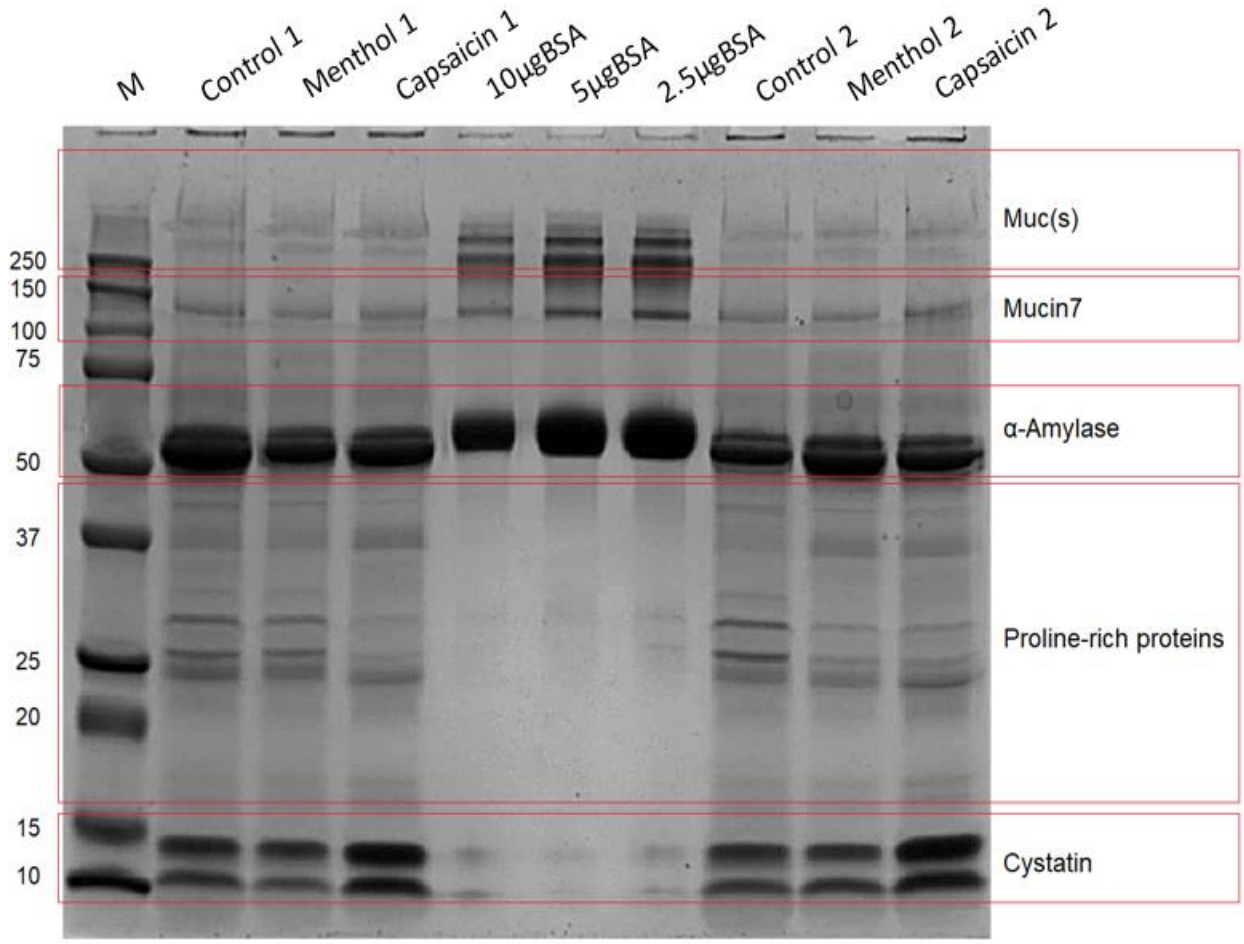


Figure 6: SDS-PAGE profile of saliva proteins stimulated by 200 µL of trigeminal solutions (0.02%). The number 1 and 2 following control, menthol, capsaicin indicate two replicates (n = 2). BSA indicate protein model with 3 different concentrations (10 µg, 5 µg, and 1.5 µg). M: biomarker.

The validation of disparities in the protein composition of stimulated saliva was undertaken through the utilization of the AUC technique. Figure 7 illustrates the discernible presence of two predominant peaks, observed at approximately 1.8S and 4.2S, which correspond to the sedimentation coefficient distribution of saliva previously reported by Dinu and co-researchers (2019). In general, the sedimentation coefficient distribution of menthol-stimulated proteins was higher than that of the control and was lower than that of capsaicin-stimulated proteins, particularly noticeable on salivary proteins at the lower end of sedimentation coefficients (approximated at 1.8S).

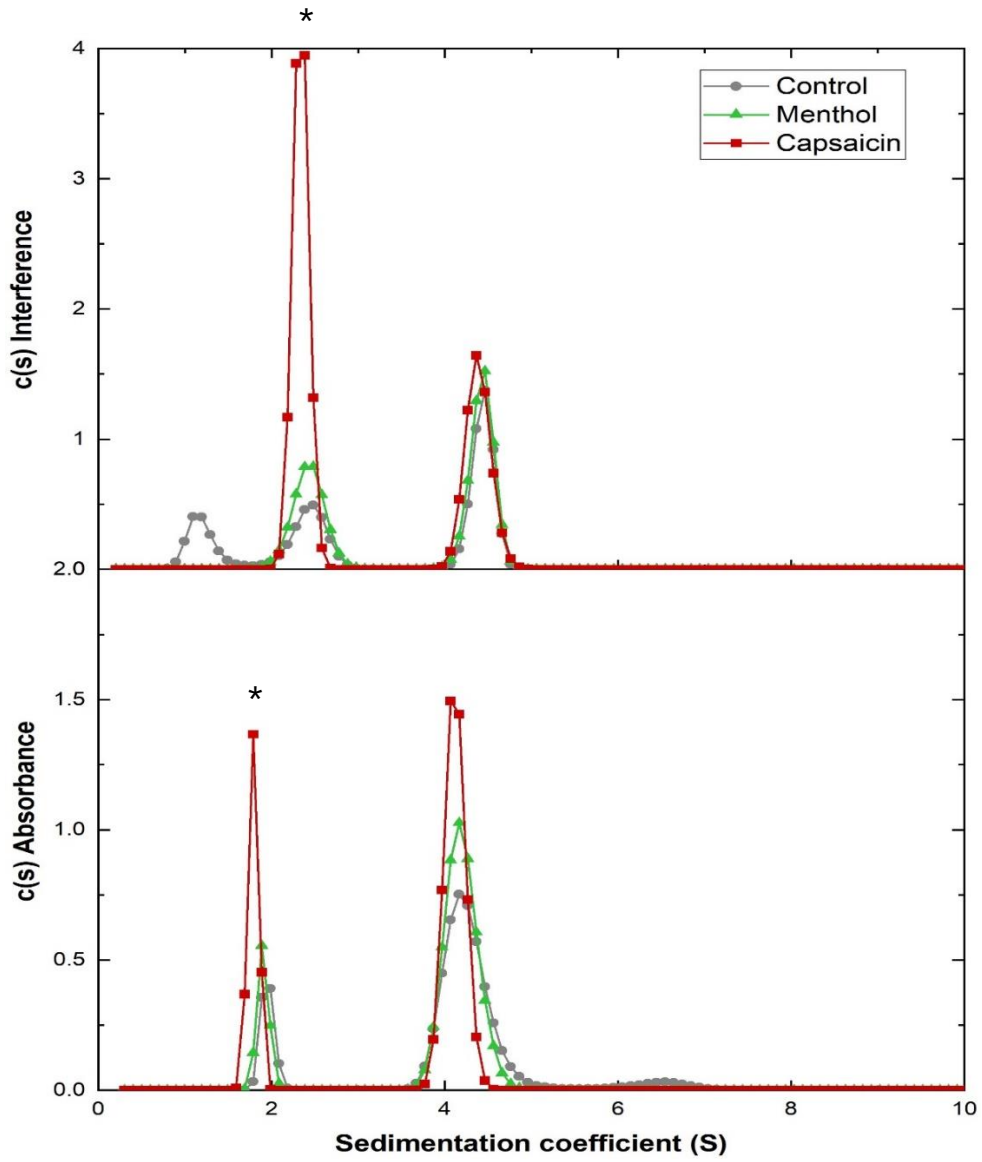


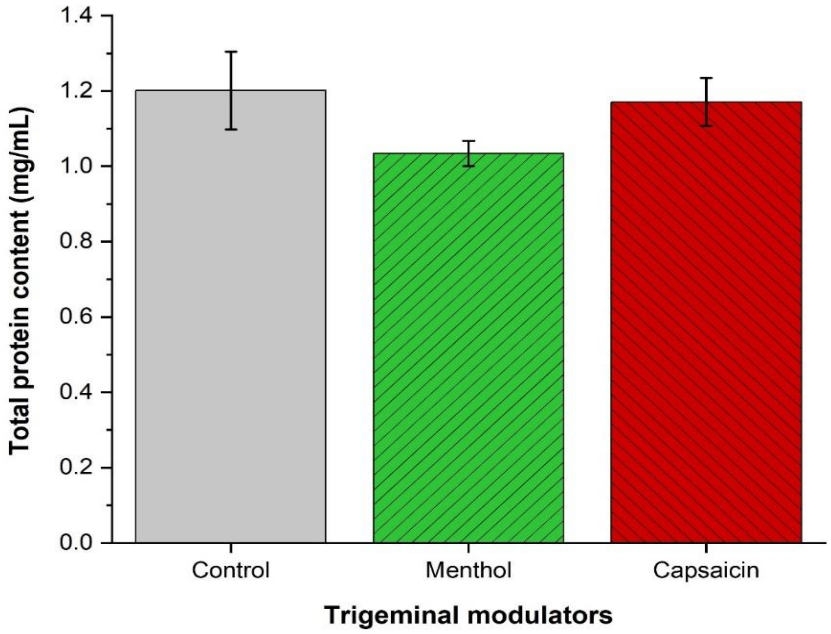
Figure 7: Sedimentation velocity of pooled saliva. The c(s) Absorbance and Interference analysis of the sedimentation species resenting in saliva stimulated by 200µL of trigeminal modulators show the sedimentation coefficient distribution using SEDFIT. Peaks are determined based on their molecular weight, which is directly linked to the sedimentation coefficient, and their relative concentration. Run 440 µL of loading volume samples at a rotor speed of 35000 rpm, 20.0 °C.

ImageJ software was applied on the SDS-PAGE gel from Figure 6, and the intensity of total salivary protein and main salivary proteins were obtained as shown in Figures 9A and 9B, respectively. In the case of capsaicin-stimulated saliva, the total salivary protein content exhibited minimal divergence from the control, manifesting values of 1.17 ± 0.06 mg/mL and

1.20 ± 0.10 mg/mL respectively. This finding aligns with the observations made by Gardner and Carpenter (2019), wherein capsaicin did not alter the total protein quantity of approximately 1.26 mg/mL. The intensity of Cystatins was also significantly different in both menthol- and capsaicin-stimulated saliva. Although capsaicin generally did not alter total salivary protein, it significantly changed the intensity of Cystatins and some PRPs. These findings are in agreement with previous research by Garner and Carpenter (2019), Garner and colleagues (2020) that the intensity of most major proteins, notably Cystatin with two-fold higher, was distinct enhancement upon exposure to capsaicin (1 ppm).

Menthol-stimulated saliva showed a lower total protein (1.03 ± 0.03 mg/mL) and also had a lower in all main proteins (Figure 8B); however, menthol only showed a reduction in the intensity of PRPs ($P < 0.05$) as compared to both the control and capsaicin stimulation. This outcome aligns with earlier findings by Gardner and Carpenter (2019), wherein a comparable total protein concentration of approximately 1.04 mg/mL was observed upon exposure to an equivalent amount of menthol (250 ppm). However, a stronger increase in the expression of salivary Cystatins "S" family when Houghton et al. (2019) applied double the concentration of menthol (500 ppm), instead of a strong change in PRPs expression.

A.



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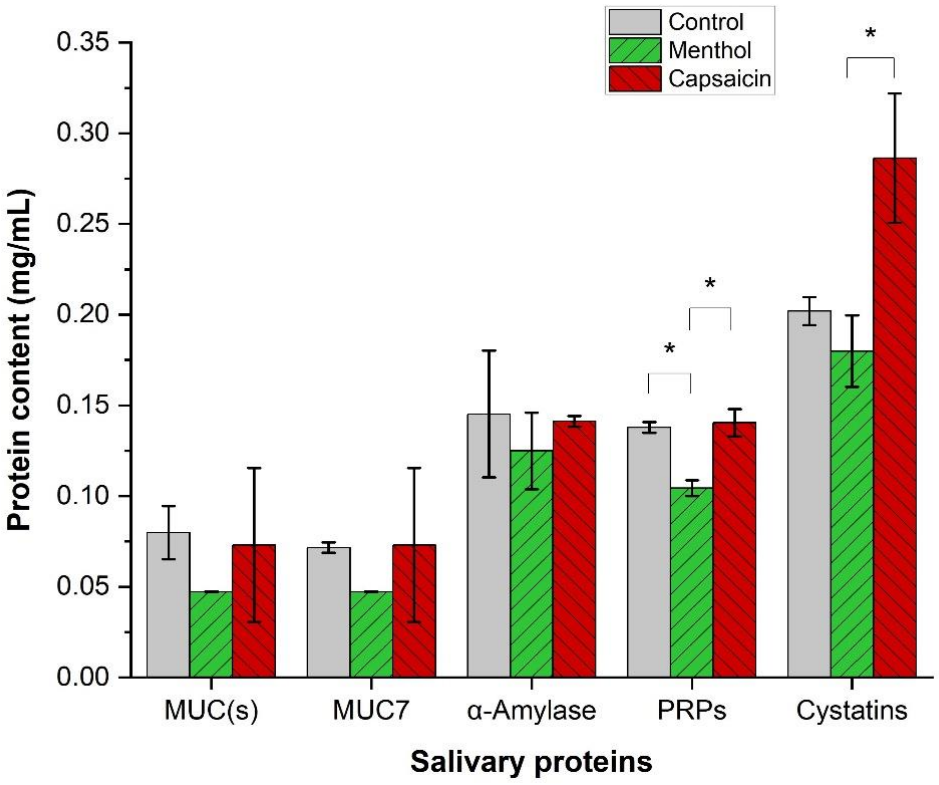

Figure 8: The changes in salivary protein band intensity stimulated by control, menthol, and capsaicin at 0.02% concentration based on SDS-PAGE densitometry. A. total salivary protein, B. main salivary proteins. Asterisk (*) indicate significant difference between groups, $P < 0.05$ (means ± SD, n = 2).

Human salivary cystatins belong to family II of the cystatin superfamily, predominantly categorized as cystatin "S" variants (including S, SA, SN) and cystatin C (Bobek and Levine, 1992). Research has shown that Cystatin S contains four phosphorylation sites that interact with hydroxyapatite (Bell et al., 1997). Its significance lies in the formation of dental pellicles, maintaining the balance of calcium and phosphate, and promoting enamel remineralization (Koopaie et al., 2021). Furthermore, it safeguards against enamel demineralization by binding it to the enamel surface (Laputková et al., 2018). Another study has indicated that *S. mutants* exhibited pronounced structural impairment, characterized by cell wall detachment, peptidoglycan, and plasma membrane disruption (Blancas et al., 2021). Moreover, there was a discernible reduction in the integrity of the plasma membrane bilayers. These findings highlight the importance of increased salivary Cystatin for bacterial reduction. Therefore, clinical trials utilizing the Enzyme-Linked Immunosorbent Assay (ELISA) technique should be conducted to assess the elevation of Cystatin levels induced by menthol and capsaicin stimulation. Salivary Cystatin is considered one of the biomarkers for evaluating the risk of early childhood caries (Hemadi et al., 2017), with the quantification of Cystatin concentrations in saliva serving as an early diagnostic tool for dental caries.

4. Conclusion

In conclusion, the results supported the hypotheses proposed in this study: menthol and capsaicin i) significantly increased saliva flow rate with menthol having the edge over the initial period of time while capsaicin provides a build-up effect throughout sampling, ii) altered the salivary protein concentrations, with significant increase on PRPs and Cystatins, iii) exerted antibacterial effects on *Bacteroides, Prevotella, Lactobacillus, Streptococcus spp.,* and other anaerobes, and iv) reduced significantly compounds responsible for oral malodour. These findings collectively contribute to our comprehension of the intricate dynamics of oral microbiota, oral malodour, saliva flow rate, and salivary protein composition, and accentuate the important effect of bioactive compounds, menthol, and capsaicin, on oral health, providing valuable insights for potential oral health applications. Further investigations should be conducted to investigate mechanisms of interactions behind the changes observed within the salivary proteome and metabolome. Furthermore, clinical trials are essential to elucidate and provide a more comprehensive perspective on the effects of menthol and capsaicin on different individual groups.

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Appendix

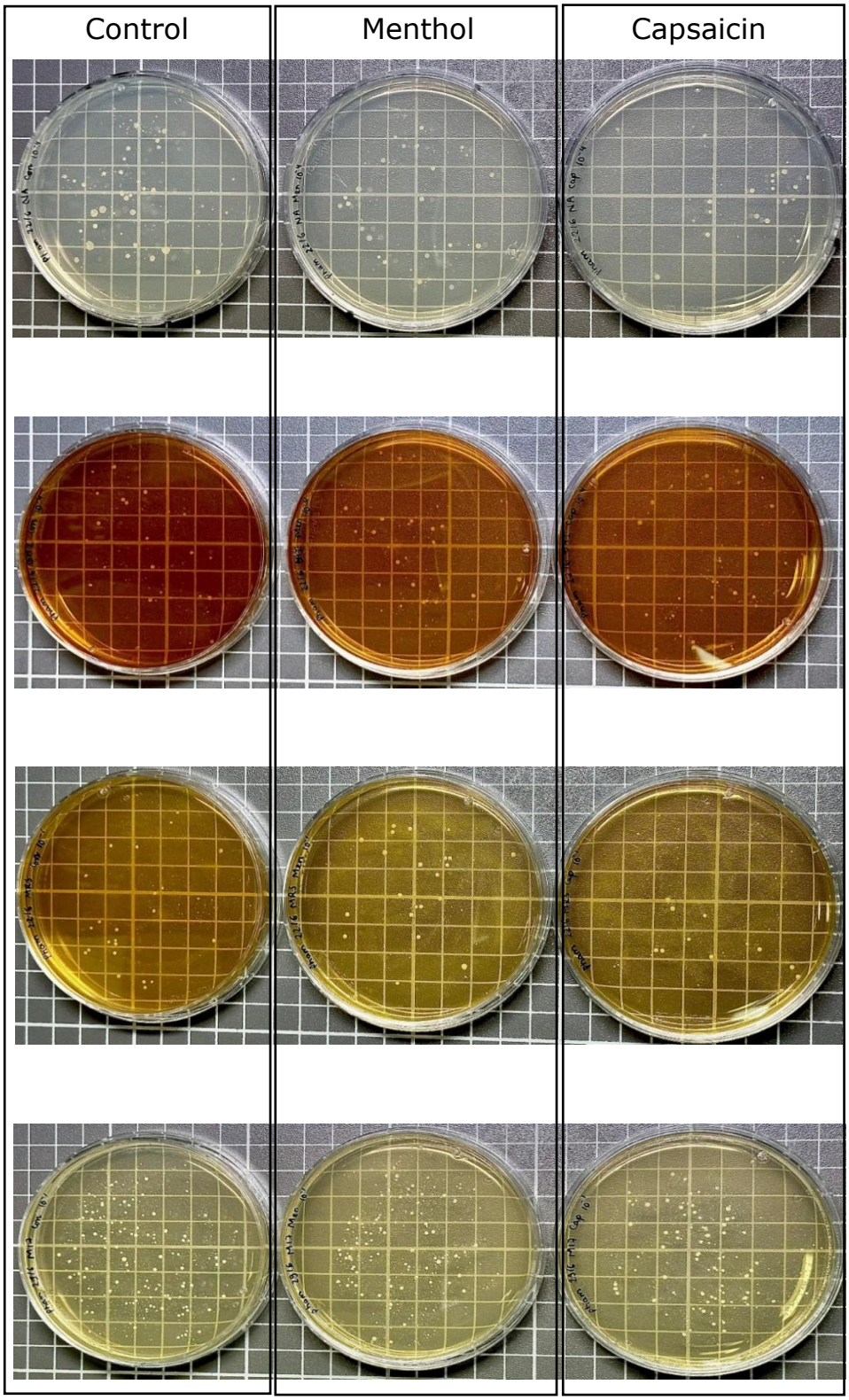

Figure 1: The changes in number of *Bacteroides spp., Prevotella spp., Lactobacillus spp., Streptococcus spp*., and other fastidious anaerobic colonies (n = 4) isolated by A. NA, B. BHI, C. MRS, and D. M17 after using 200 µL of menthol and capsaicin (0.02%). agar.

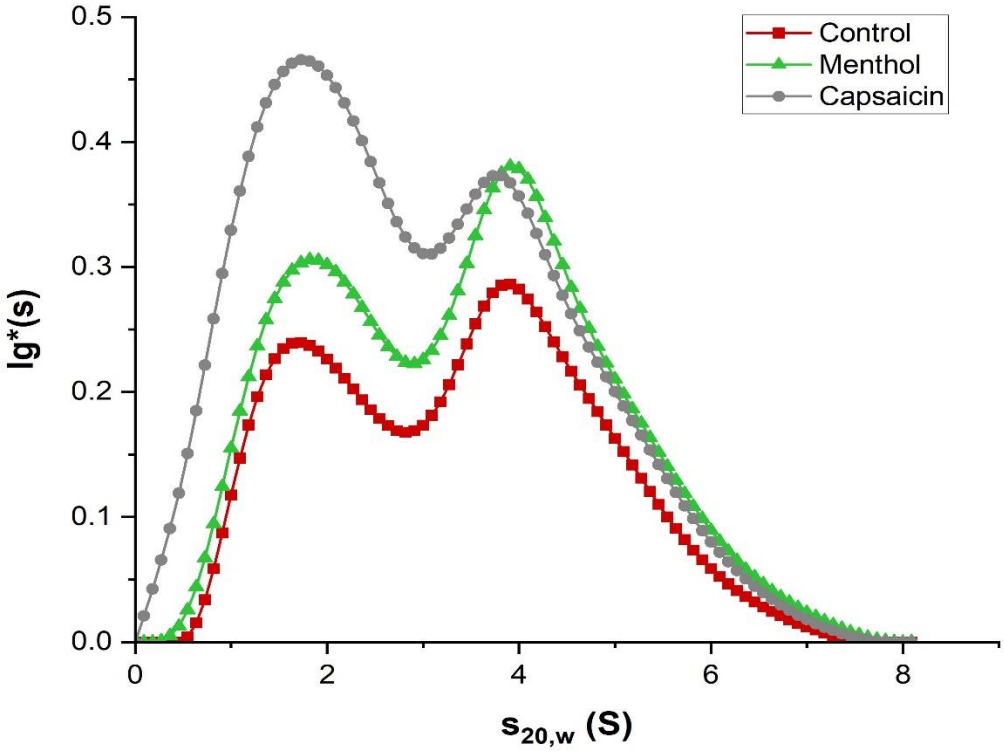

Figure 2: Sedimentation velocity of pooled saliva. The lg*(s) analysis of the sedimentation species resenting in saliva stimulated by 200µL of trigeminal modulators show the sedimentation coefficient distribution using SEDFIT. Peaks are determined based on their molecular weight, which is directly linked to the sedimentation coefficient, and their relative concentration. Run 440 µL of loading volume samples at a rotor speed of 35000 rpm, 20.0 °C.