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Antimicrobial effects in oral microenvironments by a novel herbal toothpaste

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ABSTRACT

Objective: This clinical study compared the antibacterial effects after brushing with a novel herbal toothpaste incorporating zinc [test] to a control fluoride toothpaste on anaerobic organisms, gram-negative bacteria and malodor bacteria of dental plaque, tongue scrapings and cheek surfaces.

Methods: This double-blind, two-cell study enrolled 44 adults [age range 19–63 years]. Subjects completed a 1-week washout and provided baseline oral samples i.e. dental plaque, tongue and cheek scrapings for microbiological analysis. Diluted samples for microbiological analyses were plated on agar to enumerate anaerobic organisms, gram-negative bacteria and malodor bacteria representing functional groups of organisms. Subjects were randomized to brush their teeth with either the test or control with the first brushing conducted under supervision in the dental clinic. Post-treatment samples were collected 12 h after 21 day hygiene with assigned toothpaste. After providing these samples, subjects brushed in the dental clinic with additional samples collected 4 h after brushing. Statistical analyses were conducted separately for each organism collected from each oral niche by *t*-test for within-treatment assessments and analysis of covariance (ANCOVA) for between-treatment comparisons.

Results: Treatment groups demonstrated no significant differences at baseline for anaerobic organisms, gram-negative bacteria and malodor bacteria in any oral niche ($p > 0.05$). The test demonstrated reductions between 42 and 68% for anaerobic bacteria in oral niches, 12 h after brushing with reductions increasing to 46–80%, 4 h after brushing. Similarly, the test demonstrated reductions between 49 and 61% for gram-negative bacteria of oral niches that increased to 54–69% at the 4 h post-brushing evaluation. Reductions in malodor organisms of 22–42% were noted 12 h after brushing that increased to 60–72%, 4 h after brushing.

Conclusions: In comparison to control, brushing with a novel herbal toothpaste demonstrated significant reductions in functional bacterial groups from distinct oral niches 12 h after brushing with additional microbial reductions 4 h after brushing.

1. Introduction

Clinical research has been instrumental in examining the relationship between dental plaque, a biofilm that accumulates naturally on the teeth and the onset of common oral conditions such as gingivitis and caries [1]. Based on current evidence, an unrestricted accumulation of dental plaque is reportedly associated with the initiation and progression of inflammation of the gums and tissues supporting the teeth. Research has explored the dental plaque, its components and constituent organisms including the microbiome in health along with their changes associated with disease [1,2]. Based on the available evidence, dysbiosis in the microbial composition appears associated with the initiation and progression of common oral conditions including gingivitis and dental caries [2]. Supplementing microbial dysbiosis in disease progression is the accumulation of metabolic by products by these organisms, acids, microbial toxins and residual microbial cellular components localized in the local environment that alter the antigenic and immunogenic features of the dental plaque [3].

The literature reports on dental plaque biofilm for its constituent organisms, biofilm extracellular matrix components, extracellular DNA and other structural components that serve as a scaffold to maintain biofilm integrity [1,2]. In addition, investigations have assessed sali-

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vary microbial constituents and the biofilms on the surface of the tongue, cheeks [4] and gums representing distinct oral microenvironments [5]. Organisms found in the saliva are planktonic that are dispersed within the different microenvironments of the oral cavity. The tongue surface with its papillae is coated with a complex microbial biofilm [6]. The tongue biofilm has been evaluated microbiologically with available research supporting alterations in the microbial composition amongst subjects with oral malodor. A predominance of gram-negative bacteria some of which have proteolytic and other metabolic adaptations to convert dietary constituents to malodorous by-products is reported amongst those with bad breath. Other oral niches with biofilms include the buccal mucosa with research demonstrating the presence of organisms on the surface or localized within buccal cells [4].

Effective oral hygiene representing an essential attribute for self-care is required to cleanse the dentition, remove food residues and reduce the negative influences associated with microbial accumulation [1,2] . Whereas brushing the teeth with a fluoride toothpaste and a toothbrush is reported commonly, surveys indicate that most individuals fail to maintain adequate oral hygiene [7]. The consequences of inadequate oral hygiene is readily manifest in the global incidence of oral diseases [1] and in clinical investigations that record the amount of dental plaque left behind on the teeth immediately after a brushing episode [7].

Approaches that augment daily oral hygiene reduce the negative influences of dental plaque accumulation [2]. One recognized approach to enhance oral hygiene is the use of toothpastes and mouthwashes formulated with well-characterized chemical agents demonstrating inhibitory effects on the dental plaque and oral organisms [6,8]. An additional approach seeks to provide patient relevant benefits following the use of formulations incorporating well-characterized herbs and other natural products with therapeutic features [8–11]. Described recently is a novel toothpaste formulated with herbal ingredients and zinc salts with demonstrated inhibition on dental plaque and gingivitis (manuscript prepared) in addition to reducing salivary lactate dehydrogenase [LDH] [12] representing a measure of oral mucosal integrity [45]. The purpose of this investigation was to examine the effects of oral hygiene with this toothpaste formulated with herbal ingredients and zinc salts on the microbial constituents of the dental plaque, tongue and cheeks with these samples evaluated for viable bacteria in addition to gram-negative organisms or those associated with malodor to examine antimicrobial effects.

2. Materials and methods

2.1. Study procedures and design

This was designed as a single site, parallel design study with randomized treatment assignments. The study protocol was approved by the Institutional Review Board of the University at Buffalo School of Dental Medicine, Buffalo, New York prior to study enrollment with the entire study conducted at the University at Buffalo.

Volunteers between the age of 18–70 years of either gender from the local area expressing an interest in the study were invited to a screening visit at the dental clinic conducted by a dental examiner. Subjects who voluntarily completed an informed consent form and were available for the study duration were screened for study eligibility. A study dentist completed an oral examination including the entire dentition, the tongue, palate and all soft tissue regions with all evaluations conducted under constant lighting. Subjects were interviewed for their medical history and underwent a whole mouth assessment of dental plaque and gingivitis using the Turesky-Modification of the Quigley Hein Index [13] and Loe-Silness [14] Index respectively. Subjects with atleast 20 natural teeth, in good general health and registering plaque index scores of 1.5 or more and gingival index scores of 1.0 or more were enrolled. Those presenting with dentures, orthodontic bands were excluded. Also excluded were those reporting an ongoing pregnancy, medical or dental treatments in the month preceding study screening or scheduled during the study period. Similarly, those reporting allergies of oral care or personal care products, or on prescription medications including antibiotics, anti-inflammatory were excluded. Those unable to refrain from food or drink for 4 h or reported recent participation in a clinical study were excluded. Subjects who satisfied study criteria were enrolled and underwent a washout period. Subjects were provided a tube of commercially available fluoride toothpaste [Colgate Dental Cream, Great Regular Flavor, New York, NY] and a commercially available soft-bristled toothbrush [Colgate Extra Clean, New York, NY] for twice daily oral hygiene during the one week washout phase. Subjects were instructed to refrain from sharing the provided articles with anyone or using unassigned oral hygiene formulations for the study period.

At the completion of the washout phase, subjects arrived at the dental clinic on the morning of their scheduled visit without brushing their teeth for up to 12 h and after refraining from food or drink for 4 h. All washout associated formulations were returned to study personnel and baseline samples of dental plaque, scrapings from the cheek and tongue collected for analysis using procedures described previously [15]. Each collected sample was placed in a sterile tube marked with subject identification details. After providing their baseline samples, subjects were randomly assigned a test toothpaste which was overwrapped and issued a unique code for blinded treatment assignment. Subjects were instructed to brush their teeth twice daily for the next three weeks with the assigned test toothpaste and a commercially available soft bristled toothbrush. They were scheduled to arrive at the dental clinic for post-treatment sampling conducted 12 h after brushing with subjects refraining from food for 4 h prior to their visit. Post-treatment oral samples for analysis were collected using procedures similar to those at baseline. Subjects brushed in the dental clinic with the test toothpaste used over the past three weeks and returned to the dental clinic 4 h h later to provide post-brushing samples. Samples of dental plaque, and scrapings from tongue and cheek surface were collected at this visit for analysis. At the final visit, subjects returned provided test articles to the study personnel and underwent a complete oral examination before discontinuing study participation.

2.2. Microbiological procedures

All oral samples were transferred to the microbiological laboratory upon collection. At the laboratory, samples were sonicated, serially diluted in buffer and plated on agar media to enumerate anaerobic organisms, bacteria associated with malodor and gram-negative bacteria in accordance with procedures described previously [15]. Viable organisms were enumerated after anaerobic incubation and recorded as colony forming units per ml of sample and results log transformed (log_{10}) for statistical analysis [16].

2.3. Statistical analysis

A sample size of approximately 22 subjects was enrolled to examine a difference of 0.3 between treatment groups with a standard deviation of 0.3.

Statistical analyses were conducted separately for each type of oral microorganism evaluated from each site of the human mouth sampled. Treatment groups were compared with respect to gender using a chi-square and an independent *t*-test for age. Baseline scores between treatment groups for each evaluation was evaluated using independent t-tests. A comparison from baseline to each post-treatment evaluation was conducted using a paired *t*-test. An analysis of covariance (ANCOVA) was utilized to determine the effects of treatments using baseline adjusted scores. Significant differences from each analysis is reported at $p < 0.05$.

3. Results

A summary of the study population completing the study is shown in Table 1. Twenty females and twenty four males [age range 19–63 years of age] completed all phases of the study. Twenty two subjects in the test group [average age 46 years] and an additional twenty two in the control group [average age 45 years] completed the study and provided evaluable results. Analysis indicate no statistically significant differences between the two test groups for subject age or gender by ANOVA and chi-square analyses respectively ($p > 0.05$). Over the study, subjects reported no adverse events with the clinical examiner reporting no adverse observations at the clinical examination.

Results from the study evaluating effects on anaerobic bacteria, gram-negative organisms and malodor bacteria is shown in Tables 2–4 with percent differences between these treatment groups summarized in Table 5. A summary of the mean number of viable anaerobic bacteria (log CFU/ml) collected twelve (12) hours after brushing with either the test or the control toothpaste from the oral microenvironments i.e. dental plaque, tongue and cheek surfaces is shown in Table 2. Also shown in Table 2 are corresponding mean numbers of these organisms 4 h after brushing in these oral microenvironments. The number of plaque bacteria were 7.95 and 7.59 at the baseline evaluation representing no significant differences ($p > 0.05$). At the 12 h post-brushing evaluation both the test and control groups demonstrated reductions with average scores of 7.29 and 7.53 respectively and were significantly different ($p = 0.027$) representing a 42.5% difference (Table 5). Additional reductions in these organisms were noted at the 4 h post-brushing evaluation with average scores in the test and control groups of 6.63 and 6.99 respectively representing statistically significant differences ($p = 0.026$). The test group demonstrated a 56.3% reduction in comparison to the control for dental plaque anaerobic organisms (Table 5).

Shown in Table 3 are results on dental plaque gram-negative bacteria. At baseline, no significant differences were noted for gram-negative

Table 1
Summary of Subject Demographics completing the study.

Treatment	Number of Subjects			Age[c]	
	Male	Female	Total[c]	Mean	Range
Test Toothpaste[a]	12	10	22	46.2	19–63
Control Toothpaste[b]	12	10	22	45.6	20–63

[a] Test Toothpaste (Colgate-Palmolive Co., New York, NY).
[b] Control Toothpaste (Colgate-Palmolive Co., New York, NY).
[c] No statistically significant difference was indicated between the treatment groups with respect to either gender or age ($p > 0.05$).

Table 2
Summary of anaerobic organisms (Log CFU/ml) from distinct oral microenvironments from subjects who completed the entire study (Mean ± SD).

Oral Sample	Treatment	Baseline	Post-brushing evaluation after 3 weeks of brushing[a]	
			12-Hours After brushing[a]	4 h post-brushing[a]
Plaque	Test	7.95 ± 0.41	7.29 ± 0.46	6.63 ± 0.61
	Control	7.59 ± 0.80	7.53 ± 0.74	6.99 ± 0.83
Tongue	Test	7.82 ± 0.50	7.08 ± 0.84	6.43 ± 0.83
	Control	7.54 ± 0.88	7.58 ± 0.90	7.13 ± 0.84
Cheek	Test	6.24 ± 0.45	5.74 ± 0.57	5.11 ± 0.65
	Control	5.92 ± 0.70	5.99 ± 0.53	5.38 ± 0.67

[a] Statistically significant differences between the test and control treatments for anaerobic organisms in each oral microenvironment ($p < 0.05$). Analyses were conducted separately for each oral microenvironment by analysis of covariance (ANCOVA) and Tukey-HSD multiple comparison tests.

Table 3
Summary of gram-negative organisms (Log CFU/ml) from distinct oral microenvironments from subjects who completed the entire study (Mean ± SD).

Oral Sample	Treatment	Baseline	Post-brushing evaluation after 3 weeks of brushing[a]	
			12-Hours After brushing	4 h post-brushing
Plaque	Test	7.11 ± 0.60	6.18 ± 0.83	5.67 ± 0.68
	Control	6.90 ± 0.63	6.60 ± 0.82	6.18 ± 0.95
Tongue	Test	6.76 ± 0.68	6.27 ± 0.63	5.78 ± 0.71
	Control	6.54 ± 0.82	6.61 ± 0.55	6.12 ± 0.57
Cheek	Test	5.11 ± 0.60	4.24 ± 0.70	3.81 ± 0.59
	Control	4.90 ± 0.66	4.55 ± 0.63	4.17 ± 0.76

[a] Statistically significant differences between the test and control treatments for gram-negative bacteria in each oral microenvironment ($p < 0.05$). Analyses were conducted separately for each oral microenvironment by analysis of covariance (ANCOVA) and Tukey-HSD multiple comparison tests.

Table 4
Summary of oral malodor organisms (Log CFU/ml) from distinct oral microenvironments from subjects who completed the entire study (Mean ± SD).

Oral Sample	Treatment	Baseline	Post-brushing evaluation after 3 weeks of brushing[a]	
			12-Hours After brushing	4 h post-brushing
Plaque	Test	7.18 ± 0.51	6.69 ± 0.65	5.91 ± 0.70
	Control	6.91 ± 0.46	6.92 ± 0.36	6.32 ± 0.91
Tongue	Test	7.22 ± 0.51	6.56 ± 0.47	5.94 ± 0.52
	Control	6.87 ± 0.64	6.67 ± 0.63	6.50 ± 0.57
Cheek	Test	5.50 ± 0.56	4.76 ± 0.64	4.30 ± 0.68
	Control	5.01 ± 0.99	5.00 ± 1.00	4.70 ± 0.80

[a] Statistically significant differences between the test and control treatments for malodor organisms in each oral microenvironment ($p < 0.05$). Analyses were conducted separately for each oral microenvironment by analysis of covariance (ANCOVA) and Tukey-HSD multiple comparison tests.

Table 5
Inter-group comparisons between the treatment groups for organisms in each microenvironment shown as percent differences.

Bacteria evaluated	Oral microenvironment	Evaluation conducted 12 h after 3-weeks of brushing		Evaluation conducted 4 h post brushing	
		Reduction in the test as compared to the control (shown as a %)[b]	p value[c]	Reduction in the test as compared to the control (shown as a %)[b]	p value[c]
Anaerobic bacteria[a]	Plaque	42.5	0.027	56.3	0.026
	Tongue	68.4	0.034	80	0.010
	Cheek	43.8	0.042	46.3	0.038
Gram-negative bacteria[a]	Plaque	61.9	0.046	69.1	0.031
	Tongue	54.3	0.017	54.3	0.045
	Cheek	49.9	0.047	56.3	0.044
Malodor bacteria[a]	Plaque	41.1	0.017	61.1	0.046
	Tongue	22.4	0.038	72.5	0.002
	Cheek	42.5	0.004	60.2	0.012

[a] Statistical analyses conducted separately for each organism from each oral microenvironment by analysis of covariance (ANCOVA) and Tukey-HSD multiple comparison tests.
[b] % differences between treatment groups computed using the antilog (base 10) of the difference between the control treatment and the test product subtracted from 1 and then multiplied by 100%.
[c] The test demonstrated statistically significant differences from the control for all evaluations with p values shown for each comparison.

bacteria between the treatment groups registering with the test and control groups registering scores of 7.11 and 6.90 respectively ($p > 0.05$). At the 12 h post-brushing, the treatment groups demonstrated reductions with mean scores of 6.18 and 6.60 in the test and control groups representing statistically significant differences ($p = 0.046$) (Table 5). The evaluation conducted 4 h after brushing demonstrated additional reductions with the test and control groups registering scores of 5.67 and 6.18 respectively representing statistically significant differences ($p = 0.031$). Analysis indicate that the test demonstrated a 61.9% reduction in gram-negative organisms at the 12 h post-brushing in comparison to the control treatment groups that increased to 69.1% at the 4 h post-brushing assessment representing statistically significant differencesas shown in Table 5.

The effect of treatments on malodor bacteria of the dental plaque is shown in Table 4. Average numbers of malodor bacteria in the test and control at baseline were 7.18 and 6.91 respectively with no statistically significant differences ($p > 0.05$). Average numbers of malodor bacteria in the test and control were 6.69 and 6.92 respectively with the test demonstrating a statistically significant reduction of 41.1% versus the control ($p = 0.017$) (Table 5) at the 12 h post-brushing evaluation. Additional reductions in dental plaque malodor bacteria were observed at the 4 h examination with the test and control registering scores of 5.91 and 6.32 respectively and were statistically significant ($p = 0.046$). At this evaluation, the test demonstrated a reduction of 61.1% for dental plaque malodor bacteria in comparison to the control (Table 5).

Analysis of tongue surface anaerobic bacteria is shown in Table 2. Average number of bacteria in the test and control were 7.82 and 7.54 respectively with no statistically significant differences ($p > 0.05$). While the test group demonstrated a reduction in these organisms at the 12 h post-brushing evaluation with a mean score of 7.08, the mean score in the control was 7.58. The test demonstrated a statistically significant reduction in tongue surface anaerobic bacteria in comparison to the control representing a 68.4% difference ($p = 0.034$) as shown in Table 5. Both the test and control groups demonstrated additional reductions in tongue surface anaerobic bacteria at the 4 h post-brushing evaluation with mean scores of 6.43 and 7.13 in the test and control groups respectively representing statistically significant differences ($p = 0.010$). The test demonstrated an 80% reduction in anaerobic tongue surface bacteria (Table 5) in comparison to the control at the 4 h post-brushing evaluation.

Shown in Table 3 is analysis of tongue surface gram-negative bacteria. Average scores at baseline for the test and control were 6.76 and 6.54 with no statistically significant differences ($p < 0.05$). Both the test and control demonstrated reductions in gram-negative bacteria at the 12 h post-brushing evaluation with average scores of 6.27 and 6.61 in the test and control groups respectively representing statistically significant differences of ($p = 0.017$). Gram-negative tongue surface bacteria in the test demonstrated a 54.3% reduction in comparison to the control (Table 5). Both the test and control demonstrated additional reductions in these organisms at the 4 h post-brushing evaluation with mean scores for the test and control of 5.78 and 6.12 respectively representing statistically significant differences ($p = 0.045$). At the 4 h post-brushing evaluation, the test demonstrated a reduction of 54.3% for gram-negative tongue surface bacteria as compared to the control (Table 5).

Effects on malodor tongue surface bacteria is shown in Table 4 with mean scores of 7.22 and 6.87 at baseline demonstrated no significant differences ($p > 0.05$). At the 12 h post-brushing evaluation, average scores for the test and control were 6.56 and 6.67 representing a 22.4% reduction that was statistically significant ($p = 0.038$) (Table 5). Both the test and control demonstrated additional reductions at the 4 h post-brushing evaluation with average scores in the test and control of 5.94 and 6.50 respectively representing statistically significant differences ($p = 0.002$). At the 4 h post-brushing evaluation, the test demonstrated a decrease of 72.5% malodor tongue surface organisms than the control.

The effect of evaluated treatments on cheek surface anaerobic bacteria is presented in Tables 2 and 5 At baseline, average numbers of organisms in the test and control were 6.24 and 5.92 with no statistically significant differences ($p > 0.05$). Brushing with the test demonstrated a reduction in anaerobic organisms at the evaluation conducted 12 h after 3 week of use. The average numbers of organisms at the 12 h evaluation for the test and control were 5.74 and 5.99 respectively representing statistically significant differences ($p = 0.042$). The test demonstrated a 43.8% reduction (Table 5) in anaerobic cheek surface bacteria than the control at the 12 h post-brushing evaluation. Additional reductions in anaerobic organisms were observed at the 4 h post-brushing evaluation. Average microbial scores were 5.11 and 5.38 for the test and control treatments respectively representing statistically significant differences ($p = 0.038$). The test demonstrated a reduction of 46.3% in comparison to the control (Table 5).

Results of gram-negative cheek surface organisms is presented in Tables 3 and 5 At baseline, average numbers of organisms in the test and control were 5.11 and 4.90 respectively representing results that were not statistically significant ($p > 0.05$). Post-treatment analyses conducted 12 h after 3 week brushing with the test and the control were 4.24 and 4.55 respectively representing statistically significant differences ($p = 0.047$). The test demonstrated a reduction of 49.9% in gram-negative cheek surface organisms than the control at the 12 h post-brushing evaluation (Table 5). Additional reductions in bacteria were noted at the 4 h post-brushing examination. Average numbers of organisms in the test and control were 3.81 and 4.17 respectively demonstrating statistically significant differences ($p = 0.044$). In comparison to the control, the test demonstrated a 56.3% reduction in gram-negative cheek organisms at the 4 h evaluation (Table 5).

Summarized in Tables 4 and 5 are the effects on malodor cheek surface bacteria. At baseline, average scores for the test and control were 5.50 and 5.01 respectively with no significant differences ($p > 0.05$). At the 12 h post-brushing evaluation, average scores for the test and control were 4.76 and 5.00 respectively representing statistically significant differences ($p = 0.004$). The test demonstrated a 42.5% reduction in comparison to the control at the 12 h post-brushing evaluation (Table 5). Both the test and control demonstrated additional reductions at the 4 h post-brushing evaluation with average scores in the test and control of 4.30 and 4.70 respectively representing statistically significant differences ($p = 0.012$). At the 4 h post-brushing evaluation, the test demonstrated a 60.2% decrease in malodor cheek surface organisms than the control (Table 5).

4. Discussion

Clinical evidence supports a microbiological etiology in the inception and progression of common dental diseases such as gingivitis and dental caries [1,3,8]. Despite research advances on these common oral diseases representing preventable conditions, population based epidemiological surveys report a substantial global prevalence afflicting large numbers of individuals [1–3]. This clinical study enrolled community dwelling adults to examine the effect of brushing with a novel herbal toothpaste with zinc salts to a commercially available fluoride dentifrice on oral bacteria. Assessments were conducted 12 h after brushing representing general inter-hygiene durations. Additionally, the study also examined the effects 4 h after brushing to evaluate the short-term effects of the toothpastes over the course of the day.

Herbal ingredients have a substantial history in health care with estimates suggesting more than 80% of the world's population dependent on plant based drugs [17]. Available in the literature are clinical effects of toothpastes formulated with herbs for improving oral hygiene [18,19]. Herbal ingredients and their purified extracts reportedly provide a range of effects including those associated with reducing micro-

bial binding [20], anti-oxidant and metabolic effects [21], inhibition of dental plaque and gingivitis [9,22] and reduction of tooth sensitivity [23]. A substantial literature supports the role of herbal ingredients formulated in toothpastes and mouthwashes for improving oral hygiene. Formulated in the evaluated test toothpaste were zinc salts representing an ingredient with a history of application for oral hygiene [8]. Examination of the therapeutic features of zinc describe microbial aggregation [24], stress on the microbial envelope [25], protection from microbial toxins [42], plaque reduction [26] and host membrane repair [27] Recent reports evaluate supplementary effects of zinc with other ingredients including herbal constituents [10,28,29] to augment efficacy [30–33].

This study examined the effects of toothpastes on several intra-oral microenvironments i.e. dental plaque, tongue and cheek surfaces concurrently representing a methodological feature unique to this study. Additionally, each sample was evaluated for three types of organisms examining the entire population of cultivable anaerobic organisms, those associated with malodor and gram-negative bacteria. These oral microenvironments were selected based on the routine oral hygiene conducted to reduce the tooth associated dental plaque with the soft tissue regions generally receiving no hygiene but likely serving as microbial reservoirs that seed other areas of the mouth [34]. The human mouth harbors a complex microbiome that appears to be shaped by the distinct anatomical regions and influenced by shedding and non-shedding surfaces [5,8]. Potential differences in salivary flow, nutritional supply, microbial adherence and mucosal shedding likely contribute to the microbial populations found in the distinct areas such as the tongue, dental plaque and cheek. Tooth surfaces including the interproximal regions representing non-shedding surfaces allow development of a dynamic and complex biofilm while biofilms on shedding surfaces such as the mucosa are influenced by these local physiological factors [5].

Previous reports describe the effects of oral hygiene aids including toothpastes and mouthwashes formulated with herbal ingredients or zinc on bacteria of dental plaque [43,44] and saliva [11,44]. Based on the literature, this study evaluated the effects of treatments in distinct oral microenvironments and also evaluated effects on tongue surface organisms. The tongue harbors large densities of organisms associated with malodor and gram-negative organisms capable of metabolizing dietary proteins and other components to produce compounds associated with malodor [6]. Analyses of the organisms on the cheek surface have fewer representations in the literature [10,29] with this area likely serving as a reservoir for the therapeutic components of the toothpaste.

Subjects enrolled in the study completed a washout phase and were instructed to brush twice daily with supplied toothpastes and refrain from using any other oral hygiene formulations over the study period. Antimicrobial assessments were conducted 12 h after brushing with subjects evaluated after only 21 days of brushing representing rapidly progressing effects in a study design similar to previously reported [9,35]. An additional assessment sampled subjects 4 h after brushing to examine effects over the duration of the day representing a patient relevant outcome. These features were included in the clinical study to gain additional efficacy measures from the study. Microbiological assessment examined viable organisms representing those capable of growth and metabolism, transmission between oral sites, surface re-colonization and toxin production and in contrast to techniques utilizing other approaches for microbial analysis [36]. Results from this study indicate that at the 12- hour post-brushing examination, the test toothpaste demonstrated between 42 and 68% reductions in anaerobic organisms, 49–61% reduction in gram-negative bacteria and 22–42% reductions in malodor organisms of distinct oral micro-environments. These effects increased broadly at the 4-h post-brushing evaluations with the test registering between 46 and 80% reductions in anaerobic organisms, 54–69% reduction in gram-negative bacteria and 60–72% reductions in malodor organisms of the evaluated oral micro-environments.

Enrolled subjects were from the general population not seeking any medical or dental treatments who were not asked to change their routine diet or habits nor instructed on brushing techniques. An assessment of the microbial populations in all these locations provides a comprehensive evaluation of the effects of dentifrices 12 h after brushing representing a common inter-hygiene period. Irrespective of the oral microenvironment examined several broad conclusions are evident. Brushing with the novel herbal toothpaste with zinc demonstrated significant reductions in all microbial populations evaluated in each of these oral microenvironments. Further, the observed effects improved over time demonstrating additional reductions at the 4 h post-brushing assessment conducted during the day. Taken together, the results support effects of the assigned toothpastes under general use and applicable widely to the broader population.

In summary, results from the double-blind clinical study demonstrated that twice-daily brushing for 21 days with a novel herbal toothpaste incorporating zinc, samples taken 12 h after oral hygiene had significantly greater reductions in oral bacteria of the dental plaque, cheek and tongue than those who brushed with a fluoride dentifrice. These effects were noted on several classes of oral bacteria in each of these oral microenvironments i.e. anaerobic organisms, malodor bacteria and gram-negative bacteria. Furthermore, the significantly greater reductions in all classes of oral organisms evaluated in these sites continued to be observed at the 4h post-brushing time point representing an assessment during the day.

Declaration of competing interest

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work.

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